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Caractérisation des voies de signalisation de la GTPase Rho suite aux lésions du système nerveux central

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Caractérisation des voies de signalisation de la GTPase Rho suite aux lésions du système nerveux central

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RÉSUMÉ

La petite GTPase Rho joue un rôle important dans la réponse aux protéines inhibitrices de croissance de la myéline et de la cicatrice gliale. Rho est donc un modulateur de la croissance axonale et de la régénération axonale. Rho est aussi un régulateur de l'apoptose *in vitro* et *in vivo*. Nous avons étudier les états d'activation de Rho ainsi que ses fonctions *in vivo* en réponse aux traumatismes du SNC. Pour ce faire, nous avons utilisé l'essai pull down qui détecte seulement la forme active de Rho. Nous avons pu montrer que Rho est activée suite aux lésions de la moelle épinière dans les neurones et les cellules gliales endogènes de la moelle, ainsi que dans le cortex et l'hippocampe suite aux lésions traumatiques du cerveau (TBI). En plus du TBI, Rho est activée dans l'hippocampe suite à une ischémie cérébrale ainsi que suite aux convulsions de type épileptique. Nous avons par la suite élucidé les effets produits par l'activation de Rho. Nous avons dans un premier temps montré que l'activation de Rho induit la mort cellulaire dans la moelle épinière de manière dépendante de $p75^{NTR}$ et que l'activation de Rho est dépendante de $p75^{NTR}$ 24 heures suite aux lésions de la moelle. Nous avons aussi montré que la cytokine pro-inflammatoire TNF, joue un rôle important dans l'activation de Rho. Le TNF suit aussi la même distribution que celle du Rho actif suite aux lésions de la moelle épinière. Nous avons voulu élucider les mécanismes apoptotiques dépendants de Rho. Suite aux lésions de la moelle épinière les protéines inhibitrices de la myéline sont relâchées de la matière blanche endommagée en plus de la présence de cytokines pro inflammatoires endommageantes. Nous montrons en dernier lieu que l'activation de Rho par la combinaison de la myéline et du TNF induit la mort neuronale de neurones en culture ainsi qu'*in vivo* dans les neurones cholinergiques du septum. Ces

résultats montrent pour la première fois un rôle pour les protéines de la myéline dans la mort cellulaire. Ensemble, ces résultats montrent un rôle important pour Rho dans la médiation de l'apoptose et indiquent que l'activation de Rho est une réponse clé suite aux traumatismes nerveux.

Mots Clés : Rho, SNC, moelle épinière, TBI, TNF, $p75^{NTR}$, apoptose

SUMMARY

The small GTPase Rho is a key modulator of the growth inhibitory effects of myelin derived inhibitory proteins and glial scar inhibitors. Rho is therefore an important modulator of axonal growth and regeneration after injury. Rho is also involved in the modulation of apoptosis both *in vitro* and *in vivo*. We have studied Rho activation states and its functions after CNS trauma. To do so we made use of a pull down assay that only detects the activated form of Rho. We have found that Rho is activated in response to spinal cord injury (SCI), in both neurons and glia, as well as in the cortex and hippocampus after traumatic brain injury (TBI). Furthermore we show that Rho is activated in the hippocampus after ischemic stroke as well as after epileptic type seizures. We next studied the effects of activated Rho using the SCI model. We show that Rho is activated in a $p75^{NTR}$ dependant manner 24 hours after SCI and that inactivation of Rho protects cells from apoptosis indicating that Rho regulates cell death after SCI. We also show that Rho can regulate p75 expression in the spinal cord. Next we show that the inflammatory response, TNF, can activate Rho. The distribution of TNF in the injured spinal cord correlates with that of activated Rho in the tissue. We next wanted to further study the role of Rho in apoptosis. After SCI, myelin derived growth inhibitory proteins are released from damaged white matter and pro-inflammatory cytokines such as TNF are also released. Lastly, we show that activation of Rho form the combined presence of myelin and TNF induces cell death in cultured neurons as well as *in vivo* in cholinergic neurons from the medial septum. These results show for the fist time a role for myelin derived growth inhibitory proteins in cell death. Together these results show an important

role for Rho in the regulation of apoptotic cell death. Activation of Rho is therefore a key response to CNS injury.

Key words : Rho, SNC, moelle épinière, TBI, TNF, $p75^{NTR}$, apoptosis

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LISTE DES ABRÉVIATIONS

ADF.....actin depolymerizing factor

ALS.....amyotrophic lateral sclerosis

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

AMPc.....cyclic adenosine monophosphate

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

ATP.....adenosine triphosphate

BBB..... Blood Brain Barrier

BBB.....Basso, Beattie, Bresnahan locomotor scale

C3.....C3-transferase

CSPG.....chondrotin sulphate proteoglycans

DAI..... Diffuse axonal injury

DH.....Dbl homology

DRG.....dorsal root ganglion

EAA.....excitatory amino acids

ERM.....ezrin,radaxin,moesin

GAG.....glycosaminoglycans

GAP.....GTPase-activating proteins

GDI.....guanine diphosphate

GDP.....GTPase-activating proteins

GEF.....guanine nucleotide exchange factors

GPI.....glycosylphosphatidylinositol

GTPases.....small GTP-binding proteins

KDa.....kilo Daltons

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

LPA.....lysophosphatidic acid

LRR.....leucine rich repeats

MAG.....myelin associated glycoprotein

MBS.....myosin binding subunit

MLC.....myosin light chain

MLCK.....myosin light chain kinase

MMP.....matrix metalloprotease

MT.....microtubule

MTS.....membrane transport sequence

NGF.....nerve growth factor

NgR.....Nogo receptor

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

NSAID.....non -steroidal anti-inflammatory drugs

Omgp.....oligodendrocyte myelin glycoprotein

$p75^{NTR}$.....p75 neurotrophin receptor

PH.....pleckstrin homology

PIP2.....phosphatidyl inositol biphosphate

PI-PLC.....phosphatidylinositol-specific phospholipase C

PKA.....protein kinase A

PKN.....protein kinase N

RGC.....retinal ganglion cells

RAG.....regeneration associated gene

Robo.....roundabout

SCI.....spinal cord injury

SNC.....système nerveux central

SNP.....système nerveux périférique

TBI.....Traumatic brain injury

TNF-α.....tumor necrosis factor-alpha

TNFR.....tumor necrosis factor receptor

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Chapitre 1

1 Introduction Générale

1.1 Introduction

Les Rho GTPases sont des régulateurs du cytosquelette qui régulent la polymérisation de l'actine et influencent la stabilité des microtubules. La Rho GTPase Rho a un rôle important dans les cellules nerveuses et est une molécule clé dans la réponse aux inhibiteurs de croissance présents dans le système nerveux. Rho joue aussi un rôle important dans la régulation de la mort cellulaire ainsi que dans l'apoptose suite aux traumatismes du SNC. Donc, il est important de comprendre le rôle clé de Rho suite à ces traumatismes.

1.2 Les Rho GTPases

Les Rho GTPases sont des protéines de 20 à 30 kDa de membres de la famille des protéines monomériques Ras, qui lient le GTP. Les Rho GTPases existent sous deux conformations, une forme active liée au GTP et une forme inactive liée au GDP. Il existe au moins 22 Rho GTPases différentes chez les mammifères, qui peuvent être classées en 8 sous-groupes : Rho (Rho A,B et C), Cdc42 (Cdc42, TC10, TCL, Chp, Wrch-1), Rac (Rac 1,2 et 3, RhoG), Rnd (Rnd1-Rho6, Rnd2-Rho7, Rnd3-RhoE), RhoD (RhoD et Rif), RhoH-TTF, RhoBTB (RhoBTB1 et 2) et Miro (Miro-1 et 2) (Burridge and Wennerberg, 2004; Wennerberg and Der, 2004). Les Rho GTPases affectent plusieurs aspects de la dynamique cellulaire : elles jouent un rôle dans la réorganisation du cytosquelette (Hall, 1998; Mackay and Hall, 1998; Kjoller and Hall, 1999; Etienne-Manneville and Hall, 2002), dans la régulation de la transcription, dans la progression du cycle cellulaire (Nobes and Hall, 1999; Aznar and Lacal, 2001; Benitah et al., 2004; Burridge and Wennerberg, 2004), dans la migration cellulaire (Mackay and Hall, 1998; Schmidt and Hall, 1998; Raftopoulou and Hall, 2004), ainsi que dans la migration et le guidage des

cônes de croissance des cellules nerveuses (Luo, 2002; Huber et al., 2003; Govek et al., 2005) et dans le remodelage et la régulation de la croissance axonale et dendritique (Kuhn et al., 2000; Luo, 2002; Govek et al., 2005). Les Rho GTPases les mieux caractérisées et étudiées sont Rho, Rac et Cdc42.

Chez les vertébrés, il existe 3 isoformes de la Rho GTPase Rho : RhoA, RhoB et RhoC. RhoA,B et C ont une homologie de séquence d'acides aminés de 85% (Burridge and Wennerberg, 2004; Wheeler and Ridley, 2004). Les trois isofomes ont des acides aminés conservés en N-terminale, Gly14, Phe30, Thr19, Gln63 qui sont importants pour la liaison et l'hydrolyse du GTP et pour la stabilisation des liaisons avec les régulateurs et effecteurs. Par contre, la séquence en C-terminale diffère entre RhoA, B et C et il y a des différences dans la région 'switch1' indiquant des différences de régulateurs et d'effecteurs. Malgré une similarité de fonctions (ils peuvent tous induire la contraction de l'actomyosine et induire la production de fibres de stress *in vitro*), les isoformes de Rho ont des effecteurs préférentiels, quelques fonctions différentes et une distribution tissulaire différente (Lehmann et al., 1999; Wheeler and Ridley, 2004).

1.2.1 Rho A

RhoA régule entre autre le cytosquelette d'actine, la locomotion et motilité cellulaire ainsi que la mort cellulaire. RhoA est l'isoforme la plus exprimée dans le SNC (Lehmann et al., 1999), et joue un rôle important dans le guidage axonal et dans la réponse des protéines inhibitrices de croissance (He and Koprivica, 2004; Govek et al., 2005). L'activation de Rho dans ces cellules inhibe la croissance axonale et dendritique

(Dergham et al., 2002; Winton et al., 2002). A date, aucun rôle pour RhoB et C n'a été rapporté dans les cellules nerveuses. De plus, RhoA et non RhoB et C peut être phosphorylé par la PKA, un effecteur clé de l'AMPc. Ceci indique que RhoA, mais pas B ou C, peut jouer un rôle important dans la régulation de la croissance et de la différenciation cellulaire dépendante du cAMP (Howe, 2004).

1.2.2 RhoB

RhoA et B ont le même domaine de liaison aux effecteurs ce qui indique que plusieurs de leurs effecteurs sont les mêmes et qu'ils ont potentiellement les mêmes effets. RhoB comme RhoA joue aussi un rôle dans la mort cellulaire par apoptose (Fritz and Kaina, 2001). Par contre, RhoB et non RhoA se trouve dans les endosomes ainsi que dans les membranes nucléaires (Laplante et al., 2001),. De plus, RhoB et non Rho A ou C, est modifiée post traductionellement en C-terminale par une farnesylation au C15 ou par une geranylgeranylation au C20 (Wheeler and Ridley, 2004), ce qui indique que RhoB exerce des fonctions intracellulaires distinctes de celles deRhoA. En dernier lieu, RhoB a un temps de demi-vie beaucoup plus court que RhoA et C (Wheeler and Ridley, 2004).

1.2.3 RhoC

Contrairement à RhoA et B, le rôle de RhoC est très peu connu. RhoA et RhoC sont très homologues : entre les deux molécules il y a juste six substitutions d'acides aminés tous en C-terminale (Wheeler and Ridley, 2004). RhoC induit la migration des cellules et des cellules métastasiques dans les mélanomes (Clark et al., 2000). De plus, son expression est liée au degré métastasique (mais pas à la transformation) des cellules. Dans les

neurones, les niveaux d'expression protéiques de RhoC sont beaucoup plus faibles que ceux de RhoA et B (Lehmann et al., 1999) indiquant que RhoC a un rôle mineur dans ces cellules.

1.3 Régulateurs des Rho GTPases

Les Rho GTPases sont régulées par trois familles de protéines distinctes : les GEFs, les GAPs et les GDIs. Les GEFs (Guanine nucleotide Exchange Factors) activent les Rho GTPases en induisant la liaison au GTP tandis que les GAPS et GDIs assurent l'inactivation des Rho GTPases. Les GAPs (GTPase Activating Protein) catalysent l'hydrolyse du GTP en GDP et les GDIs (Guanine nuleotide Dissociation Factor) s'associent à la forme GDP des RhoGTPases et régulent leur localisation intracellulaire en les séquestrant dans le cytosol (Figure 1). Les Rho GTPases sont synthétisées dans le cytosol et subissent par la suite des modifications post-traductionnelles en C-terminale. La modification post-traductionnelle en C-terminale de Rho, Rac et Cdc42 la plus commune est (Adamson et al., 1992; Wennerberg and Der, 2004) la géranylation par la geranyl-geranyl transférase qui catalyse l'ajout d'un géranyl-géranyl sur ces GTPases. Ceci est important, mais pas le seul déterminant, pour leur localisation intracellulaire. Les GTPases non modifiées ne peuvent pas être relocalisées à la membrane plasmique, un évènement nécessaire à leur activation par les régulateurs des Rho GTPases.

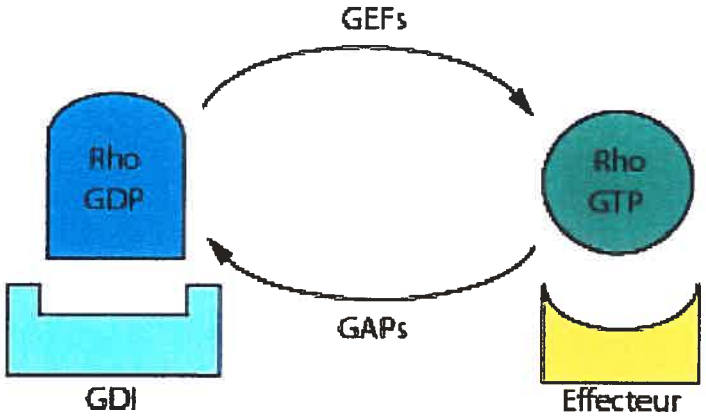

Figure 1 Régulateurs de Rho
Les RhoGTPases sont activées par les GEFs (GTP-Rho), inactivées par les GAPs (GDP-Rho) et sont séquestrées dans leur forme inactive par les GDIs. La forme active de Rho interagit par la suite avec ses effecteurs.

1.3.1 GEFs : Guanine nucleotide Exchange Factors

La fonction des GEFs est de catalyser la relâche du GDP et de promouvoir la liaison du GTP sur les GTPases. Ceci active la GTPase et permet alors son interaction avec ses effecteurs. Les GEFs se lient à la forme GDP, déstabilisant le complexe GDP-Rho, et catalysent l'échange du GDP pour le GTP (Scita et al., 2000; Rossman et al., 2005). Jusqu'à présent 69 GEFs ont été identifiés chez l'homme (Dvorsky and Ahmadian, 2004). Le premier GEF à être identifié était Dbl, un oncogène se trouvant dans les cellules B cancéreuses (Diffuse B cell lymphoma). Dbl a été identifié suite à des expériences qui visaient à transformer des cellules NIH3T3 (Eva and Aaronson, 1985). La région homologue de 200 résidus communément reconnue comme le domaine DH (Dbl homology) se trouve dans tous les GEFs et il est préssumé que toutes les protéines qui contiennent un domaine DH agissent comme un GEF pour les GTPases de la famille

Rho (Cerione and Zheng, 1996). Ce domaine DH est le site de la catalyse de l'échange GDP-GTP. La majorité des Rho GEFs contiennent aussi un domaine PH (environ 100 acides aminés) (plextrin homology) qui est adjacent au domaine DH, en C-terminale (Zheng et al., 1996; Michiels et al., 1997; Rossman et al., 2005; Rossman and Sondek, 2005). Le domaine PH régule l'activité du domaine DH, et peut aussi médier la localisation intracellulaire des GEFs, plus spécifiquement aux membranes et aux structures du cytosquelette.

Les domaines DH sont formés entre autre d'hélices alpha et ont 3 régions conservées CR1-CR3 (pour conserved regions 1-3). CR1 et CR3 ainsi que des résidus dans l'hélice alpha 6 de la partie C-terminale forme la surface majoritaire de liaison avec les GTPases (Aghazadeh et al., 1998). Le domaine DH interagit avec la région 'switch' des GTPases ce qui cause la dissociation du GDP et permet la liaison du GTP. CR1 et 3 interagissent avec la région 'switch 1' des GTPases, et la région 'switch 2' des RhoGTPases interagit avec CR3 et l'hélice alpha 6 de la partie C-terminale (Vetter and Wittinghofer, 2001; Rossman et al., 2005; Rossman and Sondek, 2005). Les GEFs ont une sélectivité variée pour les différents membres des RhoGTPases. La production d'interface de résidus non-conservés par la liaison du domaine DH du GEF à une RhoGTPase spécifique peut être déterminant dans la sélectivité du GEF aux différentes Rho GTPases (Cheng et al., 2002; Rossman et al., 2002).

Le domaine PH des GEFs se trouve dans la région C-terminale adjacsent au domaine DH et les domaines PH-DH ensemble sont plus efficaces pour induire l'échange de

nucléotides que le domaine DH seul (Liu et al., 1998; Rossman et al., 2003). La position spatiale du domaine PH par rapport au domaine DH est différente chez certains GEFs. Le domaine PH est important, puisque des mutations ou l'ablation des domaines PH dans des Rho GEFs peut abolir l'activité GEF de ces protéines (Whitehead et al., 1995a; Whitehead et al., 1995b; Zheng et al., 1996; Olson et al., 1997).

Les GEFs contiennent aussi plusieurs autres motifs tel que des domaines SH2, SH3 PDZ indiquant que ces protéines peuvent interagir avec plusieurs autres protéines possiblement pour réguler leur propre activité (Schmidt and Hall, 2002; Rossman et al., 2005). Il est possible que les GEFs aient d'autres fonctions cellulaires non identifiées tel que suggéré par la présence de ces autres domaines qui rendent possible d'autres interactions protéines-protéines (Schmidt and Hall, 2002; Rossman and Sondek, 2005).

Il est intéressant de noter que le nombre de GEFs identifiés jusqu'à ce jour soit d'environ 4 fois plus élevé que le nombre de RhoGTPases. La multiplicité des GEFs pourrait s'expliquer par une localisation tissulaire spécifique de ces derniers. (Katzav et al., 1989). Une seconde possibilité est que plusieurs récepteurs utilisent différents GEFs pour activer une même GTPase. Par exemple, plusieurs voies de signalisation activent RhoA par l'interaction de différents récepteurs avec la GTPase (ex . $p75^{NTR}$, TNFR1, Par1, intégrines, etc) (Yamashita et al., 1999; He and Koprivica, 2004). L'activation de RhoA par ces récepteurs cause des effets distincts. Il est donc concevable que chacun de ces récepteurs utilise des GEFs différents pour activer la même GTPase. La multiplicité de GEFs peut aussi causer l'activation de différentes Rho GTPases (l'activation de

différentes voies de signalisation et l'interaction avec différents effecteurs) induite par un même récepteur. Par exemple $p75^{NTR}$ peut interagir avec RhoA, Rac et Cdc42 suite à différentes stimulations. Donc non seulement différents récepteurs peuvent interagir avec différents GEFs pour activer la même GTPase, mais un récepteur peut associer différents GEFs pour stimuler l'activation de plus d'une Rho GTPase. Il reste que le rôle des GEFs est très important dans les cellules puisque des souris Knock out pour certaines de ces protéines ont plusieurs anomalies et pathologies. Par exemple, les souris 'knock out' Vav 1 (Vav1 -/-) montrent un mauvais développement anormal des cellules T et ont de sévères déficiences immunitaires (Fischer et al., 1995; Tarakhovsky et al., 1995). L'ablation du GEF Trio est létale (dû au développement anormal des tissus musculaires et nerveux) (O'Brien et al., 2000). Indépendamment de leurs fonctions, les GEFs sont une famille de protéines régulatrices essentielles.

1.3.2 GAPs : GTPase Activating Protein

Lorsque le GTP est hydrolysé en GDP, les protéines Rho sont inactivées. Bien que les GTPases aient une capacité intrinsèque d'hydrolyser le GTP en GDP, cette activité est très faible. Les GAPs catalysent l'hydrolyse du GTP en GDP par les GTPases, en augmentant la fréquence de conversion de GTP en GDP d'un facteur de 10^5. La famille de Rho GAPs compte environ 60 membres (Hall, 1990; Lamarche and Hall, 1994; Bernards, 2003; Moon and Zheng, 2003). Ces derniers sont caractérisés par un domaine conservé GAP d'environ 140-150 acides aminés mais les GAPs ont seulement 20% d'identité de séquence entre eux et la présence de ce domaine est suffisant catalyser l'hydrolyse du GTP (Moon and Zheng, 2003). P50RhoGAP, le premier RhoGAP

identifié, a été isolé du pancréas chez l'homme ainsi que des glandes adrénergiques bovines et possède une activité GAP pour Rho, Rac et Cdc 42 (Lancaster et al., 1994).

Comme pour les GEFs, les GAPs peuvent interagir avec plus d'une des GTPases de la famille Rho. Il se peut que la localisation intracellulaire des GAPs influence leur interaction avec une Rho GTPase spécifique. Il a été démontré que les modifications post-traductionnelles des Rho GTPases affectent leurs interactions avec les GAPs. Par exemple, les GAPs associées à la membrane plasmique accélèrent l'hydrolyse du GTP de Rac et Rho prénylés mais sont inactives sur les GTPases non modifiées. De plus, Rac prénylé interagit mieux avec les GAPs p50RhoGAP et p190RhoGAP pleine longueur « full lenght » (Molnar et al., 2001).

Plusieurs GAPs ont d'autres domaines que le domaine GAP (ex. SH2 et 3, CR, PH), qui pourraient affecter leur régulation, leur spécificité d'interaction et indique qu'ils pourraient avoir d'autres fonctions cellulaires (Moon and Zheng, 2003). Ensemble ces résultats indiquent qu'en plus d'être d'importants régulateurs de l'état d'activation des Rho GTPases, les GAPs pourraient aussi avoir d'autres fonctions intracellulaires.

1.3.3 GDIs : Guanine nuleotide Dissociation Factor

Les GDIs sont des protéines cytoplasmiques qui s'associent principalement à la forme inactive (GDP) des Rho GTPases (Fukumoto et al., 1990; Ueda et al., 1990). Les GDIs ont une très faible affinité pour la forme active (GTP) des Rho GTPases, mais l'association du GDI à RhoGTP stimule l'activité GTPase de Rho. Les GDIs (1) bloquent

l'activation par les GEFs, (2) inhibent l'activité GTPase intrinsèque de Rho, (3) bloquent l'échange GDP-GTP. Les GDIs contiennent un domaine régulateur dans la région N-terminale et un domaine 'immunoglobuline like' dans la région C-terminale (Nomanbhoy and Cerione, 1996; Hoffman et al., 2000). Le domaine en N-terminale se lie aux régions 'switch' I et II des GTPases, ce qui inhibe l'hydrolyse du GTP et la dissociation du GPD (donc qui interfère avec l'interaction de Rho avec les GEFs et GAPs) (Gosser et al., 1997; Keep et al., 1997). Le domaine 'Ig like' en C-terminale forme une enclave hydrophobe qui se lie avec une forte affinité à la région isoprénylée de Rho (Hoffman et al., 2000). Cette liaison peut extraire les Rho GTPases des membranes cellulaires et induit la formation du complexe Rho-GDI dans le cytosol.

Les GTPases se retrouvent majoritairement sous la forme inactive (GDP) dans le cytosol et sont activées aux membranes cellulaires : au repos, les RhoGTPases se retrouvent en complexe avec les GDI dans le cytosol et sont transloquées à la membrane suite à une stimulation. Les GDIs ont un rôle dans la régulation de la localisation intracellulaire des GTPases (Sasaki and Takai, 1998; Olofsson, 1999) (Dransart et al., 2005) (Sun and Barbieri, 2004).

Une autre fonction importante des GDIs est de décrocher les RhoGTPases des membranes (Nomanbhoy et al., 1999; Olofsson, 1999; Dvorsky and Ahmadian, 2004) et de les séquestrer dans le cytosol dans leurs formes inactives. Il a récemment été montré que les protéines FAS, RhoA, Ezrin, Moesin ainsi que RhoGDI sont recrutées aux radeaux lipidiques lors de la signalisation pro-aopotitique induite par FAS (Gajate and

Mollinedo, 2005). FAS, RhoA et Rho GDI semblent former un complexe de signalisation et ont la même localisation membranaire. Ceci pourrait appuyer le rôle de GDI à extraire Rho des membranes lipides pour réguler son activité en formant le complexe cytosolique Rho-GDI. L'extraction lipidique peut alors réguler la proportion de Rho active et inactive. De plus, les GDIs peuvent interagir avec des protéines membranaires telles que ERM, $p75^{NTR}$ et EKT. Ces interactions inhibent l'activité du GDI et engendrent aussi la dissociation du complexe Rho GTPases avec le GDI (Hirao et al., 1996; Takahashi et al., 1997) Yamashita and Tohyama, 2003) (Kim et al., 2000) (Faure et al., 1999) lors d'une stimulation des voies de signalisation engendré par ces récepteurs.

De plus, les GDIs sont régulés par leur état de phosphorylation. La phosphorylation de Rho par PKA ainsi que la phosphorylation de Cdc 42 accroit la liaison de ces GTPases au GDI (Lang et al., 1996; Forget et al., 2002) et la phosphorylation du GDI par PKCα induit l'activation de Rho (Mehta et al., 2001). L'état de phosphorylation du GDI peut aussi induire la dissociation du complexe GTPase-GDI : l'alkaline phosphatase induit la dissociation du complexe Rho-GDI dans les neutrophiles (Bourmeyster and Vignais, 1996). PAK, un effecteur de Rac et Cdc42, phosphoryle RhoGDI qui cause la dissociation du complexe Rac-GDI. Ce mécanisme semble être spécifique à Rac puisque PAK ne stimule pas la dissociation du GDI avec RhoA (DerMardirossian et al., 2004).

1.4 Les effecteurs de Rho

Les Rho GTPases existent soit sous la conformation active liée au GTP ou sous la conformation inactive liée au GDP. La différence structurale entre ces conformations est

due aux régions 'switch 1 et 2' qui sont adjacentes au site de liaison des nucléotides (Hakoshima et al., 2003; Dvorsky and Ahmadian, 2004). La structure de ces régions change en fonction du nucléotide lié (GTP ou GDP) et ces régions sont importantes dans la régulation de la liaison avec les protéines effectrices. Les changements conformationels produisent une structure en forme de poche qui permet l'interaction avec les effecteurs (Hakoshima et al., 2003; Dvorsky and Ahmadian, 2004).

1.4.1 Régulation de l'actine

Les changements morphologiques ainsi que l'adhésion et la motilité cellulaire sont dépendants du cytosquelette d'actine. Rho régule le cytosquelette d'actine par l'activation de ses effecteurs. De façon plus spécifique l'activation des effecteurs de Rho cause 1. la formation de fibres de stress 2. la formation de points d'adhésion focaux (focal adhésions) auxquels sont ancrés les fibres de stress 3. la contractilité de l'actine par la myosine 4. l'inhibition de la dépolymérisation de l'actine 5. la polymérisation de monomères d'actine.

Deux effecteurs de Rho sont responsables de la formation des fibres de stress : ROCK (Rho associated kinase, Rho kinase ou p160ROCK) et mDia (member of the formin homology family). L'activation de ROCK est nécessaire mais pas suffisante pour la formation de fibres de stress (Schmitz et al., 2000) dans les fibroblastes. ROCK, est une sérine-thréonine kinase (Matsui et al., 1996) qui se lie à la forme active de Rho et induit la formation de fibres de stress et de point d'adhésion (Leung et al., 1995; Leung et al., 1996; Amano et al., 1997). La phosphorylation de MLC (myosin light chain) (Amano et

al., 1996a) et de MLCP (myosin light chain phosphatase) (Kimura et al., 1996) par ROCK régule la contraction de la myosine et entraine la formation de fibres de stress. La phosphorylation par ROCK active MLC et inhibe MCLP. La phosphorylation de MLC stimule alors l'interaction entre la myosine II et les filaments d'actine ainsi que l'activité ATPase de la myosine, ce qui cause la contraction des fibres et la formation de fibres de stress. De plus, citron kinase et PKN, d'autres effecteurs de Rho, peuvent aussi stimuler la phosphorylation de MLC et l'activation de la myosine (Madaule et al., 1998; Takai et al., 2001; Mukai, 2003). Rho régule donc la contraction de l'actine par la phosphorylation de MLC et MLCP.

ROCK induit aussi la formation de fibres de stress par kinase LIM (LIMK) qui inhibe la dépolymérisation de l'actine. Après son activation par Rho, ROCK phosphoryle et active aussi la LIMK (Maekawa et al., 1999) qui phosphoryle et inactive cofiline. La cofiline (active) se lie et dépolymérise les filaments d'actine (Bamburg, 1999; dos Remedios et al., 2003). Son inactivation (ici par la phosphorylation par LIMK) induit donc la stabilisation de l'actine. Il existe aussi d'autres substrats de ROCK qui contribuent à l'assemblage de l'actine tel que l'adducine (qui une fois phosphorylée par ROCK renforce la liaison à la l'actine) (Kimura et al., 1998), et les protéines ERM ((ezrin-radixin-moesin) qui lient l'actine à la membrane).

Un autre effecteur de Rho, p140 mDia induit aussi la formation de filaments d'actine (Watanabe et al., 1997; Watanabe et al., 1999). L'expression de formes actives de mDia produit de minces fibres de stress et mDia stimule la polymerisation de l'actine (Li and

Higgs, 2003). mDia (Wasserman, 1998) lie la profilin (protéine liant l'actine) (Frazier and Field, 1997)et contribue à la polymérisation de l'actine et à l'organisation de l'actine en fibres de stress (Watanabe et al., 1997; Burridge and Wennerberg, 2004).

Les phosphoinositides ont aussi un rôle dans la signalisation de Rho. Rho peut stimuler la synthèse de PIP2 (phosphatidyl inositol bisphosphate ou PI4,5P_2 (phosphatidyl inositol 4,5-bisphosphate) (Chong et al., 1994; Ren et al., 1996) qui peuvent interagir avec des protéines qui lient directement l'actine tels que profilin, vinculin et gelsolin et talin (dos Remedios et al., 2003). L'extrémité où s'ajoutent les monomères d'actine (polymérisation), est masquée par des protéines qui recouvrent cette extrémité (caping protiens). Les phosphoinositides provoquent la dissociation des 'caping proteins', telle que la gelsoline, ce qui favorise la polymérisation de l'actine. De plus, l'inhibition de PIP2 empêche la formation de fibres de stress et de points d'adhésion suite à l'activation de Rho (Gilmore and Burridge, 1996). Ces résultats indiquent que Rho induit la polymérisation de l'actine, par ses effecteurs ROCK, mDIA et PIP2 par leurs effets sur les protéines qui lient et cappent l'actine. Rho a aussi d'autres effecteurs tel que Rhophilin et Rotekin, par contre leurs foncions restent encore inconnues (Reid et al., 1996; Takai et al., 2001). Par contre, le domaine de liaison de Rotekin à Rho est encore à ce jour le meilleur outil pour étudier les états d'activation de Rho (voir outils pour étudier Rho) (Reid et al., 1996).

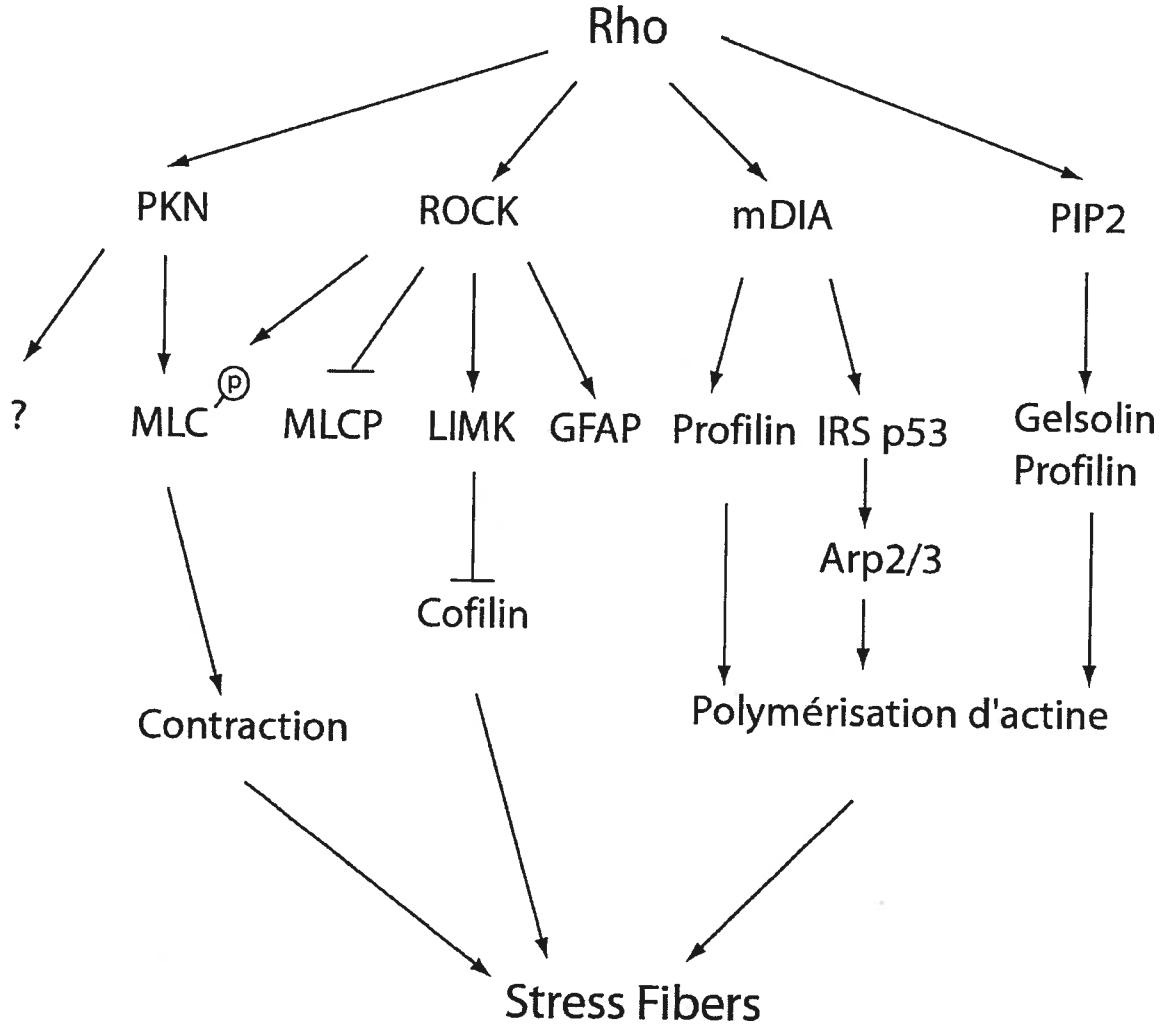

Figure 2 Régulation de l'actine par Rho
Principales voies de signalisation engendrée par Rho et ses effecteurs lors de la formation de filaments d'actine

1.4.2 Régulation de microtubules par les Rho GTPases

Les microtubules ont un rôle important dans la motilité et la migration cellulaire ainsi que dans la polarisation des cellules lors de ces phénomènes (Wittmann and Waterman-Storer, 2001; Rodriguez et al., 2003; Watanabe et al., 2005). Les microtubules sont aussi impliqués dans la croissance et le guidage axonal (Bamburg et al., 1986) (Dent and

Gertler, 2003; Dehmelt and Halpain, 2004; Gordon-Weeks, 2004; Andersen, 2005; Kornack and Giger, 2005). La dépolymérisation des microtubules induit la formation de filaments contractiles d'actine ainsi que l'augmentation des niveaux de RhoA dans les fibroblastes. De plus, la polymérisation des microtubules induit l'activation de Rac (Waterman-Storer and Salmon, 1999; Waterman-Storer et al., 1999). Ces résultats indiquent que les Rho GTPases peuvent aussi exercer un effet sur les microtubules en plus de leurs effets sur l'actine.

Les microtubules sont des structures dynamiques et Rho a un rôle dans leur régulation. Rho a un rôle dans la polymérisation et dans la dépolymérisation de ces structures par l'activation de voies de signalisation distinctes. L'activation de Rho peut induire la formation et stabilisation des microtubules par son interaction avec son effecteur mDia et le GEF-H1 (Cook et al., 1998; Palazzo et al., 2001). Il a récemment été montré que cet effet est dû à la liaison de mDia avec les protéines EB1 et APC (Wen et al., 2004). Ces protéines, des 'Tip proteins', qui font partie de la superfamille des MAP (protéines associées aux microtubules; MAPs microtubule associated proteins), s'associent à l'extrémité en croissance (plus end) et stabilisent les microtubules (Kornack and Giger, 2005; Watanabe et al., 2005). De plus, la forme active de l'effecteur Dia induit aussi l'alignement parallèle des microtubules avec les filaments d'actine sur l'axe longitudinal de la cellule (Palazzo et al., 2001; Palazzo and Gundersen, 2002).

Rho peut aussi induire la déstabilisation des microtubules. Rho active peut entraîner la phosphorylation des MAPs Tau et Map2 (Sayas et al., 1999) (Amano et al., 2003). Ces

protéines ont comme rôle de stabiliser les microtubules et sont régulées par leur état de phosphorylation. Lorsqu'elles ne sont pas phosphorylées, ces protéines s'associent aux microtubules et les stabilisent; lorsqu'elle sont phosphorylées, elles se dissocient et causent la déstabilisation des microtubules. ROCK, un effecteur de Rho, peut directement phosphoryler Tau et MAP2 (Amano et al., 2003). La phosphorylation de Tau par ROCK entraine une réduction du taux d'assemblage des MT en plus d'induire la dissociation de Tau des microtubules. De plus, il a été montré que l'activation de Rho par le GEF p115RhoGEF induit la déstabilisation et le désassemblage des microtubules (Birukova et al., 2004b; Birukova et al., 2004a) par l'activation de ROCK qui phosphoryle tau. L'inactivation de Rho ou l'inhibition de ROCK atténue ces effets. Ces résultats indiquent que l'activation de Rho peut aussi causer la déstabilisation des microtubules par la phosphorylation des MAPs. À la suite d'expériences montrant la présence de GEFs masqués ou inactivés dans les MT stables (Ren et al., 1998; Glaven et al., 1999; van Horck et al., 2001), il a aussi été proposé que l'activation de Rho puisse causer la déstabilisation des MT par un mécanisme de feedback : l'activation initiale de Rho cause la phosphorylation des MAPs ce qui induit la déstabilisation des MT. Une fois les MT déstabilisés, les GEFs associées aux MT seraient relâchés et pourraient aller activer Rho ce qui aurait comme effet d'augmenter le désassemblage des microtubules (Enomoto, 1996; Wittmann and Waterman-Storer, 2001; Watanabe et al., 2005).

Il semblerait donc que Rho puisse exercer différents effets (voir stabilisation ou désassemblage) sur les microtubules par l'activation de voies de signalisation distinctes, mDia et ROCK respectivement. Ces effets distincts pourraient aussi être dus à la

localisation cellulaire des effecteurs de Rho. A cet effet il a été montré que FAK (focal adhésion kinase) mDia et Rho sont requis pour la formation de MT stables au 'leading edge' de la cellule (Palazzo et al., 2004; Ezratty et al., 2005). Donc, il se pourrait que Rho (cascade mDia) induise la formation de MT au 'leading edge' et que l'activation de Rho à l'arrière de la cellule (rear) induise la déstabilisation/désassemblage des MT par ROCK causant la rétraction de l'arrière de la cellule (Watanabe et al., 2005).

Rac peut aussi affecter les microtubules mais contrairement à Rho, Rac induit la stabilisation et la croissance de ces structures par ses effets sur la phosphorylation des protéines stathmin/Op18 (Daub et al., 2001; Wittmann et al., 2004). Stathmin/Op18 dépolymérise les microtubules et la phosphorylation de ces protéines les inactive. Leur phosphorylation et subséquente inactivation par Rac rendent possible la croissance des microtubules. De plus, Rac interagit avec les 'Tip proteins' APC et CLIP-170, ce qui induit la stabilisation et croissance des MT (Fukata et al., 2002; Watanabe et al., 2004). Rac cause donc la polymérisation des MT par ses effets sur l'assemblage de ceux-ci et par l'inhibition de leur dépolymérisation (Kornack and Giger, 2005; Watanabe et al., 2005). Indépendamment de leurs effets (polymérisation vs déstabilisation) sur les MT, ces résultats indiquent que Rho et Rac jouent un rôle important dans la régulation des microtubules.

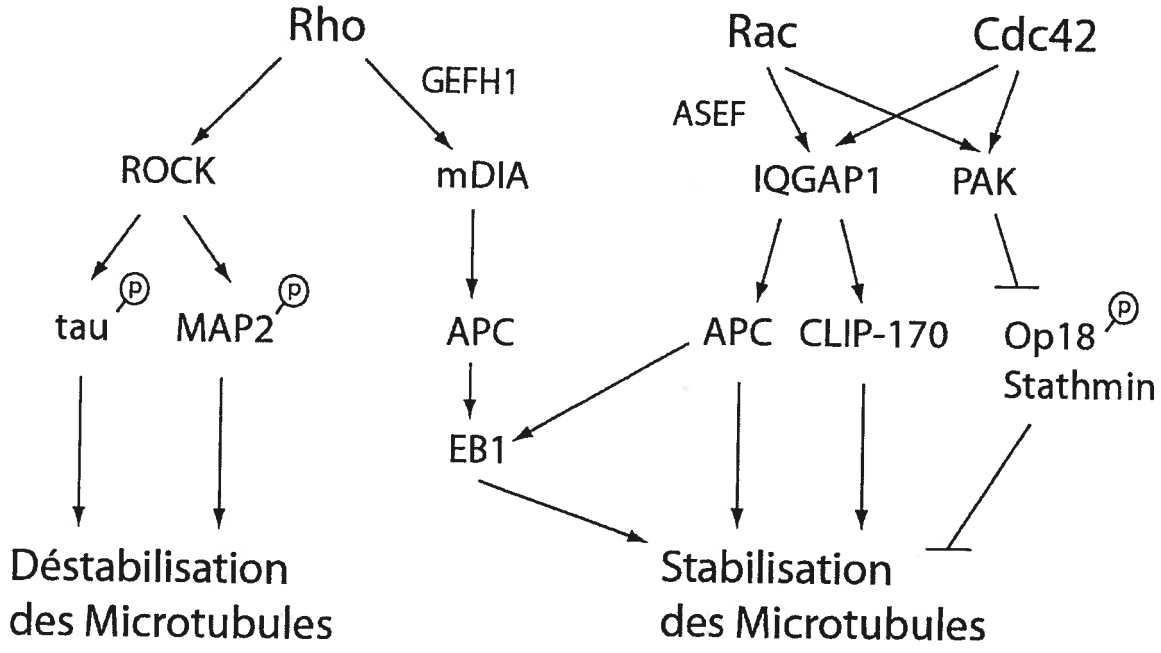

Figure 3 Régulation des microtubules par Rho
Principales voies de signalisation engendrée par Rho et ses effecteurs lors de la formation et stabilisation des microtubules

1.5 Outils pour l'étude des états d'activation de Rho

1.5.1 essai pull down

A cause du manque d'anticorps spécifiques aux différentes conformations de Rho, il est impossible de les distinguer par les techniques biochimiques normalement utilisées. La technique d'essai 'pull down' a été développée (Reid et al., 1996) et adaptée (Ren and Schwartz, 2000) pour isoler la forme active GTP de Rho. Pour ce faire, des protéines recombinantes composées du domaine de liaison spécifique pour RhoA couplé à un domaine GST, sont utilisées. La séquence de liaison provient de rhotekin, une des protéines effectrices de RhoA. Le RBD (Rho Binding Domain) est composé des 90

acides aminés en N-terminale et est spécifique à la forme active GTP de Rho. La construction, une fois couplée à des billes de glutathion, est incubée avec un lysat cellulaire ou un homogénat de tissu. Seule la forme active de Rho est retenue sur les billes. La visualisation de Rho se fait avec un anticorps contre RhoA à la suite d'un western blot.

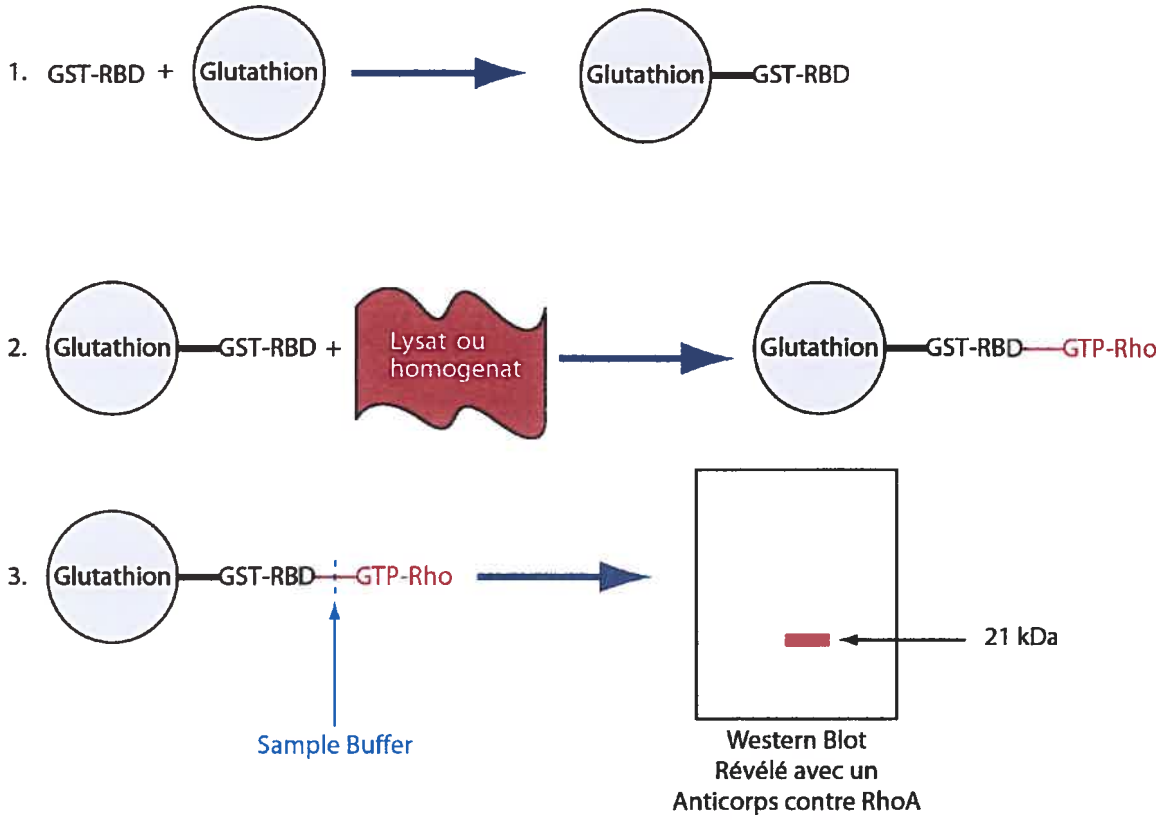

Figure 4 Shématisation de l'essai pull down

1.5.2 Toxine bactérienne : la C3 transférase

La C3 est une exoenzyme d'environ 25kDa produite par la bactérie *Clostridium Botulinum*. La C3 inactive les isoformes de Rho RhoA, B et C mais pas Rac ou Cdc42.

La C3 transfert un ADP-ribose provenant du NAD au résidu Asn41 de Rho, adjacent à la région switch, ce qui empêche l'activation de Rho par les GEFs (Aktories and Hall, 1989; Aktories, 1997; Aktories et al., 2000).

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

Cette modification bloque la signalisation par Rho mais n'affecte ni l'intéraction de Rho avec ses effecteurs, ni la liaison des GEFs. Par contre l'activation de Rho par les GEF est inhibée. La capacité de Rho à interagir avec les récepteurs membranaires est compromise par la C3. Par contre, la C3 ne traverse pas facilement les membranes cellulaires. Il faut donc utiliser de fortes concentrations, qui peuvent être toxiques ou employer des méthodes qui endommagent les cellules (ex. microinjection et 'scrape loading') pour inactiver Rho. Récemment Winton et al., ont produit une forme modifiée de la C3 qui traverse facilement la membrane cellulaire, C3-05, qui inactive Rho efficacement à de faibles concentrations (ng/ml) dans des cellules en culture (Winton et al., 2002). La C3-05 contient la même séquence de la C3 qui code pour son activité enzymatique en plus d'une séqence de transport qui facilite son entrée dans les cellules. Cette forme de C3 est donc un très bon outil pour étudier l'activation de Rho et la signalisation qui en résulte. De plus, la C3-05 peut aussi inactiver Rho dans les cellules *in vivo*.

1.6 Rho et la mort cellulaire apoptotique

1.6.1 L'apoptose

L'apoptose, ou mort cellulaire programmée, est un phénomène contrôlé caractérisé par l'activation de protéases, la fragmentation de l'ADN, la condensation de la chromatine et le 'membrane blebbing'(Kerr et al., 1972; Wyllie et al., 1980). Les caspases, qui sont des

cystéines protéases, sont les protéines effectrices dans les cascades apoptotiques. La famille des caspases peut être subdivisée en deux groupes : les caspases initiatrices (caspases 1, 2, 4, 5, 8, 9, 10, 11, 12) qui activent les caspases effectrices (caspases 3, 6, 7, 14) (Degterev et al., 2003; Yuan et al., 2003a). Ces dernières clivent des protéines intracellulaires, inactivent certaines protéines requises pour la survie, activent des protéines responsables du 'blebbing' membranaire et clivent l'ADN (Yuan and Yankner, 2000; Benn and Woolf, 2004). Chez les mammifères, il y a 2 voies apoptotiques distinctes qui activent les caspases (Nagata, 1997; Ashkenazi and Dixit, 1998; Desagher and Martinou, 2000; Wang, 2001) : la voie extrinsèque induite par les récepteurs ('death receptors' ex. TNFR et FAS) qui active la caspase 8 et la voie JNK et la voie intrinsèque induite par le stress cellulaire (ex. ROS, UV, perte de support trophique) qui entraine des dommages aux mitochondries et la libération du cytochrome c ainsi que l'activation de la caspase 9 (Strasser et al., 2000; Yuan and Yankner, 2000; Yuan et al., 2003a; Benn and Woolf, 2004).

Les protéines de la famille Bcl-2 sont aussi des régulateurs importants de l'apoptose dans les neurones. La distribution cellulaire entre ces protéines sont déterminants dans l'induction de la mort cellulaire (Merry and Korsmeyer, 1997; Gross et al., 1999). Les membres de la famille des protéines Bcl-2 peuvent inhiber (Bcl-2, Bcl-xl) ou induire (Bax, Bak) l'apoptose. Bcl-2 peut inhiber Bax (et la relâche du cytochrome c et l'activation des caspases par BAX) ce qui favorise la survie des cellules. La surexpression de Bcl-2 est aussi protectrice. Il y aussi des protéines Bcl-2, Bim, Bid, BAD, qui forment une sous catégorie de protéines pro-apoptotiques. Ces protéines

séquestrent Bcl-2 et suppriment son intéraction avec Bax. Ceci entraîne le changement de la distribution de Bax (augmentation aux membranes des mitochondries) ce qui stimule alors l'apoptose. De plus, il a récemment été montré que Bcl-2 peut aussi réguler une voie apoptotique indépendante du cytochrome c et de la caspase 9 (Marsden et al., 2002), ce qui indique que cette protéine peut réguler l'apoptose par différentes voies de signalisation. Les familles des caspases et des protéines Bcl-2 sont donc d'importants régulateurs de l'apoptose (Strasser et al., 2000; Yuan and Yankner, 2000; Yuan et al., 2003a). Rho peut influencer la mort cellulaire par l'activation de ces voies de signalisation.

1.6.2 Rho et l'apoptose

Il y a de plus en plus de preuve que Rho joue un rôle important dans l'apoptose (Aznar and Lacal, 2001; Coleman and Olson, 2002; Benitah et al., 2004). En effet la signalisation par Rho régule la mort cellulaire dans plusieurs types cellulaires. Dans certains cas l'activation de Rho engendre la mort apoptotique et dans d'autres cas l'activation de Rho protège contre l'apoptose. Cependant la régulation de l'apoptose par Rho peut parfois varier en fonction du type cellulaire. De plus, le stade développemental ou l'état pathologique peuvent parfois aussi être important dans la régulation de l'apoptose par Rho. Il a récemment été montré que contrairement aux neurones adultes dans la moelle épinière, la forme active de Rho promouvoit la survie de neurones moteurs en développement (Kobayashi et al., 2004). Dans ces expériences, l'expression de mutants dominants négatifs (forme inactive) de Rho et ROCK dans des motoneurones en développement (stages E9- E12.5) entraine l'apoptose de ces cellules (Kobayashi et al.,

2004). Ces résultats suggèrent que le stade de développement peut être un déterminant dans la régulation de l'apoptose neuronale par Rho.

La régulation de l'apoptose par Rho peut est médiée par ses effets sut l'actine et sur l'activation de la transcription (Coleman and Olson, 2002; Benitah et al., 2004). La production de 'membrane blebbs', caractéristiques de l'apoptose est induite par Rho et ROCK (par la force de contraction par MLC) (Coleman and Olson, 2002; Lai et al., 2003; Sebbagh et al., 2005). La contraction de la myosine par ROCK peut en plus causer la désintégration nucléaire dans les cellules apoptotiques (Croft et al., 2005). Ensemble, ces résultats montrent que la voie de signalisation de Rho (ici Rho, ROCKI et ROCKII) participe aussi à la mort cellulaire par apoptose en induisant la formation de 'blebbs' membranaires et la désintégration nucléaire. Rho par ses effets sur la régulation du cytosquelette d'actine, peut donc induire l'apoptose. Par contre, l'apoptose induite par Rho n'est pas exclusivement due à ses effets sur le cytosquelette. A cet effet, il a été montré qu'une diminution des quantités de filaments d'actine contribue très faiblement à l'induction de d'apoptose. Rho peut aussi induire l'activation de voies apototiques telles que celles des caspases et des protéines Bcl-2 (Esteve et al., 1998). De plus, Rho peut activer certains facteurs de transcriptions qui sont aussi impliqués dans la mort cellulaire tel que c-jun et NFκβ (Aznar and Lacal, 2001; Benitah et al., 2004).

1.6.2.1 Rho active régule la mort cellulaire apoptotique

Il y a plusieurs études *in vitro* et *in vivo* qui démontrent que l'activation de Rho induit la mort cellulaire par apoptose. Dans des fibroblastes NIH 3T3 en culture, la surexpression

de la forme active de Rho suite au retrait de sérum du milieu de culture, cause la mort de ces cellules (Jimenez et al., 1995). Dans ces mêmes cellules, la surexpression de Rho augmente les niveaux de céramides à des niveaux qui peuvent induire l'apoptose et cet effet est dépendant de la modulation (à la baisse) des niveaux de Bcl-2 par Rho (Esteve et al., 1995; Esteve et al., 1998). De plus, l'apoptose dans des cellules CHO (cellules fibroblastes), induite par FAS ou par des CTL (cytotoxic T lymphocytes qui tuent leurs cellules cibles ici les cellules CHO) est médiée par l'activation de Rho et ROCK (Subauste et al., 2000). Dans des cellules endothéliales (en présence du TNF), l'inactivation de Rho ou ROCK cause une réduction des niveaux de caspases 3,7 et 8 clivées indiquant que l'activation de Rho dans ces cellules entraine l'apoptose par l'activation de caspases (Petrache et al., 2003). De plus, dans des cellules PC-12, un type cellulaire neuronal, l'activation de Rho produit des 'blebbs'membranaires, qui sont des structures caractéristiques de la mort par apoptose (Mills et al., 1998). La thrombine, une sérine protéase, induit aussi l'apoptose par GTP-Rho, dans des neurones de l'hippocampe et dans des astrocytes en culture. En effet l'inactivation de Rho (par la C3) réduit l'apoptose de 50% dans ces deux types cellulaires (Donovan et al., 1997).

In vivo, l'inactivation de Rho (avec une C3 modifiée qui peut traverser les membranes cellulaires) suite aux lésions de la moelle épinière réduit le nombre de cellules apoptotiques d'approximativement 50% au site de lésion chez la souris et chez le rat (Dubreuil et al., 2003). GTP-Rho joue aussi un rôle dans l'apoptose des neurones suite à une ischémie cérébrale (ischemic stroke). Avec la technique de DNA array, Trapp et al ont montré que les niveaux de RhoB sont augmentés en réponse à une ischémie cérébrale

(Trapp et al., 2001). RhoB peut influencer la mort neuronale dans le modèle d'ischémie produite par MCA (middle cerebral artery occlusion). De plus, Lauf et al ont montré que l'inactivation de Rho (avec la C3) réduit le volume de la lésion dans le modèle MCA d'attaque ischémique cérébrale ce qui démontre un rôle de Rho active dans la mort neuronale suite à une ischémie cérébrale (Laufs et al., 2000). Qui plus est, Rho et ROCK sont régulées à la hausse suite à une ischémie cardiaque, et l'inactivation de ROCK réduit le volume de la lésion cardiaque ainsi que le nombre de cellules apoptotiques et améliore la fonction cardiaque (Bao et al., 2004). Ensemble, ces résultats indiquent un rôle important pour la forme active de Rho dans la régulation de l'apoptose.

Certaines expériences indiquent qu'il faut un stimulus apoptotique pour que GTP-Rho induise la mort des cellules. La surexpression de Rho n'induit l'apoptose que suite aux retrait du sérum du milieu de culture des cellules 3T3 (Jimenez et al., 1995). A cet effet il a aussi été démontré que la forme active de Rho n'active l'apoptose que suite à une stimulation par FAS (qui active aussi Rho) (Subauste et al., 2000). De plus, Winton et al., (2004, manuscrit en révision) montrent que la simple activation de Rho (ici induit par la myéline) n'est pas suffusante pour unduire la mort de ces cellules. Ce n'est que suite à la stimulation par le TNF en présence de la myéline que l'activation de Rho induit l'apoptose des neurones. Dans ces expériences, l'inactivation de Rho (par la C3) cause la survie des ces cellules. En outre, dans tous ces cas, GTP-Rho ne cause la mort cellulaire que lors d'un stress cellulaire ou d'un stimulus apoptotique. Ceci peut indiquer qu'en conditions 'normales' la forme active de Rho serait plutôt protectrice tandis qu'en conditions défavorables son activation induirait la mort des cellules.

Peu importe son état d'activation, Rho joue donc un rôle important dans la régulation de l'apoptose. Ensemble ces résultats indiquent que l'etat d'activation de Rho associée à l'apoptose peut varier en fonction du type cellulaire. Par contre, les formes GDP et GTP peuvent induire la mort dans un même type de cellule. Un exemple de ceci est le rôle de Rho dans les neurones de la moelle épinière. Dans les motoneurones de la moelle en développement, GTP Rho induit la survie de ces cellules (Kobayashi et al., 2004) tandis que dans la moelle épinière adulte suite à une lésion, GTP Rho cause la mort de ces cellules. Un autre exemple, dans des cellules vasculaires lisses GDP Rho cause la mort des cellules et suite à une ischémie cardiaque GDP Rho est protectrice (Blanco-Colio et al., 2002; Bao et al., 2004). Ces exemples montrent qu'en conditions pathologique (traumatismes, pathologie et ou lésion) la même forme qui causait la survie dans les cellules 'normales', induit alors la mort des cellules et vice versa.

1.6.2.2 Rho inactive régule la mort cellulaire apoptotique

Il a été montré que l'inactivation de Rho (par la C3) cause l'apoptose des cellules endothéliales en culture tandis que l'inactivation de Rac, Cdc 42 ou de ROCK est sans effet (Hippenstiel et al., 1997; Hippenstiel et al., 2002). Ces expériences montrent aussi que l'inactivation de Rho réduit les niveaux de la protéine anti-apoptotique Bcl-2 et augmente ceux de la protéine pro-apoptotique Bid. L'inactivation de Rho augmente aussi l'activation de la caspase9 et le caspase3 (Hippenstiel et al., 2002). De plus, l'inactivation de Rho (par les statines) dans les cellules lisses vasculaires entraine l'apoptose de ces cellules en réduisant les niveaux de Bcl-2 et en activant la caspase-9 (Blanco-Colio et al.,

2002). Il a aussi été montré que l'inactivation de Rho (par la C3,introduite dans les cellules par vecteur viral) induit la mort apoptotique dans des souches de fibroblastes et dans des lymphocytes T (Moorman et al., 1996; Bobak et al., 1997). D'autres études ont utilisé l'approche inverse pour montrer l'effet de Rho inactif dans la régulation de l'apoptose : ils montrent que l'activation de Rho (GTP) induit la survie des cellules. Dans des cellules épithéliales (cellules Hep-2) et dans des cellules T l'activation de Rho cause l'augmentation des niveaux d'expression protéique de Bcl-2 ce qui induit la survie cellulaire (Gomez et al., 1997; Fiorentini et al., 1998). Ces résultats indiquent que dans plusieurs types cellulaires, l'inactivation de Rho engendre l'apoptose en affectant la protéine anti-apoptotique Bcl-2 et les caspases.

1.7 La régénération dans le système nerveux central (SNC)

Les neurones adultes du SNC ne régénèrent pas spontanément à la suite d'une lésion. 1.Goldberg et al., ont montré que la vitesse d'extension axonale des cellules ganglionnaires de la rétine (RGC) diminue dès la naissance (Goldberg et al., 2002). 2. L'abscense de régénération des neurones du SNC est en partie dûe à la faible expression de gènes associés à la régénération (RAGs) tel que GAP-43 (Tetzlaff et al., 1991; Fernandes et al., 1999). Les axones du système nerveux périphérique (SNP), qui peuvent régénérer ont une augmentation marquée de ces gènes suite à une blessure (Plunet et al., 2002). 3. Une des autres principales raisons pour l'abscence de régénération est l'environnement inhibiteur de croissance présent suite aux traumatismes dans le SNC. Les expériences de David et Aguayo (81) ont montré que des axones lésés du SNC peuvent s'allonger que s'ils sont dans un environnement permissif à la croissance, tel

qu'une greffe de nerf périphérique (David and Aguayo, 1981). La transplantation de cellules de Schwann (cellules qui produisent la myéline) en provenance du SNP permet aussi la croissance des axones de neurones du SNC (Bunge, 2002). Ces résultants indiquent que l'environnement du SNP est favorable à la croissance alors que celui du SNC ne l'est pas. Cet environnement inhibiteur de croissance résulte de la présence de protéines inhibitrices de croissance de la myéline et de la cicatrice gliale ainsi que de l'augmentation de l'expression de facteurs de guidage répulsifs (David and Lacroix, 2003; He and Koprivica, 2004; Sandvig et al., 2004). De plus, l'inhibition de la régénération dans le SNC est associée à la forte réponse inflammatoire ainsi qu'à la mort cellulaire observée suite aux lésions (Figure 5).

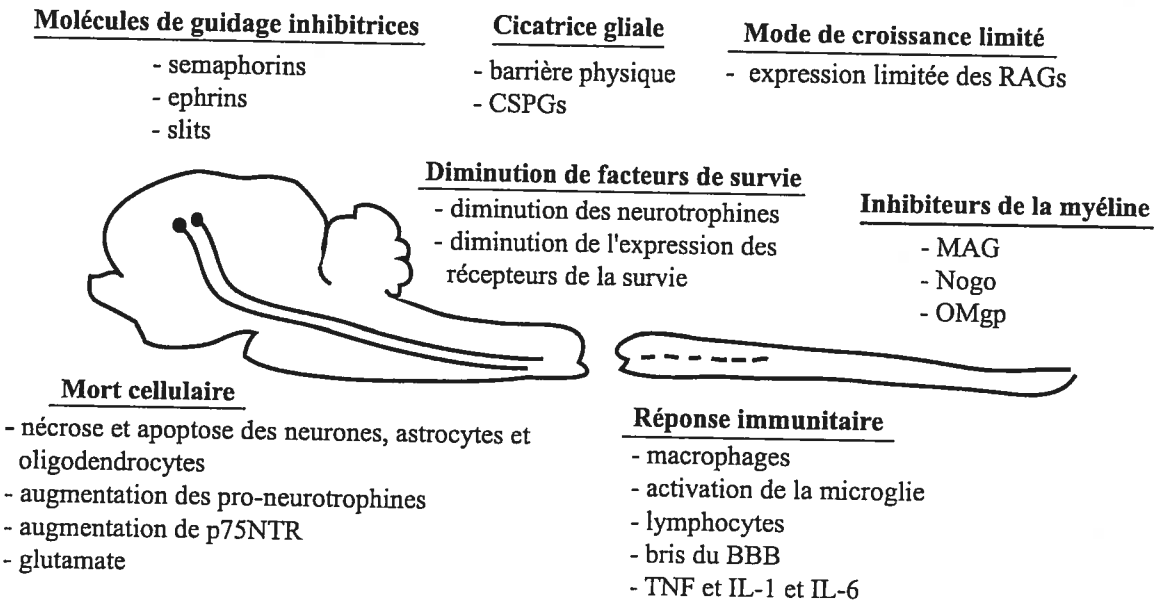

Figure 5 Facteurs associés au manque de régénération dans le SNC
Shématisation des facteurs impliqués dans l'inhibition de régénération. Le manque de régénération dans le SNC est dû à la combinaison de facteurs intrinsèques et extrinsèques

La formation de connexions appropriées entre les neurones et leurs cibles est une étape cruciale du développement du système nerveux et est essentielle à son bon fonctionnement. Le cône de croissance qui se trouve à l'extrémité d'un axone guide ce dernier afin d'innerver sa cible (Tessier-Lavigne and Goodman, 1996; Luo, 2002; Huber et al., 2003). Suite aux lésions axonales, les axones forment des bulbes de rétraction. Le cône de croissance nouvellement formé doit guider les axones à leurs cibles à travers le site de la lésion, dans l'environement inhibiteur résultant du truamatisme, Ceci a pour but de refaire les connections pour augmenter la récupération fonctionelle perdu par le bris des axones.

1.7.1 Le cône de croissance

Le cône de croissance est une structure mobile spécialisée située à l'extrémité des axones (et des dendrites) qui explore et répond aux changements de l'environnement. Les cônes de croissance dirigent les axones en répondant à des stimuli extracellulaires et à des facteurs de guidage attractifs ou répulsifs qui guident les axones vers leurs cibles (Tessier-Lavigne and Goodman, 1996; Huber et al., 2003). Les cônes de croissance sont formés de filopodes (de longs filaments minces cylindriques à l'extrémité croissante), de lamellipodes (structure aplatie en voile ou 'web' qui forme des extensions à la périphérie du cône) et de regroupements de filaments d'actine (actin bundles) contractiles qui sont régulées par les RhoGTPases (Luo, 2002; Huber et al., 2003; Govek et al., 2005). Ensemble, la régulation de la polymérisation d'actine des filopodes et des lamellipodes ainsi que la vitesse du flux rétrograde d'actine contrôlent les mouvements du cône de croissance (i.e. avancement et rétraction) (Forscher et al., 1992; Lin and Forscher, 1995;

Lin et al., 1996, 1997; Wylie et al., 1998; Wylie and Chantler, 2003). Les mouvements du cône de croissance sont dus à l'élaboration asymétrique de filaments d'actine, aux changements dans la vitesse du flux rétrograde d'actine et à la contraction par la myosine des filaments concentriques et en arc d'actine dans le domaine C du cône de croissance (Dent and Gertler, 2003; Dontchev and Letourneau, 2003; Pollard and Borisy, 2003). Le cône de croissance peut être divisé en un domaine périphérique P qui est composée majoritairement de filopodes et lamellipodes, le domaine central C, qui est riche en filaments épais d'actine, de structures en arc d'actine et de microtubules, et le domaine transitoire T qui se trouve à l'interface entre les domaines P et C (Figure 6) (Forscher and Smith, 1988; Smith, 1988; Forscher et al., 1992; Lin and Forscher, 1993, 1995; Dent and Gertler, 2003; Rodriguez et al., 2003).

Les filaments d'actine dans le cône de croissance sont constamment remodelés (multiples cycles de polymérisation et dépolymérisation) ce qui permet le mouvement du cône. Les RhoGTPases régulent ces filaments dans le cône de croissance. La motilité de ce dernier dépend alors de l'activité de ces GTPases (Luo, 2002; Govek et al., 2005). L'activation de Rac et Cdc42 mènent respectivement à la formation de lamellipodes et de filopodes. Il a été montré que Rac peut aussi participer à la formation de filopodes car un mutant inactif de Rac empêche la formation adéquate de ces structures. Rho contrôle la formation de filaments d'actine contractiles et régule la contraction de ces filaments par ses effets sur la myosine. Lors de la croissance neuritique et axonale, l'activation de Rac et Cdc42 induisent l'avancement du cône de croissance et les facteurs de guidage attractifs causent l'activation de ces 2 GTPases. Par contre, la rétraction et l'effondrement

(collapsus) du cône de croissance sont le résultat de l'activation de Rho et les facteurs de guidage répulsifs induisent l'activation de Rho.

1.7.2 Régulation de l'actine par les Rho GTPases dans le cône de croissance

Les Rho GTPases, Rho, Rac et Cdc42 régulent les cycles de polymérisation et dépolymérisation de l'actine dans le cône de croissance. Dans les neurones, l'activation de Rho promouvoit l'arrondissement des cellules, la rétraction des neurites et l'effondrement du cône de croissance (Jalink et al., 1994; Tigyi et al., 1996; Kozma et al., 1997) alors que son inactivation par la C3 empêche l'effondrement du cône de croissance et stimule la croissance neuritique (Jin and Strittmatter, 1997; Lehmann et al., 1999). Rho et Rac ont des rôles opposés dans les neurones (Li et al., 2000; Li et al., 2002; Govek et al., 2005), où l'activation de Rho cause l'effondrement du cône de croissance et inhibe la croissance axonale tandis que l'activation de Rac induit la pousse neuritique et l'extension du cône de croissance.

1.7.2.1 Rho

L'activation de Rho dans le cône de croissance induit principalement la contraction de filaments d'actine par ses effets sur la myosine. Rho, par son effecteur ROCK, induit la phosphorylation de MLC ce qui cause une tension et une force de contraction des filaments d'actine (Amano et al., 1996a; Kimura et al., 1996). De plus, il a récemment été montré que la stabilisation des structures en arc d'actine dans les domaines T et C du cône de croissance est régulée par Rho (Zhang et al., 2003). En effet, il a été proposé que les arcs d'actine sont des précurseurs ded fibres de stress dans les fibroblastes (Heath and

Holifield, 1993). Rho pourrait alors réguler la stabilisation des arcs par les mêmes voies de signalisation que pour la formation de fibres de stress. Il est intéressant de noter que malgré que ces structures semblent analogues aux fibres de stress, leur formation semble être Rho indépendante (Zhang et al., 2003). La stabilisation des filaments par Rho peut se faire par les effets de la GTPase sur les protéines de coiffe (capping proteins) (Takai et al., 2001; dos Remedios et al., 2003). Les arcs d'actine seraient alors stabilités par les effets de Rho sur les protéines de coiffe puisuq'aucun monomère ne peut être incorporé ou enlevé des extrémités une fois coiffées. Il semblerait alors par ces résultats que les effets de Rho dans le cône de croissance seraient principalement sur la stabilisation et la contraction des filaments d'actine par la myosine. L'activation de Rho pourrait aussi induire la dépolymérisation des filaments d'actine lors de l'effondrement du cône de croissance en activant des protéines tels que LIMK et cofiline (Maekawa et al., 1999). De plus, Rho peut aussi activer des protéines qui peuvent briser les filaments d'actine tel que la gelsoline.

Le groupe de Wang a montré que Rho est ubiquitinée par la protéine SMURF1 et dégradée par le protéasome au 'leading edge' pendant la migration cellulaire (Wang et al., 2003). Ces résultats montrent que l'activité de Rho est spatialement restreinte à l'arrière de la cellule en migration puisque SMURF1, une protéine qui se lie à l'ubiquitine, est localisée à l'avant de la cellule en migration. Jaffe et Hall proposent que la dégradation de Rho au 'leading edge' puisse avoir comme rôle d'assurer l'activation de Rac et Cdc42 par des GEFs qui ont des effets sur les trois GTPases (Jaffe and Hall, 2003). L'ubiquitination de Rho au 'leading edge' pourrait aussi inhiber ses effets de

dépolymérisation de l'actine qui empêcheraient l'avancement de la cellule. Il a très récemment été montré que Rho est ubiquitinée dans des cellules nerveuses, par SMURF1 *in vivo* ce qui pormouvoit la croissance axonale (neuroblastomes) (Bryan et al., 2005). L'ubiquitination de Rho par SMURF1 régule sa localisation intracellulaire en induisant la distribution asymétrique de Rho (Jaffe and Hall, 2003; Wang et al., 2003; Bryan et al., 2005). Le groupe de Forsher a simultanément montré que la régulation du flux rétrograde d'actine par la myosine dans le domaine P du cône de croissance se fait indépendamment de l'activation de Rho (Zhang et al., 2003). Donc ensemble ces études se complètent pour montrer que la régulation de l'actine au 'leading edge' se fait indépendamment de Rho puisque la dégradation de Rho au 'leading edge' régule la distribution spatiale à la base du cône dans les régions T et C. Le rôle de Rho dans le cône de croissance est donc restreint à la régulation des arcs d'actine et à la contraction de ces structures dans les domaines T et C.

1.7.2.2 Rac et Cdc42

La polymérisation de l'actine dans le domaine P du cône de croissance se fait par Rac et Cdc42. Rac induit la polymérisation de l'actine par l'activation de deux effecteurs principaux PAK et WAVE-Scar (Manser et al., 1994; Miki et al., 1998; Castellano et al., 1999; Machesky and Gould, 1999; Machesky et al., 1999; Tsujioka et al., 1999). PAK interagit avec LIMK qui a comme effet de phosphoryler et inhiber la protéine cofiline. Cofilin cause la dépolymérisation de filaments d'actine et son inactivation entraîne la production d'un plus grand nombre de filaments d'actine avec des extrémités en croissance ce qui favorise l'élongation des filaments d'actine. La protéine WAVE-Scar

interagit avec Arp2/3 qui induit la polymérisation de l'actine. Par contre, deux études récentes montrent *in vivo* et *in vitro* que la croissance axonale induite par Rac ne passe pas nécéssairement par les voies WAVE-Scar-ARP2/3 (Strasser et al., 2004) et PAK (Ng and Luo, 2004). Rac pourrait alors aussi induire la polymérisation de l'actine par ses effets sur PIP_2 qui affecte la profiline ou par une autre voie inconnue activée par le GEF sif (Ng and Luo, 2004). Cdc42 induit la polymérisation de l'actine par trois principaux effecteurs : PAK, WASP (n-WASP dans les neurones) et IRSp53-58. L'activation de PAK par Cdc42 produit les mêmes effets que son activation par Rac, c'est-à-dire l'inactivation de cofiline. L'activation de n-WASP induit la polymérisation de l'actine par l'intermédiaire de Arp2/3 et l'interaction de Cdc42 avec IRSp53-58 est responsable de l'initiation de la formation de filaments d'actine (Govek et al., 2005).

L'avancement du cône de croissance dépend des cycles de polymérisation et dépolymérisation de l'actine au 'leading edge'. Les mécanismes de motilité du cône de croissance semblent être indépendants de Rho dans le domaine P (Wang et al., 2003; Zhang et al., 2003; Govek et al., 2005) indiquant que la rétraction, un processus régulée par Rho, se fait de façon Rho indépendante dans ce domaine du cône. La rétraction des filopodes et lamellipodes pourrait donc être régulée par les voies de signalisation de Rac et Cdc42. A cet effet, il a été montré que ces deux GTPases peuvent activer des voies de signalisation qui antagonisent les voies responsables de la polymérisation de l'actine. En premier lieu, il a été montré que Rac peut positivement et négativement réguler l'actine par cofiline : 1. Rac par LIMK phosphoryle et inactive cofiline induisant la polymérisation (Edwards et al., 1999) 2. Rac peut aussi activer la protéine Slingshot (Ssh)

qui déphosphoryle et active cofiline ce qui inhibe la polymérisation de l'actine (Niwa et al., 2002; Nagata-Ohashi et al., 2004; Govek et al., 2005). De plus, Rac peut activer le p35-Cdk5 (Nikolic et al., 1998) qui inactive la voie de l'effecteur PAK (LIMK-cofilin) ce qui inhibe la nucléation et polymérisation de l'actine. Donc, Rac peut par l'activation de différents effecteurs pourrait induire l'avancement et la rétraction du cône par ses effets sur la polymérisation et dépolymérisation de l'actine respectivement. Cdc42 active directement son effecteur n-WASP mais peut aussi activer Toca-1 (transducer of Cdc42 dependant actin assembly) qui a son tour active n-WASP. L'activation de Toca-1 inhibe la protéine WIP. L'activation de WIP empêche alors l'activation de WASP et de la voie ARP2/3 inhibant la polymérisation de l'actine (Ho et al., 2004; Govek et al., 2005; Yamaguchi et al., 2005).

Ces résultats indiquent que Rac et Cdc42 peuvent réguler la durée de l'activité de leurs effecteurs par des mécanismes de feedback et d'inhibition. Ces GTPases ont aussi la capacité d'induire la polymérisation et dépolymérisation de l'actine dans le cône de croissance. Donc, dans un modèle où Rho est spatialement restreinte à la base du cône de croissance et régule la contraction de la myosine pour induire l'effondrement du cône, Rac et Cdc42 pourraient respectivement réguler la polymérisation et la dépolymérisation de l'actine dans le domaine P lors de l'avancement et de la rétraction du cône de croissance.

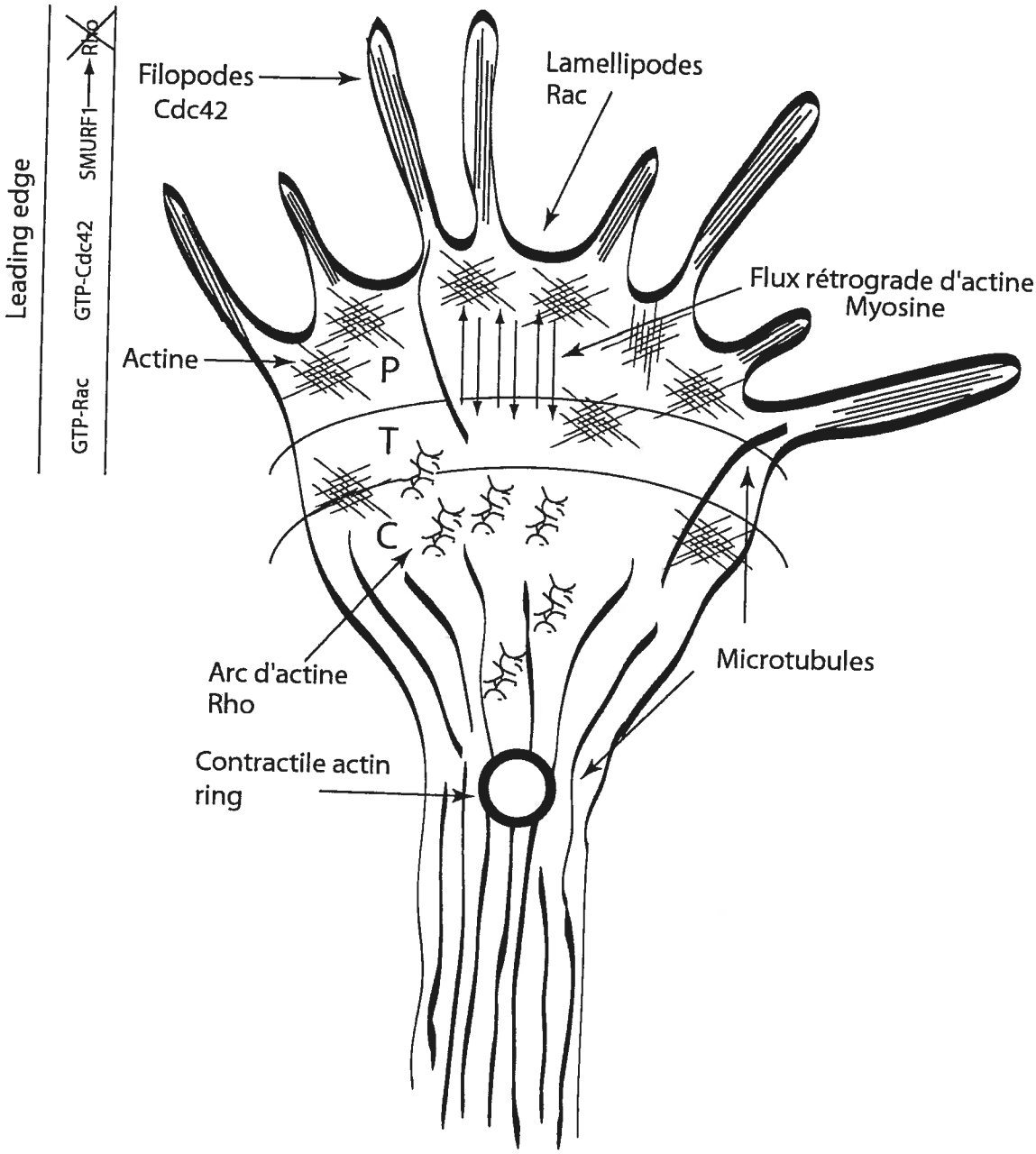

Figure 6 Le cône de coissance
Ilustration du cône de croissance et de ses composants principaux ainsi que leurs locatisation dans la structure. Représentation des domaines P, T et C du cône et du rôle des RhoGTPases, Rho, Rac et Cdc42 dans cette structure. Voir texte pour explications détaillées.

1.7.3 Régulation de la myosine dans le cône de croissance

Le rôle de la myosine dans le flux rétrograde d'actine reste encore controversé. Par contre, de récentes expériences semblent éclaircir le mécanisme de régulation du flux d'actine ainsi que le rôle de la myosine dans le flux. Puisque Rho contrôle la contraction par la myosine et que le flux rétrograde d'actine dépend de l'activité de la myosine, il a pendant longtemps été assumé que Rho régulait le flux d'actine. Par contre, tel que décrit dans la section ci-dessus, Rho est dégradée au 'leading edge', ce qui indique que Rho ne pourrait pas exercer ses effets sur le flux d'actine (Wang et al., 2003; Bryan et al., 2005). Le groupe de Forsher a montré que ceci était bien le cas : Rho ne régule pas le flux rétrograde d'actine dans le domaine P du cône de croissance (Zhang et al., 2003). La vitesse du flux d'actine reste inchangée lors de l'effondrement du cône de croissance produit par l'activation de Rho. Dans ces expériences ni l'activation de Rho (par un mutant actif), ni l'inactivation de Rho (par la C3) affectent le flux rétrograde d'actine dans le domaine P (Zhang et al., 2003). Il a récemment été montre que PAK, l'effecteur de Rac et Cdc42, peut aussi réguler la myosine (Bokoch, 2003; Rudrabhatla et al., 2003; Govek et al., 2005) : il peut induire l'inactivation de MLCK, et il peut aussi directement phosphoryler et activer MLC. Rac et Cdc42 pourraient alors réguler le flux rétrograde d'actine par leurs effets sur MLCK et par MLC qui activerait la contraction de la myosine. A cet effet, il a été montré que la formation de protrusions dendritiques par Rac dépend de la phosphorylation et l'activation de MLC par PAK. Par contre, le groupe de Forsher montre, à l'aide de mutants actifs et dominants négatifs de Rac et Cdc42, que ces GTPses ne semblent pas non plus influencer le flux rétrograde d'actine dans le domaine P.

Si la régulation du flux rétrograde d'actine se fait indépendamment de l'activation de Rho, Rac et Cdc42, il est possible que d'autres GTPases de la famille Rho tel que RhoG, TC10 et RhoT puissent jouer un rôle dans cette régulation (Neudauer et al., 1998; Katoh et al., 2000; Tanabe et al., 2000; Abe et al., 2003; Govek et al., 2005). TC10 et RhoT peuvent induire la croissance neuritique par la voie N-WASP-ARP2/3 (Abe et al., 2003) et TC10 a aussi comme effecteur PAK. Il est donc possible que TC10 régule la myosine et donc le flux d'actine par l'intermédiaire de PAK (Neudauer et al., 1998). Il existe deux autres possibilités pour expliquer la régulation du flux d'actine : les effets de ROCK indépendants de Rho et le type de myosine qui régule le flux rétrograde d'actine. En premier lieu, l'activation de ROCK et la contraction de la myosine résultante peuvent se faire de manière indépendante de Rho. L'activation de la myosine par ROCK peut se faire indépendamment de Rho lors de la formation de membrane blebbs dans des cellules épithéliales ainsi que dans des fibroblastes (Sebbagh et al., 2001). Une autre étude a montré que ROCK a des effets sur les mouvements d'attraction et de répulsion dans les neurones malgré l'inhibition de Rho (Yuan et al., 2003b). Les facteurs qui activent ROCK indépendamment de Rho restent par contre à présent inconnus.

En dernier lieu, une des raisons pour laquelle le rôle de la myosine dans le flux rétrograde d'actine reste encore controversé est associé au type de myosine. La superfamille de la myosine contient environ 18 molécules. Les membres conventionnels (myosine de classe II) et membres non conventionnels (myosine de classe I, V, VI et X) sont retrouvés dans le cône de croissance. La myosine II A et B ont des localisations différentes dans le cône

de croissance où II A est exprimée dans le domaine C et II B dans le domaine P. Il a été montré que la myosine II induit la croissance axonale et que la myosine II A est requise non seulement pour la formation de points d'adhésion (Wylie et al., 1998; Wylie and Chantler, 2001), mais aussi pour la rétraction régulée par la voie Rho-ROCK. Ceci semble indiquer que la myosine II B pourrait réguler le flux d'actine (Brown and Bridgman, 2003a; Brown and Bridgman, 2003b) dans le domaine P. L'inhibition de la myosine II B augmente le taux du flux rétrograde (Diefenbach et al., 2002) et les mêmes résultats sont obtenus dans des souris KO de la myosine II B (Lin et al., 1997; Brown and Bridgman, 2003a; Brown and Bridgman, 2003b). L'inhibition de MLCK, qui régule la myosine II, induit seulement une baisse de 40% du flux rétrograde d'actine ce qui indique que d'autres mécanismes semblent être impliqués dans cette régulation (Zhang et al., 2003). Une autre étude a montré que la myosine 1c et non II B, peut réguler le flux d'actine puisque son inhibition induit une diminution du flux (Diefenbach et al., 2002) par contre, le niveau d'expression de myosine 1c est très faible dans les neurones et le cône de croissance. Il est clair que le flux d'actine est régulé par la myosine, il semble possible par ces expériences que la myosine II B ainsi que 1c seraient impliquées et pourrait collaborer pour réguler le flux d'actine (Wylie et al., 1998; Wylie and Chantler, 2001, 2003). Il est aussi possible que d'autres types de myosine ex. V soit responsables du flux d'actine mais il reste à déterminer si ces autres formes participent à ce phénomène.

1.7.4 Régulation des microtubules par les Rho GTPases dans le cône de croissance

Les microtubules (MT), comme l'actine, ont un rôle essentiel dans la motilité du cône de

croissance et dans la croissance axonale. Les microtubules sont orientés dans l'axe longitudinal de l'axone et lorsqu'ils entrent dans le cône de croissance ils s'écartent (en forme d'éventail), ceci est connu comme le 'splaying' des microtubules. Ils se trouvent en grande partie dans les domaines C et T du cône de croissance mais sont aussi présents dans la région P. Par des cycles constants de polymérisation-dépolymérisation, les microtubules explorent constamment la région P du cône de croissance. Les microtubules sont aussi transportés de la région P à la région T par le flux rétrograde d'actine (Gordon-Weeks, 1991; Dent and Gertler, 2003; Gordon-Weeks, 2004; Kornack and Giger, 2005). Le groupe de Forsher a par la suite montré que Rho régule la conversion des microtubules provenant de la région P en microtubules moins dynamiques en induisant leur stabilisation (Schaefer et al., 2002; Zhang et al., 2003). L'association des MT avec les arcs d'actine fait en sorte que ces derniers sont transportés dans la région C du cône de croissance et ce phénomène est régulé par Rho. Rho régule (par les arcs d'actine) le transport et la distribution des MT.

La coordination entre les microtubules et l'actine est donc essentielle pour la motilité et la navigation du cône de croissance. Les microtubules s'associent à l'actine par l'intermédiaire des MAPs. En effet, les MAP MAP1B, MAP2, MAP2c et tau peuvent tous lier l'actine (Moraga et al., 1993; Dehmelt and Halpain, 2004; Roger et al., 2004; Dehmelt and Halpain, 2005) et les '+Tip proteins', CLASP, CLIP, ACP, EB1, IQGAP, GEF-H1 (Dehmelt and Halpain, 2004; Gordon-Weeks, 2004; Andersen, 2005; Kornack and Giger, 2005) peuvent interagir avec les régulateurs de l'actine. Les RhoGTPases peuvent réguler la dynamique de l'actine ainsi que les microtubules. Wittman et al, ont

proposé un mécanisme de feedback entre le rôle de Rac dans la régulation des MT et l'activation de Rac dans la polymérisation de l'actine dans le domaine P (Waterman-Storer and Salmon, 1999; Waterman-Storer et al., 1999; Wittmann and Waterman-Storer, 2001; Wittmann et al., 2003). Dans un premier temps, la stabilisation des MT par CLIP-170 et IQGAP activent Rac. Rac va induire la formation des filopodes ainsi que la phosphorylation et inhibition de OP18-Stathmin (par l'effecteur PAK), qui a comme rôle de déstabiliser les MT. Les MT stables peuvent ensuite causer l'activation de Rac par CLIP-170. Une autre voie impliquant un mécanisme de feedback avec Rac est celle de APC. APC stabilise les MT et induit la liaison avec l'actine. APC peut aussi interagir avec le GEF spécifique pour Rac Asef (pour APC stimulated GEF) ce qui active la GTPase. L'activation de Rac peut alors influencer la croissance des MT par ses effets sur OP18-Stathmin et les MT stables peuvent ensuite causer l'activation de Rac (Daub et al., 2001; Wittmann et al., 2004; Andersen, 2005; Kornack and Giger, 2005).

De plus, il se pourrait que le transport des MT par le flux rétrograde d'atine produit une amplification de signal de la voie de Rho. Le flux rétrograde cause le bris des MT ce qui pourrait libérer des GEFs pour Rho p190RhoGEF, GEF H1 et Lcf qui colocalisent avec les MT mais sont masqués et/ou inactivés par les MT stables (Ren et al., 1998; Glaven et al., 1999; van Horck et al., 2001). L'activation de Rho par ces GEFs peut induire la stabilisation des MT par l'activation de la voie mDIA-APC (Wen et al., 2004). APC peut donc induire la liaison des MT avec les arcs d'actine dans la zone T. Cette association et le transport des MT par les arcs au domaine C sont dépendants de l'activation de Rho (Zhang et al., 2003). Donc la formation des arcs d'actine dans la zone T dépend de la

forme active de Rho. L'activation de Rho stabilise aussi les microtubules dans la zone T provenant du flux d'actine par mDia-APC, APC qui par la suite pourrait réguler l'association des MT aux arcs d'actine. Ici l'amplification du signal proviendrait de la libération des GEF lors du bris des MT par le flux d'actine ce qui aurait alors comme conséquence l'amplification de l'activation de Rho qui peut alors induire la stabilisation et le transport des MT ainsi que ses effets sur l'actine.

Ensemble ces résultats indiquent que les RhoGTPases peuvent non seulement réguler l'actine mais aussi les microtubules pour moduler la motilité du cône de croissance. Ces GTPases, par leur régulation de la navigation du cône de croisance, pourraient alors avoir un rôle important dans la régénération axonale.

1.8 Protéines inhibitrices de croissance

En 1928 Ramon y Cajal a démontré que les axones lésés du SNC pouvaient produire une extension neuritique si ils étaient dans un environnement permissif comme le nerf périphérique. Nous savons depuis les années 1980 que la croissance axonale dépend de l'environnement inhibiteur (David and Aguayo, 1981; Benfey and Aguayo, 1982). Schwab et al., ont montré que l'inhibition de croissance dans le SNC est non seulement due au manque de facteurs trophiques, mais aussi à la présence de protéines inhibitrices (Schwab and Thoenen, 1985). Au cours du dévelopement du système nerveux, la fin de la période d'extension axonale corespond à la période de différenciation des oligodendrocytes et de la myélinisation des axones. En 1982, Berry (Berry, 1982) a été un des premier a émettre l'hypothèse que la myéline du SNC est inhibitrice, suite à ses

observations que des axones non-myélinisés peuvent régénérer suite à une axotomie tandis que des fibres myélinisées n'ont pas cette capacité. Le groupe de Schwab ont directement montré pour la première fois que la myéline du SNC inhibe la croissance axonale (Caroni et al., 1988; Caroni and Schwab, 1988a, b; Schwab and Caroni, 1988; Savio and Schwab, 1990). L'inhibition du développement des oligodendrocytes ou un délai de la myélinisation sont permissifs à la croissance axonale (Savio and Schwab, 1990; Keirstead et al., 1992). Suite à une lésion, des souris immunisées avec un homogénat de moelle épinière montrent un taux élevé d'axones qui régénèrent (Huang et al., 1999). L'environnement inhibiteur présent suite aux lésions de la moelle épinière est aussi dû à la présence de la cicatrice gliale et à la production d'un autre groupe de molécules inhibitrices de croissance : les CSPGs (chondroitin sulfate proteoglycans). De plus, les lésions de la moelle induisent aussi l'expression de facteurs répulsifs de croissance tels que les sémaphorines, les ephrines et les slits (qui peuvent induire l'effondrement du cône de croissance et la rétraction des axones; voir section Rho dans les neurones, facteur de guidage) qui peuvent participer à l'inhibition de la régénération (David and Lacroix, 2003; He and Koprivica, 2004).

1.8.1 Les protéines inhibitrices de la myéline

Les protéines inhibitrices de croissance présents dans la myéline les mieux caractérisées sont MAG, Nogo et Omgp. Il est possible que la myéline contienne d'autres protéines qui puissent inhiber la croissance axonale.

1.8.2 MAG (Myelin Associated Glycoprotein)

MAG est une protéine transmembranaire qui fait partie de la superfamille des immunoglobulines. MAG se trouve dans le SNC ainsi que dans le SNP, et elle est localisée dans la membrane périaxonale ainsi que dans des régions où la myéline n'est pas compactée telle que les 'paranodal loops' et les 'schmidt-Lanterman incisures' (Filbin, 1996; Filbin, 2003; He and Koprivica, 2004) (Shen et al., 1998). Simultanément en 1994, les groupes de McKerracher et de Filbin ont pour la première fois identifié MAG, une protéine déjà connue de la myéline, comme étant une protéine inhibitrice de croissance (McKerracher et al., 1994; Mukhopadhyay et al., 1994). *In vitro*, MAG inhibe la croissance axonale des cellules neuronales en culture. De plus, il a récemment été montré qu'une forme de MAG, dMAG (le domaine extracellulaire de la protéine) en plus d'être inhibiteur est présent dans le tissu lésé de la moelle (Tang et al., 2001). Par contre, pendant le développement neuronal, MAG peut promouvoir la croissance axonale. Les effets inhibiteurs de MAG dépendent donc de l'âge et peuvent aussi varier en fonction du type de neurone (DeBellard et al., 1996; Cai et al., 2001).

1.8.3 Nogo

Suite aux expériences de fractionnement de la myéline par le groupe de Schwab, deux protéines inhibitrices de croissance de 35 kDa et de 250 kDa, nommées NI-35 et NI-250 respectivement, ont été identifiées. L'anticorps monoclonal IN-1, produit pour réagir contre ces protéines, stimule la croissance axonale en présence de ces protéines (Caroni and Schwab, 1988a, b). La purification et le séquençage de la protéine NI-250 (Spillmann, 1999) ont permis à trois groupes indépendants d'identifier cette protéine,

nommée Nogo et de cloner le gène correspondant (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). Il existe trois formes de Nogo, A,B et C, provenant d'épissage alternatif du gène. Nogo est membre de la famille des réticulons qui est souvent associée au réticulum endoplasmique mais peut atteindre la surface cellulaire. Nogo-A est enrichie dans les oligodendrocytes et dans les neurones du SNC, mais pas dans les cellules de Schwann. Dans les oligodendrocytes, Nogo se trouve dans les boucles internes de la myéline où il peut être en contact avec les axones (He and Koprivica, 2004). Dans les neurones, Nogo est exprimé durant le développement et les niveaux d'expression de Nogo diminuent dans le système nerveux adulte (Josephson et al., 2001; Huber et al., 2002).

Les trois isoformes de Nogo partagent un domaine C-terminal de 188 acides aminés et un domaine conservé en N-terminale de 66 acides aminés (Nogo 66). Le domaine N-terminale de Nogo-A contient deux domaines inhibiteurs : Nogo 66 qu'on trouve dans les trois isoformes de Nogo et amino Nogo qui est spécifique à Nogo-A. Nogo 66 inhibe la croissance axonale et amino-Nogo empêche la propagation et la migration de cellules non-neuronales en plus de bloquer la croissance axonale (Fournier et al., 2001; Fournier et al., 2002; Oertle et al., 2003). La topologie exacte de Nogo reste à déterminer, mais plusieurs études localisent Nogo 66 de façon extracellulaire entre deux domaines transmembranires avec le reste des parties N et C-terminale qui serait intracellulaire (Chen et al., 2000; Prinjha et al., 2000). Une seconde topologie possible est la portion amino-Nogo placée de façon extracellulaire. Il est possible que ces deux formations co-existent dans les cellules et que le dommage de la myéline suite à une lésion expose les

deux domaines inhibiteurs de Nogo (Filbin, 2003; He and Koprivica, 2004; Schwab, 2004).

1.8.4 Omgp (Oligodendrocyte Myelin Glycoprotein)

Omgp, la plus récente protéine inhibitrice à être caractérisée (Wang et al., 2002c), semble être un composant mineur de la myéline. Omgp est une protéine liée à la membrane par un lien GPI, et est exprimée non seulement dans les oligodendrocytes mais aussi dans les neurones. Omgp est localisée à proximité des nœuds de Ranvier dans les sections de la myéline adjacente aux axones. *In vitro,* Omgp induit l'effondrement du cône de croissance et inhibe la croissance axonale (Kottis et al., 2002; Wang et al., 2002c).

1.8.5 Signalisation par les protéines inhibitrices de la myéline

1.8.5.1 Récepteurs

1.8.5.1.1 NgR

Les protéines inhibitrices de la myéline MAG, Nogo et Omgp se lient à un récepteur commun NgR (Nogo-66 receptor). Le groupe de Strittmatter a d'abord identifié le récepteur NgR comme étant le récepteur pour Nogo 66. Il faut noter que ce récepteur ne lie pas amino-Nogo. NgR est une protéine de 473 acides aminés ancrée à la membrane par un lien GPI. Ce récepteur contient aussi une séquence amino-terminale de translocation à la membrane, et 8 motifs LLR (leucine riche repeats) (Fournier et al., 2001) (GrandPre et al., 2000). Cette protéine est fortement exprimée dans le cerveau et est aussi détectée dans le cœur et dans les reins. La transfection de NgR dans des cellules qui sont insensibles à Nogo, cause l'effondrement du cône de croissance de ces cellules.

Le traitement de neurones avec le PIPLC, qui clive les liens GPI de la surface membranaire, bloque les effets inhibiteurs de Nogo (Fournier et al., 2001).

Il a été montré que NgR est aussi un récepteur pour MAG et Omgp. Wang et al., ont montré que Omgp se lie à NgR et que cette liaison est suffisante pour inhiber la croissance axonale (Wang et al., 2002c). De plus, deux groupes indépendants ont monté que NgR lie MAG (Domeniconi et al., 2002; Liu et al., 2002). Ces deux groupes ont montré que cette liaison inhibe la croissance axonale en présence de MAG et que l'inhibition du récepteur permet la croissance axonale malgré la présence de MAG. Les sites de liaison précis des 3 protéines inhibitrices de la myéline sur NgR restent encore inconnus. Par contre, il a été suggéré que ces sites soient distincts mais qu'ils se chevauchent (Fournier et al., 2001; Domeniconi et al., 2002; Fournier et al., 2002; Wang et al., 2002c; Wang et al., 2002d; He and Koprivica, 2004).

1.8.5.1.2 $p75^{NTR}$

Étant une protéine GPI, NgR ne possède pas de domaine intracellulaire de signalisation et doit alors former un ou des complexes avec d'autres protéines transmembranaires pour initier des cascades de signalisation. Les groupes de He et Poo ont identifié le récepteur $p75^{NTR}$ comme étant un co-récepteur de NgR (Wang et al., 2002b; Wong et al., 2002). Le groupe de Yamashita avait préalablement montré que des neurones provenant de souris knock out pour $p75^{NTR}$ ($p75^{NTR}$ -/-) n'étaient pas inhibés par MAG (Yamashita et al., 1999; Yamashita et al., 2002). Il a par la suite été montré que $p75^{NTR}$ s'associe au récepteur NgR par leurs portions extracellulaires et que cette association se fait en

présence de protéines inhibitrices. De plus l'ablation du domaine intracellulaire de $p75^{NTR}$ induit la croissance axonale de neurones en présence de MAG, Omgp et de myéline (Wang et al., 2002b). Ces résultats indiquent donc que p75 peut agir comme co-récepteur avec NgR pour médier les effets inhibiteurs des protéines de la myéline (He and Koprivica, 2004; Schwab, 2004).

1.8.5.1.3 LINGO-1

Récemment un second co-récepteur de NgR a été identifié : LINGO-1 (LRR and Ig domain containing Nogo receptor interacting protein) (Mi et al., 2004). LINGO-1 est une protéine transmembranaire spécifique au système nerveux. LINGO-1 forme un complexe de récepteur avec NgR et $p75^{NTR}$ et se lie aux deux récepteurs. La présence d'une forme dominante négative de LINGO bloque les effets inhibiteurs de Omgp, ce qui suggère que la présence de LINGO dans le complexe NgR- $p75^{NTR}$ est requise pour produire les effets inhibiteurs de la myéline.

1.8.5.1.4 TROY

Le récepteur $p75^{NTR}$ est exprimé dans le système nerveux, mais son niveau d'expression est faible chez l'adulte et certaines populations neuronales ne semblent pas exprimer ce récepteur. Ceci conduit alors à questionner le rôle de $p75^{NTR}$ dans le complexe de récepteurs NgR- LINGO- $p75^{NTR}$ dans les effets des protéines inhibitrices pour les neurones n'exprimant pas $p75^{NTR}$. Très récemment deux groupes ont indépendamment identifié le récepteur TROY-TAJ (aussi nommé TROY) comme co-récepteur de NgR et LINGO-1 dans des neurones dépourvu de $p75^{NTR}$ (Park et al., 2005; Shao et al., 2005). Le

récepteur TROY est un récepteur de la superfamille des récepteurs TNF, comme l'est $p75^{NTR}$, et est exprimé dans le système nerveux post-natal et adulte. De tous les récepteurs de la superfamille du TNF, TROY est le plus semblable à $p75^{NTR}$. De plus, l'interaction entre TROY et NgR est plus forte que celle entre $p75^{NTR}$ et NgR. Il a aussi été montré que TROY peut participer à la mort cellulaire de façon caspase indépendante (Wang et al., 2004). Puisque $p75^{NTR}$ joue un rôle important dans la modulation de l'apoptose (Barrett, 2000; Kaplan and Miller, 2000; Dechant and Barde, 2002; Roux and Barker, 2002), le rôle de TROY dans la mort cellulaire indique des similarités de fonctions entre ces 2 récepteurs en plus de leurs rôles dans la régulation de la signalisation des protéines inhibitrices de la myéline.

Plusieurs expériences et techniques ont été utilisées pour identifier la fonction inhibitrice de TROY. En utilisant des techniques de 'cell binding assays' ou d'ELISA, il a été montré que TROY lie NgR1 et LINGO-1. Les études qui ont identifié TROY dans la voie de signalisation des protéines inhibitrices de la myéline montrent en premier lieu, que TROY est exprimé dans le SNC adulte. Pour confirmer l'effet inhibiteur, il a été démontré que la croissance axonale de neurones de souris $p75^{NTR}$ +/+ et -/- est inhibée par Nogo et Omgp. Il a par la suite été montré qu'une forme tronquée de TROY, et pas la forme complète, induit une croissance axonale de neurones en présence de Nogo 66 indiquant que TROY peut médier l'inhibition par Nogo. Dans des neurones souris de $p75^{NTR}$ -/-, l'inhibition de TROY augmente la croissance axonale de ces cellules en présence de Nogo (Park et al., 2005) indiquant que TROY peut médier l'inhibition en abscence de $p75^{NTR}$. Le groupe de Mi montre TROY se lie à NgR en abscence de $p75^{NTR}$

suggèrant un même site d'interaction de TROY et $p75^{NTR}$ sur NgR. Par la suite ce groupe montre que la présence de TROY inhibe la croissance axonale en présence de Nogo 66, Omgp et de la myéline indiquant que TROY peut être récepteur fonctionnel des protéines inhibitrices de la myéline. Pour confirmer ces résultats, des souris TROY-/- ont été générées. Des neurones de ces souris ont des axones plus longs que ceux provenant de souris témoins (contrôles négatif) malgré la présence de myéline, Nogo 66 ou Omgp (Shao et al., 2005). Ensemble, ces études montrent que TROY peut remplacer $p75^{NTR}$ pour former un complexe fonctionnel avec NgR et LINGO pour médier les effets des protéines inhibitrices de la myéline.

Des neurones provenant de souris TROY -/- ou $p75^{NTR}$ -/- ont des axones plus courts que chez les contrôles positifs. Ceci pourrait être du à la présence de p75 dans les neurones TROY-/- et vice versa (Mandemakers and Barres, 2005). Cette croissance axonale résiduelle pourrait aussi être due aux autres protéines inhibitrices de la myéline tels que Sema 4D et amino-Nogo qui induisent leurs effets par des voies indépendantes du complexe NgR. Il se pourrait en dernier lieu que cette croissance soit aussi médiée par une voie de signalisation encore inconnue.

1.8.5.1.5 Gangliosides

Avant la découverte de NgR, les gangliosides GD1a et GT1b avaient été identifiés comme étant les récepteurs de MAG. Ces gangliosides sont exprimés dans les neurones et jouent un rôle dans la médiation des effets inhibiteurs de MAG (Vinson et al., 2001). Des anticorps dirigés contre GT1b neutralisent les effets inhibiteurs de MAG (Vinson et al.,

2001; Vyas et al., 2002a, b). Le groupe de Yamashita a par la suite montré que des neurones provenant de souris knockout pour $p75^{NTR}$ n'étaient pas inhibés par MAG et que GT1b pouvait s'associer au récepteur $p75^{NTR}$ suggèrant que ces deux récepteurs forment un complexe de signalisation inhibiteur (Yamashita et al., 2002). Il reste à déterminer si ces gangliosides sont impliqués dans la signalisation par les autres inhibiteurs de la myéline. Puisque l'absence TROY, $p75^{NTR}$ ou NgR, ne résulte pas en l'abolition complète de l'inhibition de croissance axonale par la myéline, il se peut que ce mécanisme d'inhibition par GT1b soit valide.

1.8.5.2 Signalisation intracellulaire

Les protéines inhibitrices de la myéline exercent leurs effets inhibiteurs de croissance axonale en activant la GTPase Rho. Lorsque Rho est inactivée (GDP) il y a croissance neuritique malgré la présence de protéines inhibitrices (Dergham et al., 2002; Winton et al., 2002). Lorsque la GTPase est active (GTP) les axones perdent cette capacité. Rho joue un rôle prédominant dans la voie de signalisation activée par ces inhibiteurs. Lehmann et al., (Lehmann et al., 1999) ont montré que l'expression d'une forme dominante-négative de Rho ou que l'inactivation de Rho par la C3 stimule la croissance axonale de neurones en présence de MAG ou de myéline dans des cultures de neurones. Des études biochimiques subséquentes ont directement montré que la myéline active Rho et que cette activation est inhibée par la C3. Cette inactivation de Rho dans les cellules entraine la croissance axonale même en présence de myéline (Winton et al., 2002). Par la suite, plusieurs autres groupes ont montré que Nogo 66, MAG, Omgp et amino-Nogo activent aussi Rho (Niederost et al., 2002; Fournier et al., 2003; Sivasankaran et al.,

2004). L'effecteur de Rho, ROCK est impliquée dans la réponse aux protéines inhibitrices de la myéline : l'inhibition de ROCK avec le Y27632 bloque la réponse inhibitrice à la myéline (Dergham et al., 2002; Fournier et al., 2003) et l'inactivation de Rho ou de ROCK induit la croissance des axones lésés.

Comme préalablement décrit, puisque NgR est un récepteur GPI, il doit former des complexes avec des récepteurs transmembranaires pour induire une signalisation intracellulaire. Deux complexes ont été décrits : NgR-LINGO- $p75^{NTR}$ et NgR-LINGO-TROY. La surexpression des récepteurs TROY, LINGO ou $p75^{NTR}$ induit l'activation de Rho en présence de protéines inhibitrices de la myéline. Les études de Yamashita ont été les premières à montrer que la surexpression de $p75^{NTR}$ active Rho, indiquant un lien entre ces deux protéines (Yamashita et al., 1999). *In vivo*, l'activation de Rho 24 suite à la lésion de la moelle épinière est dépendante de $p75^{NTR}$. Yamashita a montré que ce récepteur peut activer Rho en présence de MAG mais pas en présence du NGF. De plus, il montre que $p75^{NTR}$ active Rho mais ne peut pas directement lier la GTPase (Yamashita et al., 2002). $P75^{NTR}$ interagit directement avec RhoGDI permettant l'activation de Rho en présence de la myéline (MAG et Nogo A) mais pas du NGF. La liaison entre $p75^{NTR}$ et RhoGDI permet la dissociation de Rho avec le GDI et permet alors son activation par les GEFs (qui restent encore inconnus dans ce mécanisme) (Yamashita and Tohyama, 2003). Le site d'interaction entre $p75^{NTR}$ et RhoGDI est la $5^{ième}$ hélice alpha du domaine intracellulaire du récepteur, et le peptide inhibiteur de cette région (Pep5) bloque complètement l'activation de Rho par les protéines de la myéline (Yamashita and Tohyama, 2003). La signalisation de MAG induite aussi le clivage protéolitique de p75

ce qui active Rho pour médier l'inhibition de croissance (Domeniconi et al., 2005). Les études sur le récepteur TROY montrent aussi qu'il active Rho. La présence des trois récepteurs TROY, LINGO et NgR induit l'activation de Rho tandis que l'expression d'un seul ou deux de ces récepteurs n'a pas cette capacité (Park et al., 2005; Shao et al., 2005). Ces résultats indiquent que l'activation de Rho est une étape cruciale dans la production des effets inhibiteurs des protéines inhibitrices de la myéline, puisque chaque protéine inhibitrice de la myéline (MAG, Nogo, Omgp, amino-Nogo) indépendamment du complexe de récepteur pour ces protéines (NgR-LINGO- $p75^{NTR}$ ou TROY ainsi que GT1b) active Rho.

Pour induire la signalisation des protéines inhibitrices et l'activation de Rho, les composants de ces voies doivent être recrutés aux radeaux lipidiques. A cet effet, il a été montré que les protéines GPI, telles que NgR sont localisées aux radeaux lipidiques membranaires et que la signalisation de NgR est inhibée par la perturbation de ces structures (Vyas et al., 2002a, b; Vinson et al., 2003). De plus, MAG interagit avec des radeaux lipidiques qui contiennent $p75^{NTR}$, NgR, GT1b et Rho (Vinson et al., 2003). D'autres études ont montré que la localisation de $p75^{NTR}$ aux radeaux lipidiques est médiée par la phosphorylation du récepteur par PKA (Higuchi et al., 2003).

Le calcium semble aussi être important pour initier les effets inhibiteurs des protéines de la myéline. MAG peut entrainer l'augmentation du Ca^{+2} dans le cône de croissance et l'augmentation de niveaux de Ca^{+2} induit l'effondrement de cette structure (Bandtlow et al., 1993). L'interaction de MAG avec des cellules qui expriment $p75^{NTR}$ et NgR cause

aussi cette augmentation (Wong et al., 2002) suggèrant que la signalisation par $p75^{NTR}$ pourrait être suffisante pour causer l'influx de Ca^{+2} par la myéline (He and Koprivica, 2004). De plus, dans le guidage axonal, le Ca^{+2} influence la motilité du cône de croissance. Il a été montré que la signalisation par calcineurin-NFAT (Graef et al., 2003) et par les calpaines, des protéines activées par le Ca^{+2} influencent la motilité du cône de croissance. Il reste à voir si ces voies de signalisation induites par le Ca^{+2} sont aussi impliquées dans les effets de la myéline.

Le groupe de He a montré que PKC (α et β) médie les effets inhibiteurs de la myéline. L'inhibition de PKC induit la croissance axonale de neurones en présence de protéines inhibitrices de la myéline et stimule la régénération des axones suite aux lésions de la moelle épinière. De plus l'inhibition de PKC inactive Rho en présence de MAG et de Nogo 66, ce qui indique que PKC est requis pour l'activation de Rho par les protéines inhibitrices de la myéline (Sivasankaran et al., 2004). A cet effet, il a déjà été montré que PKC, comme Rho, NgR et $p75^{NTR}$, est localisé aux radeaux lipidiques des neurones (Brown and London, 1998; Vinson et al., 2003) et peut interagir avec Rho (Slater et al., 2001). De plus, les formes de PKC qui induisent l'inhibition de la croissance axonale par la myéline, dépendent du calcium. L'influx du Ca^{+2} produit par les protéines inhibitrices de la myéline pourrait stimuler l'activation de PKC. PKC pourrait par la suite, de concert avec les récepteurs et les GEFs, activer Rho et inhiber la croissance axonale.

1.8.5.3 Modulation de la signalisation de la myéline

La myéline peut inactiver la GTPase Rac (Niederost et al., 2002) en plus d'activer Rho. Il a aussi été montré que la PI3K peut bloquer les effets de MAG dans le cône de croissance (Ming et al., 1999). PI3K se trouve dans la voie de signalisation de Rac et ensemble ils pourraient moduler la réponse des protéines inhibitrices de croissance et favoriser la croissance axonale.

Durant le développement neuronal, MAG peut stimuler l'élongation des axones. Par contre, avec l'âge MAG devient inhibiteur de croissance. Ce changement est modulé par les niveaux d'AMPc (Cai et al., 2001). Le traitement de neurones avec l'AMPc, ou avec des agents pour stimuler son augmentation, bloque l'inhibition de croissance causée par MAG dans des neurones post-nataux et augmentent la régénération de neurones lésés *in vivo* (Cai et al., 2001; Neumann et al., 2002b; Qiu et al., 2002b, c; Qiu et al., 2002a). L'augmentation des niveaux d'AMPc et l'activation de son effecteur PKA sont responsables de ces effets puisque l'inactivation de PKA inhibe la croissance axonale en présence de MAG. Puisque PKA inactive Rho (Lang et al., 1996), il semble donc que l'augmentation de l'AMPc et de l'activation de PKA résultante soit suffisante pour inactiver Rho et favoriser la croissance axonale même en présence de MAG.

L'effet de l'AMPc sur la croissance axonale peut aussi s'expliquer par ses effets sur l'arginase et les polyamines. L'AMPc, par un mécanisme dépendant de la transcription, entraine l'augmentation de l'expression de l'aginase 1 (Arg1) ce qui augmente la synthèse de polyamines (Cai et al., 2002). De plus, Arg1 est fortement exprimé lors du

développement quand les neurones ne sont pas inhibés par MAG, et ces niveaux diminuent lorsque la croissance axonale devient inhibée par MAG. L'augmentation des niveaux de Arg 1 ou des polyamines est suffusant pour bloquer les effets inhibiteurs de MAG et de la myéline. Dans une étude récente, le groupe de Filbin a montré que les effets de l'AMPc nécessitent l'activation du facteur de transcription CREB pour bloquer la signalisation des protéines inhibitrices de la myéline. Des cellules qui expriment une forme dominante-négative de CREB sont inhibées par la myéline tandis que des cellules exprimant la forme active de CREB ne le sont pas. L'expression de CREB dans des neurones lésés est aussi suffisante pour promouvoir la régénération des axones (Gao et al., 2004). De plus ces études montrent que l'activation de CREB est requise pour induire l'augmentation des niveaux de Arg1 par l'cAMP. Ces mécanismes pourraient aussi moduler la voie de signalisation de Rho par phosphorylation de la protéine.

1.8.5.4 Rôle des protéines inhibitrices de la myéline dans la régénération

Afin de déterminer les effets des molécules inhibitrices de croissance de la myéline *in vivo* suite à une lésion, plusieurs animaux transgéniques et knockout (KO) : des souris KO pour MAG, $p75^{NTR}$, Nogo, et NgR ont été générés. Dans plusieurs cas, des résultats contradictoires ont été trouvés avec ces animaux par des groupes distincts. Premièrement, chez les souris MAG -/- ,deux groupes ont montré de faibles améliorations dans la croissance axonale (Li et al., 1996; Shen et al., 1998). Des souris qui surexpriment MAG ont des taux plus faibles de régénération dans le SNP (Shen et al., 1998) et la régénération dans le SNP est augmentée dans les souris MAG -/- (Schafer et al., 1996).

Par contre, Bartch et al., montrent aucune croissance axonale et aucune régénération après une lésion de la moelle épinière dans les souris MAG -/- (Bartsch et al., 1995).

1.8.5.4.1 $p75^{NTR}$

Song et al., ont récemment montré que les souris KO pour le récepteur $p75^{NTR}$ ne montrent aucune régénération dans le tract corticospinal (CST) suite aux lésions de la moelle épinière (Song et al., 2004; Zheng et al., 2005) malgré qu'*in vitro* des cellules de ces souris soient inhibées par MAG (Yamashita et al., 2002). Plusieurs études montrent un rôle important pour ce récepteur dans la mort cellulaire suite à ces lésions et les souris KO ont un niveau plus faible de cellules apoptotiques suite aux lésions de la moelle (Frade and Barde, 1999). Ces résultats indiquent que le rôle de $p75^{NTR}$ suite aux lésions de la moelle épinière serait plutôt dans la régulation de l'apoptose que dans la régénération.

1.8.5.4.2 Nogo

Beaucoup d'efforts ont été consacrés à comprendre le rôle de Nogo et de son récepteur NgR dans l'inhibition de la régénération *in vivo*. Puisque Nogo et la signalisation par NgR inhibent la croissance axonale, la suppression ou l'ablation de ces protéines devrait induire une augmentation du taux d'axones qui régénèrent suite aux lésions du SNC. Plusieurs groupes indépendants ont alors produit des antagonistes, des souris transgéniques et des souris KO de Nogo et NgR mais les résultats obtenus avec ces outils sont contradictoires. Trois groupes indépendants ont produit des souris KO de Nogo : le

groupe de Tessier-Lavigne a produit des souris KO de Nogo A,B et C, ainsi qu'une souris KO pour Nogo A et B (cette souris exprime encore Nogo-C) (Zheng et al., 2003), le groupe de Schwab a produit une souris KO de Nogo-A (cette souris exprime encore Nogo-B et C) (Simonen et al., 2003) et le groupe de Strittmatter a produit une souris KO de Nogo-A et B (cette souris exprime encore Nogo-C) (Kim et al., 2003). Zeng et al ne trouvent aucune régénération dans leurs deux souris, Nogo-A, B, C -/- ou Nogo-A, B -/-. Simonen et al., montrent un très faible 'sprouting' dans leur animaux Nogo-A -/-, où quelques axones seulement ont une amélioration dans leur croissance. Ces souris ont une augmentation compensatoire de Nogo-B et les auteurs discutent que la faible régénération observée est possiblement dûe à cette expression (Simonen et al., 2003). En dernier lieu, Kim et al, contrairement aux autres groupes, montrent une amélioration de régénération malgré qu'elle soit limitée et seulement chez de jeunes animaux (Kim et al., 2003). Les différences dans ces résultats peuvent provenir de la souche de souris utilisée pour produire les KO ainsi que de la méthodologie utilisée pour enlever le gène Nogo (Filbin, 2003). Malgré les différences de résultats, ces études suggèrent que l'implication de Nogo dans l'inhibition de la régénération *in vivo* est faible.

1.8.5.4.3 NgR

Plusieurs efforts ont aussi été mis pour identifier le rôle de NgR *in vivo,* puisque ce récepteur engendre la signalisation de MAG, Nogo et Omgp, il est possible que son inhibition cause un degré plus élevé de régénération que l'inhibition de MAG ou Nogo seul. En premier lieu, GrandPre et al., ont produit un inhibiteur de NgR ,NEP1-40, un peptide contenant les résidus 1-40 de Nogo-66 qui inhibe la liaison de Nogo-66 à NgR

donc inhibant la signalisation de NgR par Nogo-66. Suite aux lésions de la moelle épinière, le traitement avec NEP1-40, induit l'amélioration de la récupération de la fonction motrice et de la régénération axonale comparativement aux animaux non traités (GrandPre et al., 2002). Dans la même optique, le groupe de Strittmatter ont aussi administré un autre peptide qui inhibe la fonction de NgR : l'ectodomaine soluble de NgR NgR(310)ecto, suite aux lésions de la moelle épinière. Dans une première étude, l'administration de NgR(310)ecto (par une pompe intrathécale) suite aux lésions de la moelle épinière induit le 'sprouting' axonal de fibres des tracts CST et raphespinal ainsi que l'amélioration de la locomotion et de la conduction électrique dans les animaux traités (Li et al., 2004). Dans une seconde étude, ce même groupe ont produit des souris dont les actrocytes sécrètent NgR(310)ecto (sous le promoteur GFAP). Suite aux lésions de la moelle ces souris montrent du 'sprouting' axonal de fibres des tracts CST et raphespinal ainsi qu'une amélioration de la locomotion (Li et al., 2005). Une autre étude par le groupe de Benowitz a montré que l'inhibition de NgR par l'application virale d'une forme dominant-négative du récepteur induit seulement la régénération de axones des cellules ganglionnaire de la rétine lorsque le programme de croissance intrinsèque de ces cellules est activé (ici par une 'conditional lésion') (Fischer et al., 2004a). Ces résultats indiquent que NgR semble participer à l'inhibition de la régénération dans le SNC.

Récemment deux groupes ont produit des souris KO pour NgR, dans les deux cas, ils ont remplacé l'exon 2 (l'exon codant primaire) et la même souche de souris a été utilisée (Kim et al., 2004; Zheng et al., 2005). Dans la première étude sur ces souris, Kim et al., montrent que des neurones (DRG, dorsal root ganglion) des souris NgR -/- ne sont pas

inhibés (aucun effondrement du cône de croissance) par la myéline, MAG, Nogo ou Omgp contrairement à l'étude de Zheng qui montrent que la croissance axonale de neurones (DRG et CGN (cerebral granule neurons)) de leur souris NgR-/-, est inhibée par la myéline. Les deux groupent montrent indépendamment qu'il n'y a aucune régénération spontanée du CST dans souris NgR -/- suite a une transection complète au niveau T8 (kim 04) ou suite à une hémisection au niveau T8 de la moelle (Zheng et al., 2005). Le groupe de Kim montre par contre une amélioration de la récupération fonctionnelle (mesurée par le test de locomotion BBB) suite aux lésions de la moelle chez les animaux NgR -/-. Puisqu'aucune régénération n'est détectée dans le CST, Kim et al., ont examiné si d'autres populations de neurones de la moelle sont affectées. Ils montrent une régénération des axones des neurones rubro-spinaux et de neurones sérotoninergiques du raphé (raphespinal axons). De plus, l'induction de la mort des neurones raphespinaux et l'épuisement de l'apport de fibres sérotoninergiques à la région lombaire de la moelle, inhibe l'amélioration de locomotion (mesuré par le test BBB) des souris NgR-/-. Ceci indique que l'amélioration de la récupération fonctionnelle dans les souris NgR -/- est associée à la régénération des neurones sérotoninergiques du raphé (raphespinal axons) (Kim et al., 2004). Ensemble, les résultats sur les fonctions de NgR *in vivo,* indiquent que la régénération dans le SNC nécessite plus que contrer la signalisation des protéines inhibitrices de la myéline.

1.8.5.4.4 Rho

Les Rho GTPases jouent un rôle important dans les neurones car elles régulent la croissance et le guidage axonal par leurs effets sur le cytosquelette (Govek et al., 2005).

En effet le réarrangement du cytosquelette est requis pour la migration neuronale et la croissance neuritique. Dans les neurones, l'activation de Rho promouvoit l'arrondissement des cellules, la rétraction de neurites et l'effondrement du cône de croissance (Jalink et al., 1994; Tigyi et al., 1996; Kozma et al., 1997) alors que son inactivation par la C3 empêche l'effondrement du cône de croissance et stimule la croissance neuritique (Jin and Strittmatter, 1997; Lehmann et al., 1999). *In vivo*, Rho est activée en réponse aux traumatismes du CNS (lésion de la moelle épinière, ischémie, convulsions épileptiques et trauma au cerveau) et la forme active de Rho peut être détectée dans les neurones et les cellules gliales, ainsi que dans les fibres de la matière blanche (Madura et al., 2004). Ceci indique que Rho est activée dans les corps cellulaires et dans les axones suite aux lésions de la moelle épinière. Le traitement avec la C3 induit la régénération des axones suite aux lésions de la moelle épinière et du nerf optique (Lehmann et al., 1999; Dergham et al., 2002; Bertrand et al., 2005) ainsi que la récupération motrice chez les souris. De plus, l'inactiation de Rho inhibe la mort apoptotiques des cellules de la moelle épinière endommagé. L'inhibition de ROCK avec le Y27632 ou avec du fasudil hydrochloride induit aussi la croissance de axones lésés de la moelle épinière des tracts CST (Hara et al., 2000; Dergham et al., 2002; Fournier et al., 2003) et d'axones sérotoninergiques (Ramer et al., 2004). De plus l'inhibition de Rho (Dergham et al., 2002) ou de ROCK (Hara et al., 2000) augmente la récupération fonctionelle motrice des animaux suite aux blessures de la moelle épinière (déterminée par le test BBB et des tests de réflexes et d'activité motrice coordinée respectivement). Il y a par contre aussi d'autres études qui obsevent aucune récupération fonctionelle suite à l'inhibiton de Rho (Fournier et al., 2003) ou de ROCK (Fournier et al., 2003; Sung et al.,

2003), la différence dans les niveaux de récupération peut être due à la méthode d'application des drogues (pompes et administration orale). De plus, plusieurs autres protéines inhibitrices autre que MAG, Nogo et Omgp tel que les facteurs de guidage répulsifs, animo-Nogo, les proéines inhibitrice de la cicatrice gliale et les CSPG (Monnier et al., 2003; Schweigreiter et al., 2004; Sivasankaran et al., 2004), peuvent activer Rho et induire l'inhibition de croissance axonale. Il a de plus été montré que l'inhibition de Rho par la C3 réduit la formation de la cicatrice gliale suite aux lésions de la moelle épinière (Fournier et al., 2003) et induit la régénération des axones à travers la cicatrice gliale (Shearer et al., 2003). Ensemble, ces résultats indiquent que l'activation de Rho est une réponse clé aux traumatismes du SNC et son inactivation, ou l'inactivation de la voie de signalisation de Rho, augmente le taux de régénération des axones lésés et est protecteur.

1.8.6 La cicatrice gliale

La formation de la cicatrice gliale est spécifique au SNC (Fawcett and Asher, 1999). Elle constitue une barrière physique et chimique à la régénération des axones suite à une lésion (Properzi et al., 2003; Matsui and Oohira, 2004; Sandvig et al., 2004; Silver and Miller, 2004; Carulli et al., 2005). La cicatrice mature est complètement formée 2 semaines après une lésion de la moelle épinière chez le rat (Berry et al., 1983). Les astrocytes réactifs sont le composant majoritaire de la cicatrice et ils migrent au site de lésion suite au traumatisme de la moelle épinière. Les astrocytes réactifs expriment plusieurs CSPGs (chondroitin sulfate proteoglycans) inhibiteurs de croissance axonal tel que versican, phosphocan, neurocan, agrecan, brevican et NG2 (McKeon et al., 1991; Fawcett and Asher, 1999). Suite à l'invasion de macrophages, des cellules des méninges

ainsi que des cellules précurseurs d'oligodendrocytes migrent au site de la lésion (Berry et al., 1983). Les cellules des méninges produisent des protéoglycans et des sémaphorines 3A, et les cellules précurseur d'oligodendrocytes expriment le protéoglycan inhibiteur NG2 (Levine, 1994; Tang, 2003; Tang et al., 2003).

Les CSPGs sont des protéoglycans qui ont un noyau protéique avec des chaînes latérales de glycosaminoglycan (GAG) attachées de façon covalente au noyau potéique. Les CSPGs et la cicatrice gliale peuvent inhiber la croissance axonale *in vitro* (Rudge and Silver, 1990; Snow et al., 1990; Snow et al., 1996; Sango et al., 2003). L'expression des CSPGs versican, neurocan et brevican augmente après quelques jours et atteint son maximum 2 semaines après une lésion de la moelle épinière. L'expression de versican et neurocan reste élevée jusqu'à 4 semaines tandis que l'expression accrue de brevican persiste jusqu'à 2 mois suite à la lésion. Les niveaux d'expression de phosphocan diminuent immédiatement après la lésion et augmentent par la suite pour atteindre leurs maximum 2 mois suite au traumatisme (Jones et al., 2003a). Les effets inhibiteurs de croissance des CSPGs sont principalement dûs aux chaînes GAGs et dans certains cas au noyau protéique (ce qui semble être le cas de NG2) (Ughrin et al., 2003). Les CSPGs activent la GTPase Rho et son effecteur ROCK pour induire l'inhibition de croissance (Schweigreiter et al., 2004) (Borisoff et al., 2003; Monnier et al., 2003; Sivasankaran et al., 2004)et que cette activation est dépendante de PKC (Sivasankaran et al., 2004). Par contre, les récepteurs et voies exactes de signalisation des CSPGs restent encore inconnus.

1.8.6.1 Les CSPGs et la régénération dans le SNC

Les CSPGs sont des inhibiteurs de croissance et sont un obstacle à la régénération suite aux lésions du SNC (Properzi et al., 2003; Matsui and Oohira, 2004; Silver and Miller, 2004; Carulli et al., 2005). Les études du groupe de Davies ont montré que des axones du SNC peuvent croître sur la matière blanche intacte (pas endommagée) du SNC en absence de cicatrice gliale (Davies et al., 1997). Les axones arrêtent de croître dès qu'ils sont mis en contact avec des régions de tissus riches en CSPGs (Davies et al., 1997). Ces études montrent non seulement que la myéline non endommagée ne semble pas être inhibitrice, mais qu'en plus des protéines inhibitrices de la myéline (libérées de la myéline endommagée), la cicatrice gliale et l'expression des CSPGs sont des obstacles environnementaux à la régénération axonale dans le SNC (Fitch and Silver, 1997; Lemons et al., 1999; Moon et al., 2002; Jones et al., 2003a; Jones et al., 2003b). En effet, la dégradation des CSPGs au site de lésion par le traitement avec l'enzyme chondroitinase ABC, qui dégrade spécifiquement les chaînes GAGs, augmente le taux de régénération axonale *in vivo* et stimule la récupération fonctionnelle (Moon et al., 2001; Bradbury et al., 2002). En plus des GAGs, le noyau protéique des CSPGs peut aussi inhiber la croissance axonale. Le traitement avec la chondroitinase ABC n'affecte pas le noyau protéique qui est le domaine inhibiteur de certains CSPGs tel que NG2 (Dou and Levine, 1994; Jones et al., 2002; Jones et al., 2003a; Ughrin et al., 2003). L'effet inhibiteur de croissance de NG2 peut être bloqué par l'application d'anticorps spécifiques, mais il reste à déterminer si cette inhibition a des effets sur la régénération *in vivo* (Chen et al., 2002). Une autre méthode utilisée pour inhiber les CSPGs est la suppression de leur synthèse protéique. Il a récemment été montré que l'administration de

decorin, qui inhibe la production de protéoglycans en inhibant certains facteurs de croissance, supprime l'expression des CSPGs neurocan, brevican, phosphocan et NG2 et induit la régénération des axones dans la moelle épinière (Davies et al., 2004). Le groupe de Silver a utilisé une technique différente pour inhiber la synthèse protéique des CSPGs. Ils ont utilisé une enzyme qui dégrade l'ARNm de l'enzyme xylosyltransfarase-1 qui induit la synthèse des GAGs sur le noyau protéique des CSPGs. L'application de cette enzyme autour du site de lésion entraine une diminution des CSPGs dans la région de la lésion et stimule la régénération axonale dans la moelle (Grimpe and Silver, 2004). Ensemble, ces résultats indiquent que cibler la cicatrice gliale suite à une lésion de la moelle épinière peut induire la régénération des axones lésés. Il reste à déterminer si l'inhibition de la cicatrice gliale (et des CSPGs) combiné à l'inhibiton de la signalisation des protéines inhibitrices de la myéline induit une meilleure régénération *in vivo* que le ciblage d'un seul de ces inhibiteurs.

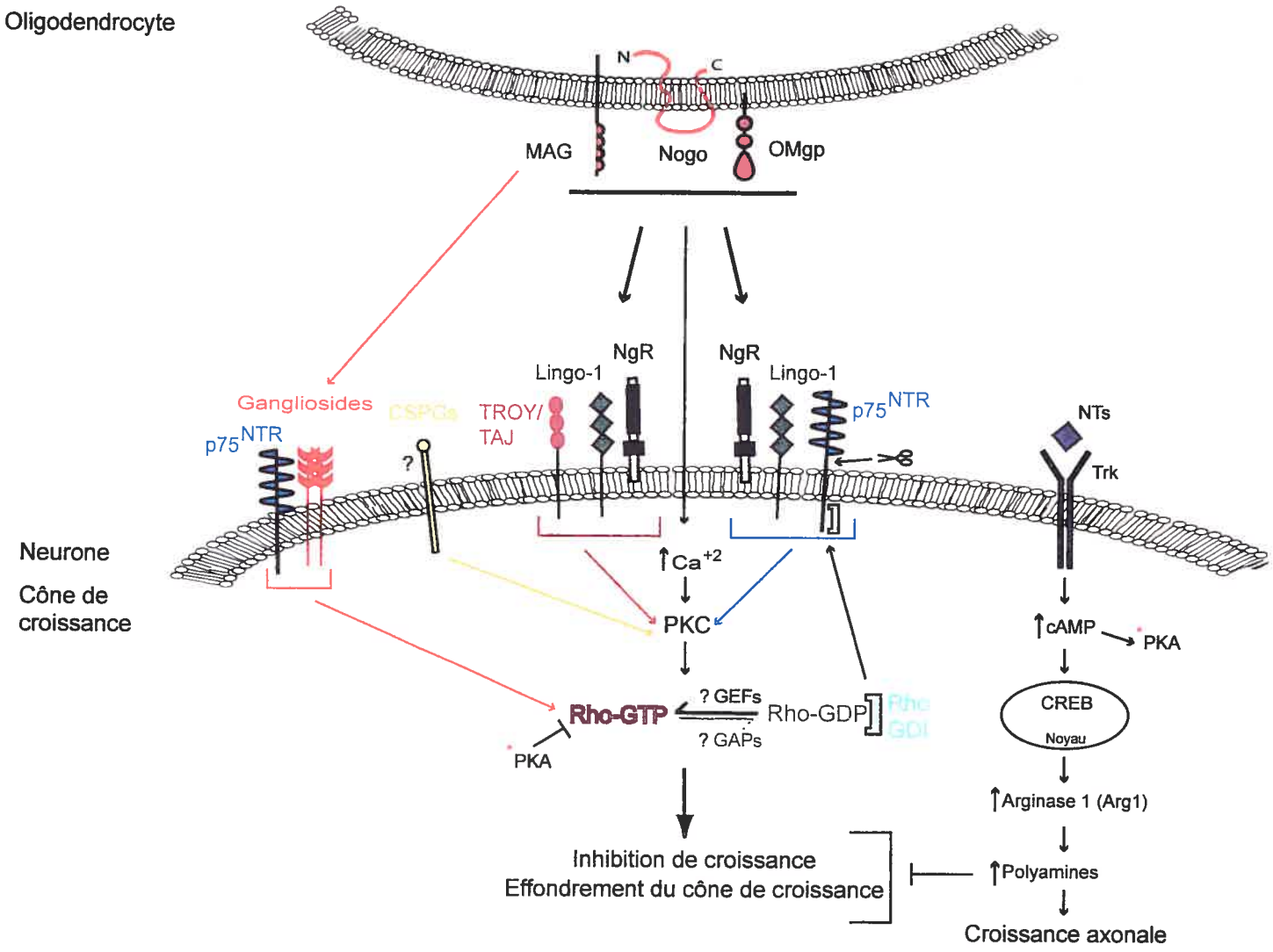

Figure 7 Signalisation des protéines inhibitrices de croissance et la myéline et des CSPGs. Voir texte pour plus de détails. Représentation des protéines de la myéline et des CSPGs et des voies de signalisation engendrées pour inhiber la croissance axonale et la régénération. Ces protéines activent la GTPase Rho pour médier leurs effets inhibiteurs

1.8.7 Facteurs de guidage

Pour faire les connexions appropriées et innerver leurs cibles, les neurones en développement projettent leurs axones sur de longues distances. Le cône de croissance à l'extrémité de l'axone sonde l'environnement, détecte et répond aux facteurs extracellulaires qui le guide vers ses cibles appropriées. Les facteurs extracellulaires de guidage axonal peuvent attirer ou repousser le cône de croissance (Tessier-Lavigne and Goodman, 1996; Dickson, 2001; Guan and Rao, 2003; Huber et al., 2003). De plus, certains doivent entrer en contact avec le cône de croissance pour agir (attraction et répulsion par contact) alors que d'autres, principalement des molécules diffusibles distribuées selon un gradient peuvent agir à plus longues distances (chimoattractant ou chimorepulsifs). Les mouvements d'attraction ou de répulsion du cône de croissance causés par les facteurs de guidage sont régulés par les Rho GTPases et leurs effets sur l'actine et les microtubules (décrits ci-dessus) (Dickson, 2001; Luo, 2002; Huber et al., 2003; Govek et al., 2005). Les facteurs de guidage axonal les plus étudiés sont les sémaphorines, les éphrines, les slits et les nétrines et les plus récemment décrits sont des protéines de la famille des morphogènes ainsi que des chémokines.

1.8.7.1 Sémaphorines

Les sémaphorines sont des facteurs de guidage membranaires et sécrétés (Guan and Rao, 2003; Huber et al., 2003) qui ont un effet majoritairement de répulsion médié par les Rho GTPases (Jin and Strittmatter, 1997). Le premier membre de la famille à être identifié chez les vertébrés est Sema3A ou collapsine qui cause l'effondrement du cône de

croissance. Les sémaphorines induisent les cascades de signalisation par une famille commune de récepteurs les plexines. Ce récepteur s'associe et forme alors des complexes avec d'autres récepteurs pour médier les effets des différent sémaphorines. En présence de Sema 3A, Plexin A1 s'associe au récepteur neuropilin 1 (NP1) et à la molécule transmembranaire d'adhésion L1 pour induire la réponse de répulsion du cône de croissance. Sema 3A cause l'effondrement du cône de croissance en activant la GTPase Rac (Jin and Strittmatter, 1997) Par contre, Sema 3A cause l'activation de LIMK et l'inactivation PAK (l'effecteur de Rac) (Dontchev and Letourneau, 2002) (Aizawa et al., 2001), ce qui indique que Sema3A peut induire des effets répulsifs indépendamment de Rac. L'activation de LIMK par Sema3A pourrait impliquer un rôle pour Rho dans sa signalisation inhibitrice. Sema 4D, une autre sémaphorine, induit aussi l'effondrement du cône de croissance mais par des voies de signalisation distinctes de celles induites par Sema3A. Les effets de Sema4D sont causés par l'activation du récepteur plexin B qui forme un complexe avec le récepteur MET (Giordano et al., 2002). L'activation des cascades de signalisation par plexin B supprime les effets de Rac (Vikis et al., 2002) et active Rho (Hu et al., 2001), par les GEFs PDZ-RhoGEF et LARG (Aurandt et al., 2002; Driessens et al., 2002; Hirotani et al., 2002; Perrot et al., 2002; Swiercz et al., 2002).

1.8.7.2 Slits

Les slits sont des facteurs de guidage répulsifs. Ils se lient aux récepteurs transmembranaires Robo (Roundabout) (Guan and Rao, 2003; Huber et al., 2003). La portion intracellulaire de Robo comprend 3 motifs conservés CC0, CC2 et CC3. Les effets répulsifs de slits-Robo se font par l'activation de Rac et de son effecteur PAK (Fan

et al., 2003) et par l'inactivation de Rho et Cdc42 (Wong et al., 2001). Le motif CC3 lie le RhoGAP srGAP qui interagit avec et inactive Cdc42 et Rho, mais pas Rac. La surexpression du GEF spécifique pour RhoGEF64C inhibe l'effet de slit et induit l'attraction du cône de croissance (Bashaw et al., 2001).

1.8.7.3 Ephrines

Les éphrines sont des protéines transmembranaires (ephrines B) ou ancrées dans la membrane par un lien GPI (ephrin A). Elles s'associent à leurs récepteurs Eph et ont un effet de répulsion sur le cône de croissance (Flanagan and Vanderhaeghen, 1998; Huber et al., 2003; Huot, 2004). La répulsion par les ephrines se fait par l'activation de la voie Rho-ROCK, puisque l'inhibition de Rho ou ROCK abolit la répulsion induite par les éphrines. L'activation de Rho par les ephrines se fait grâce à l'activation du GEF pour Rho ephexin (Wahl et al., 2000; Shamah et al., 2001; Sahin et al., 2005). Les éphrines ephrinA1-EphA induisent aussi l'inhibition de la voie de signalisation Rac par l'inhibition de l'activation de PAK par Rac. Par contre, Jurney et al., ont montré que l'activation de Rac semble requise pour induire la répulsion par ephrine A2 (Jurney et al., 2002). De plus, les ephrines A et B peuvent induire une signalisation inversée par leurs domaine intracellulaire (Holland et al., 1996; Bruckner et al., 1997)ou par l'association de ces protéines GPI avec des intégrines. Contrairement aux effets caractéristiques des ephrines, l'activation de la signalisation inversée par ephrine A induit l'attraction plutôt que la répulsion du cône de croissance (Holland et al., 1996; Flanagan and Vanderhaeghen, 1998; Davy et al., 1999; Davy and Robbins, 2000; Huai and Drescher, 2001; Huot, 2004).

1.8.7.4 Nétrines

Les nétrines sont des protéines sécrétées qui peuvent induire l'attraction ainsi que la répulsion du cône de croissance dépendamment du complexe de récepteurs qui est activé. Les récepteurs des nétrines sont DCC, UNC-40 et UNC-5. La signalisation par UNC-5 ou DCC seul ainsi que le complexe DCC-UNC-5 induisent la répulsion tandis que DCC peut aussi causer l'attraction (Serafini et al., 1994; Goodman, 1996; Deiner et al., 1997; Culotti and Merz, 1998; Stein et al., 2001; Gitai et al., 2003). Les effets d'attraction par DCC se font via l'activation de Rac et Cdc42 et l'inactivation de Rho. Par contre, il a très récemment été démontré que les nétrines, indépendamment du récepteurs DCC, peuvent aussi activer Rho, qui par ROCK cause aussi des effets d'attraction par une voie de signalisation indépendante de Rac et Cdc42 (Govek et al., 2005).

1.8.7.5 Autres facteurs : morphogènes et chémokines

Il a récemment été montré que les morphogènes BMP, Wnt et Shh ont aussi un rôle dans le guidage axonal. Originalement, ces protéines ont été caractérisées pour leurs rôles dans les étapes du développement neuronal qui précèdent le guidage des axones, tels que la spécification cellulaire neuronale (cell fate) et 'l'embryonic patterning'. Dodd et Butler (Butler and Dodd, 2003) ont montré que BMP7 est un agent qui induit la répulsion des neurones commissuraux (Augsburger et al., 1999; Butler and Dodd, 2003) et Wnt4 et 5 peuvent causer la redirection des axones des neurones commissuraux (Lyuksyutova et al., 2003; Yoshikawa et al., 2003). Le groupe de Tessier-Lavigne a recemment montré que comme pour les netrines, Shh peut induire l'attraction des neurones commissuraux et ce

par la voie de signalisation classiques de Shh (Charron et al., 2003). Les voies de signalisation de ces protéines dans le guidage axonale restent cependant à être clarifiées. Il est possible que ses protéines exercerent différents effets par des voies de signalisation distinctes à différents temps lors du dévelopemment. De plus, il a été suggéré que ces protéines aurait un rôle dans le guidage sur de longues distances contrairement aux semaphorines, netrines, ephrines et slit (Schnorrer and Dickson, 2004).

La chémokine SDF1 et son récepteur Cxcr4 se trouvent dans les leucocytes ainsi que dans des neurones où ils peuvent activer les Rho GTPases. Récemment, il a été montré que SDF1 a aussi un rôle dans le guidage axonal et la migration neuronale. De plus, Sdf1 peut moduler la signalisation par les ephrines, les slits et les sémaphorines (Lu et al., 2001; Lu et al., 2002). Il a été suggeré que les RhoGTPases et l'AMP cyclique seraient responsables du 'crosstalk' entre les voies de signalisation (de Sdf1 et les facteurs de guidage) et les effets qui en découlent (Wu et al., 2001; Chalasani et al., 2003).

1.9 Les lésions de la moelle épinière

Les lésions de la moelle épinière endommagent et brisent les axones des neurones provenant du SNC, ce qui cause l'interruption du flux nerveux et la perte de fonction motrice des individus affectés. De plus, ces lésions peuvent aussi endommager et causer la mort des cellules dans la région entourant le site de la lésion. Chez l'homme, les types de lésions les plus fréquentes sont induites par la compression ou la transection de la moelle épinière. Les expériences sur les lésions de la moelle se font majoritairement dans des modèles de compression (contusion de la moelle) ou de transection chez les rongeurs

ou chez le chat. La recherche dans le domaine des lésions de la moelle épinière cherche à contrer la mort des cellules affectées et à induire la régénération des axones endommagés et la récupération de la fonction motrice

1.9.1 Réponse inflammatoire suite aux lésions de la moelle épinière

Les lésions de la moelle épinière engendrent une forte réponse inflammatoire causée par l'infiltration de cellules immunitaires périphériques (macrophages, lymphocytes, neutrophiles) au site de lésion et par l'activation des astrocytes et de la microglie. Les lymphocytes peuvent activer les macrophages (Cohen and Schwartz, 1999; Yoles et al., 2001; Bethea and Dietrich, 2002; Hausmann, 2003; Popovich and Jones, 2003). Les macrophages et les neutrophiles phagocytent les débris cellulaires et les débris de myéline au site de la lésion. Les macrophages ainsi que la microglie secrètent des cytokines et des chémokines qui sont responsables du recrutement des cellules immunitaires périphériques et de la régulation de l'inflammation dans la moelle épinière. Ces facteurs peuvent aussi participer à la mort des cellules au site de lésion.

1.9.2 Effets des cellules immunitaires dans la moelle épinière

Les neutrophiles sont les premières cellules immuniatiares présentes au site de lésion suite à une blessure de la moelle épinière. Ils s'accumulent généralement autour de l'endothélium vasculaire aux endroits adjacents à l'hémorragie, 3-6 heures après la lésion (Chatzipanteli et al., 2002). Ces cellules ont comme rôle primaire d'éliminer les débris cellulaires (cellules mortes, myéline, débris axonal). Elles peuvent aussi produire certaines protéases endommagentes. Les macrophages sont aussi des cellules

phagocytaires périphériques qui se rendent au site de la lésion pour éliminer aussi les débris cellulaires. Les macrophages secrètent aussi des cytokines tels que des interlukines (Il-1,6,10) et du TNF. Contrairement aux autres cellules périphériques, les lymphocytes T peuvent entrer dans le SNC même en abscence de lésion, car ils peuvent traverser la barriere hemato-encéphalique (BBB; blood brain barrier). Par contre dans un état normal (non lésé) ces cellules sont inactives. Une fois activés, les lymphocytes T peuvent tuer des cellules endommagées, monter une immunité cellulaire ainsi que produire des cytokines et des chémokines (Bethea and Dietrich, 2002; Hausmann, 2003; Popovich and Jones, 2003; Dietrich et al., 2004). Le bris de la BBB est responsable de l'infiltration des cellules immunitaires périphériques dans la moelle lésée avec l'exception d'une faible population de lymphocytes T. Par contre, le bris du BBB fait en sorte qu'un plus grand nombre de lymphocytes T peuvent être recrutés. Toutes ces cellules sont attirées au site de la lésion par les cytokines et chémokines sécrétées entre autre par les cellules immunitaires périphériques, mais aussi par la microglie activée dans le tissu. La microglie représente environ 13% de la population gliale, et est rapidement activée en réponse à une lésion ou un traumatisme du SNC (Watanabe and Raff, 1988). Une fois activée la microglie secrète et libère des cytokines ainsi que certaines chémokines. Ces cellules peuvent aussi sécréter des facteurs trophiques et produire des radicaux libres. Une population significative de microglie activée peut être détectée 24 heures à 7 jours suite à une lésion. Par contre une augmentation des niveaux de cytokines (Il-1 et TNF) quelques heures suite à la lésion indique que la microglie est déjà activée à ce temps, puisque l'invasion de cellules immunitaires périphériques qui sécrètent aussi ces

cytokines ne se fait que quelques jours suite à la blessure (Popovich et al., 1997; Bethea and Dietrich, 2002; Hausmann, 2003; Popovich and Jones, 2003; Dietrich et al., 2004).

1.9.3 Effets de l'inflammation

La réponse inflammatoire suite aux lésions du SNC a comme rôle premier de restaurer l'homéostasie du tissu endommagé. Ceci indique donc que l'inflammation dans ces tissus aurait un but ultimement réparateur. Par contre, les cytokines sécrétées suite à l'activation des macrophages et de la microglie peuvent aussi causer des dommages cellulaires et même causer l'apoptose de cellules endogènes de la moelle épinière (Popovich et al., 1997; Cohen and Schwartz, 1999; Yoles et al., 2001; Bethea and Dietrich, 2002; Hausmann, 2003; Popovich and Jones, 2003; Dietrich et al., 2004).

Les macrophages et la microglie activés peuvent sécréter des facteurs trophiques tels que des neurotrophines et IL-10, qui sont essentiels pour la survie neuronale (DeKosky et al., 1994). Le groupe de David a montré que les macrophages peuvent jouer un rôle bénifique (Miller et al., 1994) suite à une lésion de la moelle épinière. L'implantation de macrophages au site de lésion augmente le nombre d'axones qui régénèrent au-delà du site de la lésion. La présence de molécules inhibitrices provenant de la dégradation de la myéline empêche la régénération des axones suite à une lésion de la moelle épinière et l'augmentation des niveaux de macrophages accélère l'élimination de ces débris. L'élimination d'inhibiteurs de croissance favoriserait donc une croissance axonale plus rapide menant à la régénération des axones lésés. Une approche de vaccination pour augmenter la réponse immunitaire suite à une lésion a aussi été adoptée. Pour ce faire,

des souris et des rats ont été inoculés avec des homogénats de moelle épinière ou avec des protéines inhibitrices de croisance de la myéline pour activer la réponse immunitaire chez ces animaux. L'innoculation cause la production d'anticorps et un grand nombre d'axones en régénération et une récupération fonctionnelle motrice a été observée chez les animaux vaccinés (Huang et al., 1999; Ellezam et al., 2003; Sicotte et al., 2003). Le groupe de Schwartz a utilisé une approche différente : l'administration des cellules T activées dirigées spécifiquement contre des protéines de la myéline (Hauben et al., 2001). Ceci induit une diminution du dommage cellulaire ainsi qu'une augmentation de la récupération dans les animaux traités comparativement aux contrôles suite aux lésions de la moelle épinière. Collectivement ces études indiquent que l'élimination de débris cellulaires et de myéline par les cellules immunitaires augmente la récupération suite aux lésions, indiquant un rôle protecteur de la réponse inflammatoire. De plus, une cytokine qui peut protéger les cellules environnantes est Il-10. Il-10 est une cytokine anti-inflammatoire produite par des lymphocytes T «helper», des macrophages, des astrocytes et la microglie. Il-10 peut bloquer la production de métalloprotéases, de chemokines et de plusieurs autres cytokines, comme le TNF. Il a été montré que l'administration de Il-10 suite à une lésion de la moelle épinière diminue l'inflammation, diminue le volume de la lésion et améliore la récupération fonctionnelle motrice indiquant que Il-10 a un effet neuroprotecteur (Crisi et al., 1995; Bethea et al., 1999; Dietrich et al., 1999; Bachis et al., 2001). Il-10 diminue aussi la mort neuronale en diminuant l'activation de caspases et de NFκβ. Dans les cellules T, Il-10 induit un effet protecteur en augmentant l'expression des protéines anti-aopotitiques BCL-2 et XIAP. Ceci indique que cette cytokine a plusieurs

effets protecteurs, et des traitements qui visent à augmenter les niveaux de Il-10 peuvent être bénifiques (Hausmann, 2003; Popovich and Jones, 2003; Dietrich et al., 2004).

Plusieurs autres cytokines produites en réponse aux lésions de la moelle épinière ont des effets néfastes. Les cytokines et chémokines peuvent réguler la réponse inflammatoire en contrôlant la quantité de cellules immunitaires périphériques qui sont attirées au site de lésion (Moser and Willimann, 2004). Malgré que les cellules inflammatoires périphériques puissent avoir des effets protecteurs, en plus grande quantité elles peuvent aussi augmenter le volume et la grosseur de la lésion (Klusman and Schwab, 1997). De plus, une fois les débris de myéline phagocytés, les cellules peuvent attaquer le reste de la myéline intacte (puisq'elle n'est pas reconnue comme une protéine du 'soi') ce qui inhibe la récupération fonctionnelle. Les cytokines peuvent induire la production d'agents toxiques par les astrocytes, les macrophages et la microglie, tels que le NO et des acides aminés excitotoxiques (comme le glutamate). En plus, certaines cytokines tels que le TNF et Il-1 et 6 peuvent causer la mort de neurones et de cellules gliales suite aux lésions du SNC (Bethea and Dietrich, 2002; Hausmann, 2003; Dietrich et al., 2004).

TNF

Le TNF (Tumor necrosis factor)est une cytokine pro-inflammatoire de 17 kDa produite par des macrophages activés, des astrocytes activés et la microglie activée. Cette cytokine est une des plus étudiée suite aux lésions de la moelle épinière (Selmaj and Raine, 1988; Perry et al., 1995; Schnell et al., 1999; Shohami et al., 1999; Yune et al., 2003; Varfolomeev and Ashkenazi, 2004). Le TNF peut d'abord causer l'augmentation de la

perméabilité de cellules endothéliales, ce qui engendre la migration de cellules immunitaires périphériques dans la moelle épinière lésée. Les niveaux de TNF augmentent 1 heure après une lésion de la moelle et cette augmentation peut persister jusqu' à 7 jours après la blessure initiale. Le TNF présent dans les premières heures après la lésion provient majoritairement de la microglie activée puisqu' à ce temps les autres cellules immunitaires ne sont pas encore présentes en quantité suffisante au site de lésion. Le TNF présent 3-7 jours suite à la lésion est plutôt produit par les macrophages (Perry et al., 1995; Pan et al., 1999; Lee et al., 2000; Yan et al., 2001; Wang et al., 2002a).

Le TNF induit une signalisation intracellulaire par sa liaison à ses récepteurs TNFR 1 et 2 (Locksley et al., 2001; MacEwan, 2002a, b). La signalisation par TNFR1 a un effet plutôt néfaste tandis que celle du TNFR2 est protectrice et/ou réparatrice (Fontaine et al., 2002). Les niveaux d'expression des deux récepteurs sont significativement augmentés en réponse à une lésion de la moelle (Yan et al., 2003). Le rôle exact du TNF en réponse aux lésions du SCN reste controversé puisqu'il peut être protecteur ou pro-apoptotique. Plusieurs études montrent des effets bénifiques du TNF : entre autre, le TNF induit une protection contre des insultes excitotoxiques, et des souris transgéniques TNFR -/- ont un niveau plus élevé de cellules apoptotiques et montrent une diminution de récupération fonctionnelle suite aux lésions de la moelle (Fontaine et al., 2002; Raivich et al., 2002). Plusieurs effets néfastes du TNF sont aussi documentés (Selmaj and Raine, 1988; Simmons and Willenborg, 1990; Chao and Hu, 1994; Talley et al., 1995; Sipe et al., 1996; Akassoglou et al., 1998; Gary et al., 1998; MacEwan, 2002a, b; Deng et al., 2003; Yune et al., 2003) : le TNF cause l'apoptose de neurones et d'oligodendrocytes, et le

blocage de TNF avec des anticorps est neuroprotecteur. De plus, l'injection de TNF dans la moelle et dans le nerf optique cause la démyélinisation des axones (Jenkins and Ikeda, 1992). L'augmentation des niveaux d'expression de TNFR1 in vivo dans la moelle et sa relocalisation membranaire suite aux lésions traumatiques au cerveau cause une augmentation du nombre de cellules apoptotiques (Lotocki et al., 2004). TNFR1 contient des domaines intracellulaires de mort (death domains) et la mort cellulaire médiée par TNFR1 est dépendante de la voie des caspases, plus spécifiquement par l'activation de la caspase 3.

1.9.5 La mort cellulaire suite aux lésions de la moelle épinière

Les lésions de la moelle épinière induisent la mort nécrotique et apoptotique des neurones et des cellules gliales. L'apoptose ne se limite pas au site immédiat de la lésion. La mort des cellules par apoptose peut se produire à quelques millimètres du site de lésion. La condensation de la chromatine et la fragmentation de l'ADN caractéristique de l'apoptose sont détectées dans les neurones dans les premières heures qui suivent la lésion de la moelle épinière et la mort neuronale apoptotique atteint son maximum 24 heures suite au traumatisme (Katoh et al., 1996; Crowe et al., 1997; Liu et al., 1997; Casha et al., 2001; Grossman et al., 2001a). Les cellules gliales de la moelle meurent aussi par apoptose suite aux lésions, à des temps différents des neurones. En effet, la majorité de la mort apoptotique des astrocytes est après environ 3 jours et 7-8 jours suite aux lésions pour les oligodenrocytes. Par contre, ces périodes peuvent s'étendrent car des cellules gliales apoptotiques peuvent être détectées jusqu'à 28 jours suite au traumatisme (Katoh et al., 1996; Crowe et al., 1997; Liu et al., 1997; Shuman et al., 1997; Casha et al., 2001).

Plusieurs événements qui contribuent à la mort cellulaire apoptotique produisent le phénomène de lésion secondaire (secondary injury) (Schwartz and Fehlings, 2002; Jacobs and Fehlings, 2003; Park et al., 2004). Ces événements incluent la relâche d'acides aminés excitateurs tel que le glutamate, l'augmentation des niveaux intracellulaires de calcium, la réponse inflammatoire, la production de protéines cytotoxiques tel que le TNF, la production de radicaux libres et la formation de la cicatrice gliale (Figure 8). Il a été montré que des traitements neuroprotecteurs visant à contrer les effets produits par lalésion secondaire peuvent augmenter la récupération fonctionnelle (Liu et al., 1997), et que la protection d'aussi peu que 10% des neurones peuvent induire une amélioration fonctionnelle (Fehlings and Tator, 1995).

Plusieurs facteurs qui peuvent induire l'apoptose ainsi que des récepteurs pro-apoptotiques sont régulés à la hausse suite aux lésions de la moelle épinière dont le TNF et son récepteur TNF-R1, FAS ligand et son récepteur FAS, le glutamate et ses récepteurs NMDA et AMPA-Kinate, p75 ^{NTR} et les pro-neurotrophines. L'inflammation produite par les lésions de la moelle ont des effets importants, particulièrement la production de cytokines induites par les cellules immunitaires tel que le TNF qui peuvent avoir plusieurs effets néfastes dans le tissu et peut même induire la mort des cellules (voir section sur le rôle de l'inflammation dans les lésions de la moelle épinière). Il existe plusieurs traitements qui visent à contrer l'inflammation dans le but d'augmenter la survie et la récupération fonctionnelle. En effet, présentement le seul traitement approuvé (non- expérimental) chez l'humain pour traiter les blessés médullaires est l'administration de drogues anti-inflammatoires.

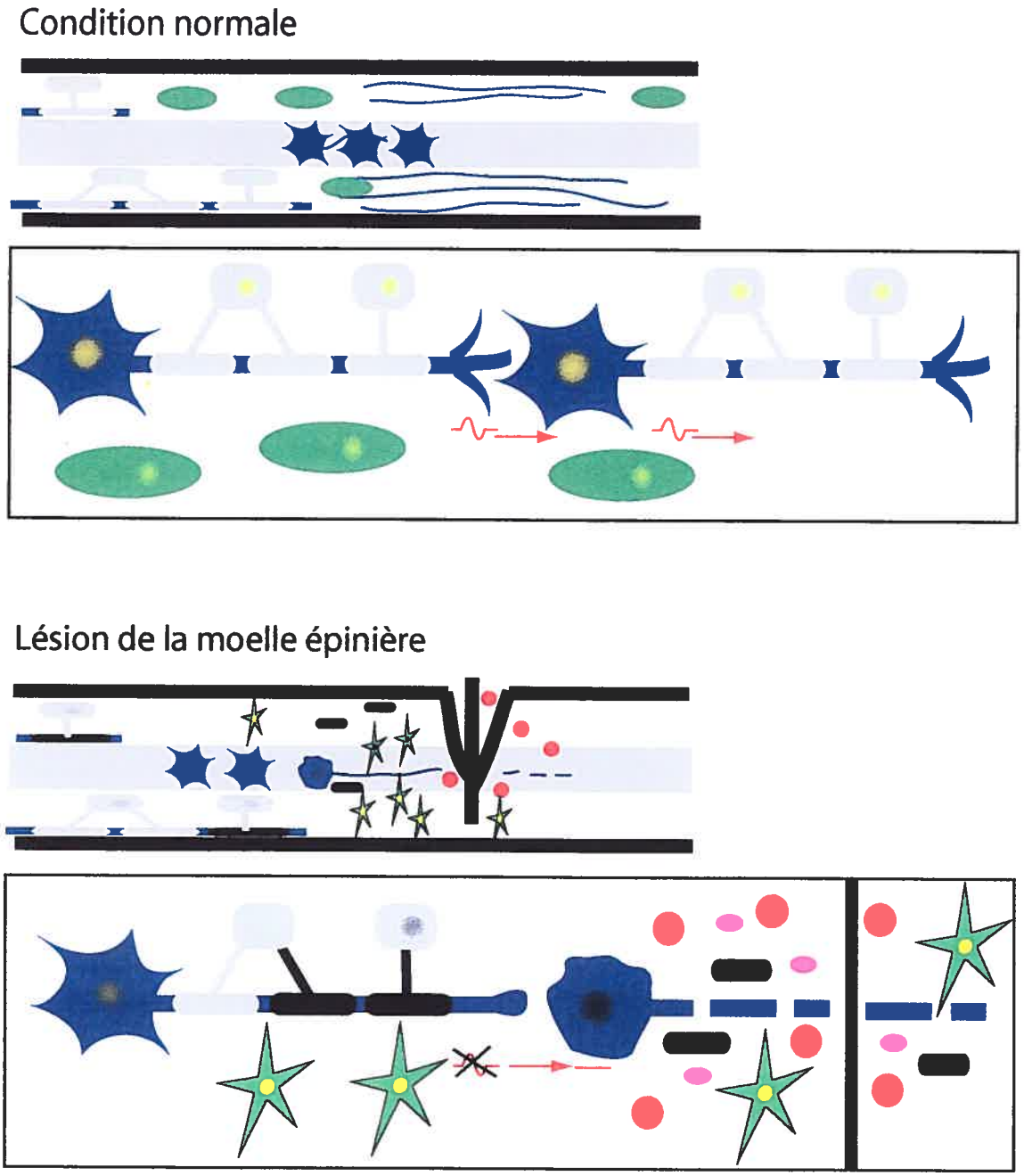

Figure 8 Illustration des évènements de la lésion secondaire
En condition normale : les **neurones** ( ), **astrocytes** ( ), et oligodendrocytes ( ). Suite aux lésions de la moelle, les **neurones** et les oligodendrocytes au site de lésion deviennent apoptotiques ( ) et ne sont plus fonctionnels. La myéline se dégrade induisant la **relâche des protéines inhibitrices de croissance** ( ), il y a la relâche de **cytokines pro-inflammatoires** ( ) et **d'acides aminés excitateurs** ( ) et es **astrocytes deviennent réactifs** ( ) et migrent au site de lésion pour former la cicatrice gliale

1.9.5.1 Signalisation apoptotique suite aux lésions de la moelle

1.9.5.1.2 FAS

Les récepteurs de la famille TNF (TNFR) ont des domaines de mort (death domains) qui sont impliqués dans l'initiation des cascades apoptotiques. FAS et p75 ^{NTR} sont des récepteurs de la superfamille des TNFR qui sont impliqués dans la mort cellulaire apoptotique. Les lésions de la moelle causent aussi une augmentation des niveaux du ligant FAS ligand et de son récepteur FAS dans les cellules neuronales et gliales (Zurita et al., 2001; Demjen et al., 2004). La surexpression de FAS suite aux lésions de la moelle épinière engendre la mort des neurones et oligodendrocytes. De plus, il a recemment été démontré que la neutralisation de FAS diminue non seulement la mort des ces cellules, mais induit aussi une augmentation du nombre de fibres en régénération et de la récupération fonctionnelle des animaux (Demjen et al., 2004).

1.9.5.1.3 p75^{NTR}

Le récepteur p75 ^{NTR}, en plus de ses fonctions dans la régulation de la signalisation des protéines inhibitrices de croissance, joue un rôle important dans la modulation de l'apoptose (Barrett, 2000; Kaplan and Miller, 2000; Dechant and Barde, 2002; Hempstead, 2002; Roux and Barker, 2002; Gentry et al., 2004; Teng and Hempstead, 2004; Zampieri and Chao, 2004) ainsi que dans la mort apoptotique suite aux lésions du SNC (Cheema et al., 1996; Frade and Barde, 1999; Dechant and Barde, 2002). Le niveau d'expression de p75 ^{NTR} est augmenté suite aux lésions de la moelle (Casha et al., 2001;

Beattie et al., 2002) et cette augmentation est corrélée avec une augmentation du nombre de cellules apoptotiques. De plus, la diminution des niveaux de p75 ^{NTR} réduit la mort des neurones suite à une axotomie (Cheema et al., 1996) et diminue le nombre de cellules apoptotiques dans la moelle épinière suite à une lésion par contusion (Brandoli et al., 2001). Les souris knock out pour p75 ^{NTR} montrent aussi une diminution du nombre de cellules apoptotiques dans la moelle épinière (Frade and Barde, 1999). Il a récemment été montré que les pro-neurotrophines (pro-NGF et pro-BDNF), protéines précurseurs des neurotrophines, induisent l'apoptose par leur association avec p75 ^{NTR} (Beattie et al., 2002; Harrington et al., 2004; Fayard et al., 2005; Teng et al., 2005). De plus, le pro-NGF induit la mort des neurones et des oligodendrocytes de la moelle de façon p75 ^{NTR} dépendante. Ces résultats indiquent un rôle important pour p75 ^{NTR} dans la modulation de la mort apoptotique des cellules de la moelle épinière lésée.

1.9.5.1.4 Le glutamate

Le glutamate est un acide aminé excitateur pour les cellules nerveuses. Les lésions de la moelle épinière induisent une forte augmentation de glutamate (Panter et al., 1990; Liu et al., 1999; Xu et al., 2004) qui cause la mort par apoptose des neurones et ded cellules gliales, particulièrement des oligodendrocytes (Liu et al., 1999; Xu et al., 2004). Il existe 2 classes de récepteurs pour le glutamate, les récepteurs métabotropes (MgluRs) et les récepteurs ionotropiques (NMDA et AMPA-Kainate). L'expression des deux classes de récepteurs est augmentée suite aux lésions de la moelle épinière, ce qui induit la mort des cellules par le glutamate (Grossman et al., 2001b; Mills et al., 2001). Le traitement avec des antagonistes de ces récepteurs réduit le dommage et la mort des cellules neuronales et

gliales suite aux lésions de la moelle (Teng and Wrathall, 1996; Wrathall et al., 1997; Grossman et al., 1999; Rosenberg et al., 1999a; Rosenberg et al., 1999b; Xu et al., 2004). L'inhibition du récepteur AMPA par un antagoniste spécifique augmente la survie des oligodendrocytes de la moelle épinière (Wrathall et al., 1997; Rosenberg et al., 1999a; Rosenberg et al., 1999b) en inhibant la mort de ces cellules par le glutamate (Xu et al., 2004). De plus, les récepteurs ionotropiques sont couplés à des canaux ioniques tels que les canaux calciques. L'augmentation de la signalisation par ces récepteurs dus à l'excès de glutamate, peut donc aussi entrainer des dommages cellulaires (Choi, 1992) en causant l'aumentation de la concentration intracellulaire de calcium, qui est aussi impliqué dans l'induction de l'apoptose.

1.9.5.1.5 La GTPase Rho

La GTPase Rho a aussi un rôle important comme médiateur de l'apoptose (section Rho et l'apoptose). Rho est significativement activée dans la moelle suite à une lésion. L'inactivation de Rho diminue le nombre de cellules apoptotiques dans la moelle épinière de manière $p75^{NTR}$ dépendante et dans les cellules ganglionnaires de la rétine (Fischer et al., 2004b; Bertrand et al., 2005). Tous les facteurs décrits ci-dessus (le TNF, FAS, $p75^{NTR}$ et le glutamate) en plus de la thrombine qui est présente suite aux lésions de la moelle épinière et qui peut aussi induire la mort apoptotiques des neurones et astrocytes, peuvent activer Rho. Cette GTPase est donc une cible importante pour contrer l'apoptose suite aux lésions de la moelle épinière.

Les traitements qui visent à diminuer la mort ou à augmenter la survie des cellules sont non seulement importants pour sauver la population cellulaire affectée, mais aussi pour

protéger les cellules qui ne sont pas directement affectées par la lésion. La survie des oligodendrocytes pourrait aussi influencer le passage de l'influx nerveux et pourrait avoir comme conséquence de diminuer la perte de fonction motrice suite aux lésions de la moelle épinière. Ceci peut mener à une augmentation de la régénération des axones et une récupération fonctionnelle motrice.

1.10 Lésions au cerveau (TBI: Traumatic Brain Injury)

En Amérique et en Europe, les lésions au cerveau sont la cause principale de mort et de séquelles permanentes chez les jeunes adultes de 45 ans et moins. Ces blessures ont des effets dévastateurs : elles entraînent des pertes de fonctions motrices et cognitives et contribuent au développement de l'épilepsie chez les personnes affectées. Il est estimé que 2% de la population américaine souffre de ce type de lésion et que 5-50% de ces gens ont des convulsions post-traumatiques de type épileptique (Golarai et al., 2001; Beghi, 2003; Bruns and Hauser, 2003). Les 2 causes majeures du TBI sont des accidents résultants d'impact mécanique (contact ex. impact au sol, accident sportif) et des mouvements de la tête (accélération-décélération ex. accident d'automobile). Dans les deux cas, le TBI peut causer des fractures du crâne, la déformation du tissu cérébral, la rupture de la matière blanche et grise ainsi que la rupture de vaisseaux sanguins dans le cerveau. Des dommages hypoxiques et ischémiques sont aussi très communs suite au TBI et causent l'enflure et l'oedème des tissus et des cellules ainsi que des dommages cellulaires (Bramlett and Dietrich, 2004; Gaetz, 2004; Thompson et al., 2005a; Thompson et al., 2005b).

Les premières études sur des tissus affectés suite au TBI causée par des mouvements d'accélération-décélération, ont rappoté de la dégénéresence dans la matière blanche. Puisque des commotions cérébrales et la perte de connaissances sont des effets de ce type de TBI, il a initialement été suggèré que le tronc cérébral (plus particulièrement les noyaux réticulaires et les neurones cholinergiques de cette région) était la région la plus affectée (Gaetz, 2004). La perte de mémoire associée au TBI indique aussi des effets dans l'hippocampe puisque ce dernier est le siège de l'apprentissage et de la mémoire (Gaetz, 2004). Des études plus récentes montrent que le cortex, l'hippocampe et le tronc cérébral sont très affectés par des lésions traumatiques au cerveau. En effet toutes ces régions montrent des cellules axotomisés ainsi que des neurones apoptotiques (voir section mort cellulaire associé au TBI ci-dessous) (Royo et al., 2003; Thompson et al., 2005a; Thompson et al., 2005b).

1.10.1 Lésions diffuses axonales et accumulation de protéines dans le TBI

Le TBI cause des lésions diffuses axonales ou DAI (diffuse axonal injury). La rupture des axones et des vaisseaux sanguins dans le cerveau est due au traumatisme primaire de la lésion. Il a été proposé que le DAI est un processus patho-physiologique dûe à la lésion secondaire causée par l'entrée d'ions proche des nœuds de Ranvier qui endommagent le cytosquelette et les microtubules du neurone. Le DAI est caractérisé par l'accumulation de protéines et d'organelles dans les axones des neurones dû à l'altération du transport axonal. Ceci résultant en l'endommagement du cytosquelette (Bruns and Hauser, 2003; Smith et al., 2003b; Bramlett and Dietrich, 2004). Aussi tôt qu'une heure suite au traumatisme, les axones des neurones atteints enflent et s'engorgent d'organelles

(mitochondries et RE lisse), à ce temps, aucune rupture de la gaine de myéline ou du cylindre axonal n'est détectée. Dans les heures qui suivent le TBI, les axones continuent à s'enfler et environ 3 heures suite à la lésion il y a déconnection de l'axone (de la cible ou de la synapse). Environ 4-6 heures suite au TBI, les neurofilaments sont désorganisés et commencent à se fragmenter. À ce temps une forte expression de neurofilament (de 68kDa) est détectable. Quelques jours suite au TBI, les neurites sont très gonflées et ne contiennent plus de myéline. De plus, il y une forte accumulation d'organelles et de protéines à l'extrémité distale de l'axone. Cette extrémité montre des signes de dégénérescence et contient des bulbes de rétraction axonal (Smith et al., 2003b). Il y aussi une population grandissante de macrophages qui s'accumulent à cette extrémité. Plusieurs facteurs sont impliqués dans l'initiation de l'enflure des axones endommagés. En premier lieu, la diminution du flux sanguin induit une ischémie et l'enflure des cellules. La déformation de l'axone induit l'augmentation de l'entrée de calcium dans les cellules. Le calcium active des protases (les calpaines) qui vont dégrader les protéines neuronales tel que la tubuline, les MAPs et les neurofilaments. De plus, l'entrée de calcium a comme effet d'activer des phospholipases et la PKC qui vont respectivement attaquer les membranes et induire la production de glutamate neurotoxique. Le glutamate peut en son tour produire un feedback en augmentant les niveaux de calcium intracellulaire.

Une augmentation des niveaux protéiques de βA et de APP sont retrouvés dans ces axones et l'identification immunohistochimique de ces protéines est utilisée pour identifier le DAI suite au TBI (Smith et al., 2003a; Smith et al., 2003b; Olsson et al.,

2004). Cette accumulation est dûe à l'altération du transport axonal résultant de l'endommagement du cytosquelette. L'APP est normalement transportée par la machinierie du transport axonal rapide et suite aux lésions il y a une diminution et une perte du transport axonal ce qui induit l'accumulation de la protéine. De plus, la rupture ou la fuite des axones enflés peut induire la relâche d'agrégats de protéines accumulées qui peuvent augmenter le dommage aux cellules environnantes comme peut en être le cas pour la βA qui forme des plaques extracellulaires (Smith et al., 2003a; Smith et al., 2003b). D'autres protéines tels que les neurofilaments et la synucléine s'accumulent aussi dans les axones endommagés, ces accumulations sont aussi dues aux déficits de transport axonal (Smith et al., 2003b).

1.10.2 Mécanismes impliqués dans la pathologie secondaire suite au TBI

Le TBI induit plusieurs effets qui contribuent au dommage et à la mort des neurones (Morganti-Kossmann et al., 2001; Morganti-Kossmann et al., 2002; Thompson et al., 2003; Bramlett and Dietrich, 2004; Thompson et al., 2005a). En premier lieu, le TBI cause souvent des hémorragies dans le cortex. Ceci peut induire une ischémie du tissu (ischémie hémorragique) dûe au manque d'oxygène induit par la perte de sang dans le tissus. L'ischémie résultante induit alors la mort apoptotique des neurones. Un autre effet du TBI est l'œdème des tissus et des cellules. L'enflure des cellules peut induite la mort cellulaire par la simple augmentation de la pression intracellulaire. L'échec des pompes de Na-K induisent l'accumulation de (sodium) Na et d'eau dans la cellule (Thompson et al., 2003; Thompson et al., 2005a). De plus, l'augmentation des niveaux de gluatamate et de glycine extracellulaire (Sihver et al., 2001), produit en réponse à la lésion, induisent

non seulement l'augmentation du Ca^{+2} mais aussi du (sodium) Na intracellulaire. Cet influx de Na cause l'augmentation du niveau d'eau dans la cellule induisant l'enflure de cette dernière. De plus, la production de radicaux libres par la cellule est aussi liée à l'œdème cellulaire.

Comme pour les lésions de la moelle épinière, le TBI induit aussi une forte réponse inflammatoire dans les tissus affectés. Immédiatement suite à la lésion, les lymphocytes polymorphonucléaires (PML) infiltrent le site de la lésion (Royo et al., 1999), suivi des macrophages. Les macrophages et la microglie activée produisent des cytokines pro-inflammatoires tel que le TNF et IL-1 (Marciano et al., 2002). Il y a une augmentation bilatérale du TNF dans le cortex et l'hippocampe dans des modèles animaux de TBI (Fan et al., 1996; Shohami et al., 1997; Knoblach et al., 1999; Shohami et al., 1999; Lenzlinger et al., 2001; Morganti-Kossmann et al., 2001; Kinoshita et al., 2002b; Kinoshita et al., 2002a; Lotocki et al., 2004; Vitarbo et al., 2004; Marklund et al., 2005; Thompson et al., 2005a). Ces cytokines peuvent par la suite produire des effets toxiques. Les macrophages et la microglie produisent aussi des neurotrophines (TNF et BDNF) dans le cortex et l'hippocampe en réponse au TBI (Marciano et al., 2002). Ce type de lésion induit aussi la production du glutamate et une augmentation de l'expression de ses récepteurs, ce qui ensemble induit la mort des cellules.

1.10.3 Mort cellulaire suite au TBI

Chez l'homme, suite au TBI, il y a des preuves de mort cellulaire apoptotique dans le cortex, l'hippocampe, le cervelet et le thalamus (Royo et al., 2003; Raghupathi, 2004).

Ces mêmes structures sont aussi affectées dans les modèles animaux de TBI. Il y a du dommage bilatéral dans le cortex et de la mort neuronale bilatérale dans l'hippocampe dans la matière grise et blanche. Suite au TBI les neurones et les cellules gliales (astrocytes et oligodendrocytes) meurent par apoptose. Les neurones sont le premier type cellulaire à mourir suivi des astrocytes et des oligodendrocytes (Conti et al., 1998; Newcomb et al., 1999; Clark et al., 2000). Dans les 6-48 heures suivant le traumatisme, les neurones du cortex cérébral et de l'hippocampe sont apoptotiques. 1-2 semaines suivant le TBI la vague apoptotique affecte les neurones principalement du thalamus. Immédiatement suite au TBI, les cellules meurent par nécrose. Des neurones nécrotiques sont détectés dans le cortex, dans les régions CA1,2 et 3 de l'hippocampe et dans le gyrus dentelé et ce entre 10 minutes et 24 heures post-TBI. La mort apoptotique se produit entre 12-72 heures post-lésion où les neurones TUNEL+ sont détectés dans le cortex, la matière blanche subcorticale et dans la région CA3 de l'hippocampe. L'apoptose dans le cortex est biphasique : la mort neuronale augmente 24 heures suite à la lésion, diminue et remonte après 7 jours. Dans l'hippocampe, l'apoptose est détectée après 12 heures et atteint son maximum 48 heures suite à la lésion; les niveaux de cellules apoptotiques dans l'hippocampe diminuent constamment après ce temps. Dans le thalamus, la vague d'apoptose augmente après 7 jours et est encore significativement élevé 14 jours suite au traumatisme (Conti et al., 1998; Newcomb et al., 1999; Clark et al., 2000; Royo et al., 2003). De plus, il y a aussi une augmentation des niveaux de capsases8 et de caspase3 dans les neurones et oligodendrocytes du cortex et de l'hippocampe. L'inhibition de la caspase3 suite au TBI induit une baisse de la fragmentation de l'ADN, une diminution de

cellules TUNEL+ et augmente la fonction neurologique suite à la lésion (Royo et al., 2003).

1.11 Objectifs

Nous avons étudié le rôle de l'activation de Rho suite aux traumatismes du SNC. Notre principal focus a été l'étude de Rho suite aux lésions de la moelle épinière et suite au TBI. Il y a plusieurs aspects neuro-patho-physiologiques communs suite au traumatisme de la moelle et du cerveau tel que la relâche de glutamate et de protéines inhibitrices de croissance ainsi que la forte réponse inflammatoire. Il y a aussi plusieurs effets uniques associés à chacune de ces lésions. Les lésions de la moelle épinière causent un dommage sévère à la matière blanche tandis que dans le TBI, les dommages à la matière grise sont plus étudiés. De plus ce dernier type de lésion induit aussi des effets contralatéraux. Dans cette thèse, nous montrons que Rho est activée suite à ces deux types de lésions (de la moelle et TBI) et nous avons examiné les conséquences de cette activation.

1.11.1 Objectif 1

Notre premier objectif était de directement étudier les états d'activation de Rho. Pour ce faire la technique d'essai pull down a été utilisée puisque aucun anticorps commercial discernant les formes actives et inactives de Rho n'existe. Lehmann a montré que l'inactivation de Rho par la C3 induit la croissance axonale dans le nerf optique. Par la suite, Dergham a montré que la myéline inhibe la croissance axonale de neurones en culture. Nous avons alors émis l'hypothèse que les protéines inhibitrices de la myéline activent Rho. Avec l'essai pull down nous avons montré pour la première fois que ces

protéines activent Rho et que l'inactivation spécifique de Rho par la C3 perméable (confirmé par pull down) induit la croissance axonale en présence de ces inhibiteurs. Ces résultats sont publiés dans Winton et al., 2002 (Winton et al., 2002)(en annexe) et Dubreuil (chapitre 2 de la thèse) (Dubreuil et al., 2003).

1.11.2 Objectif 2

Les lésions axonales dans le SNC induisent la formation de cône de croissance et leur navigation est régulée par Rho. Suite à l'étude de Lehmann montrant que l'inactivation de Rho induit la croissance axonale dans le nerf optique nous avons émis l'hypothèse que les lésions de la moelle épinière activent Rho. A cet effet nous avons montré que Rho est activée suite à une lésion de la moelle chez la souris et chez le rat. Nous avons aussi montré que Rho est activée dans les neurones et les cellules gliales endogènes de la moelle suite à ces lésions. Ces résultats sont publiés dans Dubreuil (chapitre 2) (Dubreuil et al., 2003).

1.11.3 Objectif 3

Nous avons voulu examiné si l'activation de Rho est une réponse spécifique aux lésions de la moelle épinière ou si cette activation est commune aux lésions su SNC. Nous avons émis l'hypothèse que due aux similaritées dans l'effet des lésions aux axones, que Rho serait activée suite à d'autres types de lésions du SNC. Nous avons en premier lieu montré que Rho est activée suite à une ischémie cérébrale. Ces résultats sont soumis pour publication dans le journal MCN (chapitre 3 du corps de la thèse). Nous avons par la suite montré que Rho est activée dans le cortex et l'hippocampe suite TBI. De plus nous

avons trouvé que Rho est aussi activée du côté contralatéral suite au TBI. Ces résultats sont soumis pour publication dans le journal Expérimental Neurology (chapitre 4 du corps de la thèse). En plus nous avons aussi trouvé que Rho est activée bilatéralement dans le cortex et l'hippocampe suite aux attaques de types épileptique. Puisque les convulsions épilpetiques sont communes suite au TBI, ces dernières peuvent expliquer l'activation contralatérale de Rho suite aux TBI. Ces résultats sont soumis pour publication dans le journal Expérimental Neurology (chapitre 4 du corps de la thèse).

1.11.4 Objectif 4

En dernier lieu, nous avions comme objectif d'examiner les voies de signalisation en aval et amont de Rho et nous avons utilisé le modèle de lésion de la moelle épinière pour étuder ces effets. Les études de Dergham ont montré que la récupération fonctionelle 24 suite aux lésions des animaux traités avec la C3, est significativement plus élevée que chez les animaux non traités. A ce temps, puisque les axones n'ont pas le temps de régénérer, nous avons émis l'hypothèse que cet effet était du à une augmentation de la survie des cellules suite aux lésions de la moelle. En effet, l'inactivation de Rho augmente de facon significative la survie cellulaire suite aux lésions de la moelle épinière chez les souris et les rats. Nous avons montré que l'activation de Rho et la mort cellulaire résultantes sont dépendantes du récepteur $p75^{NTR}$ et que Rho peut aussi réguler l'expression de ce dernier dans la moelle. Ces résultats sont publiés dans Dubreuil et al.(chapitre 2) (Dubreuil et al., 2003). De plus nous avons aussi montré que la distribution de Rho active dans la moelle épinière peut être corrélée à la préscence du TNF. Ces résultats sont soumis pour publication dans le journal MCN (chapitre 3 du corps de la

thèse). Pour mieux comprendre le rôle de Rho dans la mort cellulaire apoptotique, nous montrons aussi que la présence combinée des protéines de la myéline et du TNF induisent la mort neuronale in vivo et in vitro de faççon dépendante de l'activation de Rho. Ces résultats sont en révision pour la publication dans Nature Neurosceince et son présenté en anexe dans Winton et al., troisième article de l'anexe.

Chapitre 2

2 Premier article

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

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2.1 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.

2.2 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., 1996). 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 promotes axon regeneration and functional recovery after spinal cord injury (SCI) in rats and mice (Dergham et al., 2002; Hara et al., 2000; Lehmann et al., 1999) However, it is not known how CNS injury may affect Rho expression and activation.

The mechanism where growth inhibitory proteins may affect Rho signalling are beginning to be understood. It has recently been shown that NgR can activate Rho in a

p75 neurotrophin receptor ($p75^{NTR}$) dependent manner (Wang et al., 2002). First, $p75^{NTR}$ null mutant mice are not inhibited by MAG, showing a key role of $p75^{NTR}$ in growth inhibitory 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 as TNF (Neumann et al., 2002) can alter Rho activation levels. Levels of Rho expression are altered in malignant disease (Clark et al., 2000; Fritz et al., 1999; Suwa et al., 1998) however, little is known about how traumatic injury and disease in the CNS alter Rho activation states *in vivo*.

Rho activation can be studied by probing cell homogenates with the Rho binding domain (RBD) from the Rho-GTP interacting protein, rhotekin (Reid et al., 1996). We use this pull down assay to detect a significant increase in active Rho in CNS tissue 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.

2.3 Results

2.3.1 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., 1996). 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., 2002).

2.3.2 Rho is activated after traumatic SCI injury

To investigate RhoA activation states after traumatic CNS injury, we measured active RhoA levels in rodent tissue homogenates by Rho pull down assay. We studied tissue isolated from regions of traumatically injured spinal cords of both rats and mice because of their different responses to injury. Rats develop an extensive necrotic lesion cavity after SCI whereas mice do not (Steward, 1999). In rats, we examined spinal cord regions after transection or contusion injury. Experiments are shown as paired control and injured CNS samples (Figures 2 and 3). Homogenates from different animals were not 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 the experiments in Figure 2B were probed with a monoclonal antibody raised against MAG. MAG levels remain unchanged; minimal protein degradation was visible in overexposed blots (Figure 2D). The sustained activation of Rho after SCI may result, in part, from contact of damaged cells with myelin-derived inhibitory proteins.

2.3.3 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 (Figure 3C) showing that endogenous cells from the spinal cord take up C3-05 *in vivo*.

2.3.4 Rho is active in neurons and glial cells after SCI

Although the location of C3-05 can indicate the potential to suppress Rho activation, it does not permit us to determine which cells have increased Rho activation after SCI. To further examine increased GTP-Rho after SCI, we used a modified 'in situ' pull down

method, omitting cell transfection with recombinant Rho (Li, 2002) to detect 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.

2.3.5 Treatment with C3-05 protects cells from apoptosis after injury

Both neurons and glia undergo apoptosis after spinal cord injury in rat, which leads to the formation of a large lesion cavity (Grossman et al., 2001; Liu et al., 1997; Shuman et al., 1997). Even though mice do not develop cavitation at the site of SCI, we detected

apoptotic neurons, astrocytes and oligodendrocytes by double staining with cell-specific markers and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (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., 1997).

2.3.6 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 present at the lesion sites (Figure 2D) . In order to determine the mechanism by which Rho is activated after injury, we examined if Rho activation was $p75^{NTR}$ dependant. We first examined if $p75^{NTR}$ was present in cells containing active Rho. We found that $p75^{NTR}$ colocalizes with active Rho in both grey (Figure 7A, top panel) and white matter (Figure

7A, bottom panel) 24 hours after SCI. We then examined levels of active Rho in mice lacking the $p75^{NTR}$ gene ($p75^{NTR}$-/-). No change in Rho activation was detected 24 hours after SCI. Rho activation was, however, detected in these animals 3 days after injury (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., 2002; Casha et al., 2001; Widenfalk et al., 2001). Treatment with C3-05 not only blocked the increase in $p75^{NTR}$ protein levels after SCI, as detected by Western blot (Figures 8C) but also reduced the number of $p75^{NTR}$ -TUNEL labeled cells (Figure 8B). These results suggest that early on, Rho activation after SCI is

mediated, at least in part, by $p75^{NTR}$. These results also indicate that Rho activation is important for $p75^{NTR}$ upregulation after SCI.

2.4 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., 2000). 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 from apoptotic cell death. We further show a role for $p75^{NTR}$ in mediating Rho activation after SCI.

2.4.1 Mechanisms for sustained Rho activation after SCI

Activation of Rho in CNS tissue after SCI injury likely results from changes in the local inhibitory and inflammatory environment. Both neurons and glial cells show increased Rho activation. There is evidence that the inhibitory environment of the CNS 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., 2002). 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; Schwartz and Fehlings, 2002). Our failure to detect Rho activation in $p75^{NTR}$ null mutant mice early after SCI (24 h) suggests activation of NgR signaling to $p75^{NTR}$ is an early event in CNS injury. However, 3 days after injury in these mice, Rho activation was observed, a finding that indicates that at later time points Rho activation is $p75^{NTR}$ independent. Many factors activate Rho independently of $p75^{NTR}$ such as semaphorins, ephrins and thrombin that are know to be present after SCI (De Winter et al., 2002;

Donovan et al., 1997; Shirvan et al., 2002; Swiercz et al., 2002; Wahl, 2000). The $p75^{NTR}$ dependence of early Rho activation is interesting because thrombin and TNF, both 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.

2.4.2 Rho activation leads to apoptosis after SCI

Rho GTPases are known regulators of apoptosis in various cell types. In non-neuronal cells such as epithelial cells (Fiorentini et al., 1998a; Fiorentini et al., 1998b), endothelial cells (Hippenstiel et al., 2002), T cells (Gomez et al., 1997; Moorman et al., 1996), and some fibroblasts (Bobak et al., 1997), inactivation of Rho causes apoptosis through a Bcl-2 dependant mechanism. In other cells, such as PC-12 cells (Mills et al., 1998) and endogenous cells of the spinal cord, as we show here, inactivation of Rho protects cells

from apoptosis. In NIH 3T3 fibroblasts, over expression of active Rho induces cell death upon serum withdrawal (Jimenez et al., 1995). Thus, the cell background 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). In neurons, TNF activates Rho (Neumann et al., 2002) and antibody mediated blocking of TNF reduces apoptosis after SCI (Lee et al., 2000). These data support our direct evidence that Rho activation contributes to apoptosis after traumatic spinal cord injury. Rho activation in neurons alone may not be sufficient to cause cell death, such as when neurons are plated on inhibitory substrates in culture. Our data suggest that *in vivo* the combination of multiple Rho activating factors, including the myelin derived inhibitory factors, contribute to apoptotic signaling cascades.

2.4.3 Mechanism of Rho activation

Our results show that blocking the activation of Rho after SCI prevents an increased synthesis of $p75^{NTR}$ protein (Figure 8C), implicating Rho activation in the transcriptional changes in $p75^{NTR}$ expression. This result of C3-05 can be explained by a mechanism in which the change in transcriptional factor activation is Rho-dependant (Figure 9). It is known that Rho is involved in the activation of transcription factors in the nucleus that control synthesis of pro-apoptotic mRNAs such as c-jun and NFκβ, members of the

$p75^{NTR}$ 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., 2002; 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., 2002). 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 SCI, and that inactivation of Rho is cell protective.

2.5 Materials and methods

2.5.1 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.

2.5.2 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.

2.5.3 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).

2.5.4 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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2.8 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., 2002) 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.

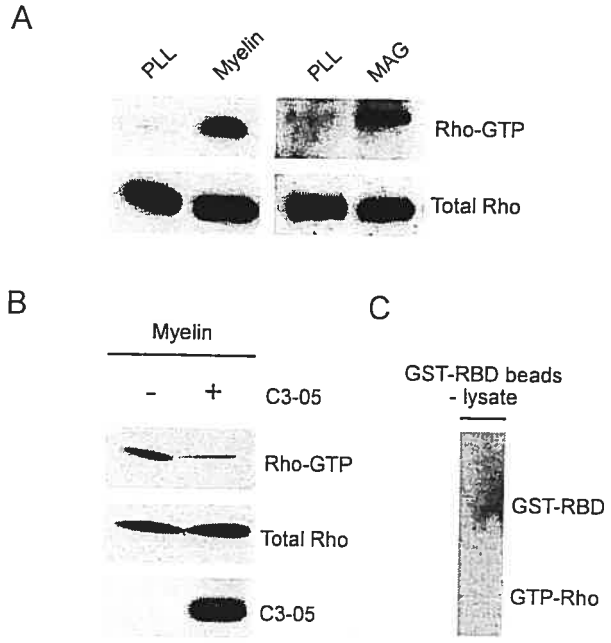
A
PLL
Myelin
PLL
MAG
Rho-GTP
Total Rho
B
Myelin
-
+
C3-05
Rho-GTP
Total Rho
C3-05
C
GST-RBD beads
- lysate
GST-RBD
GTP-Rho

Figure 1

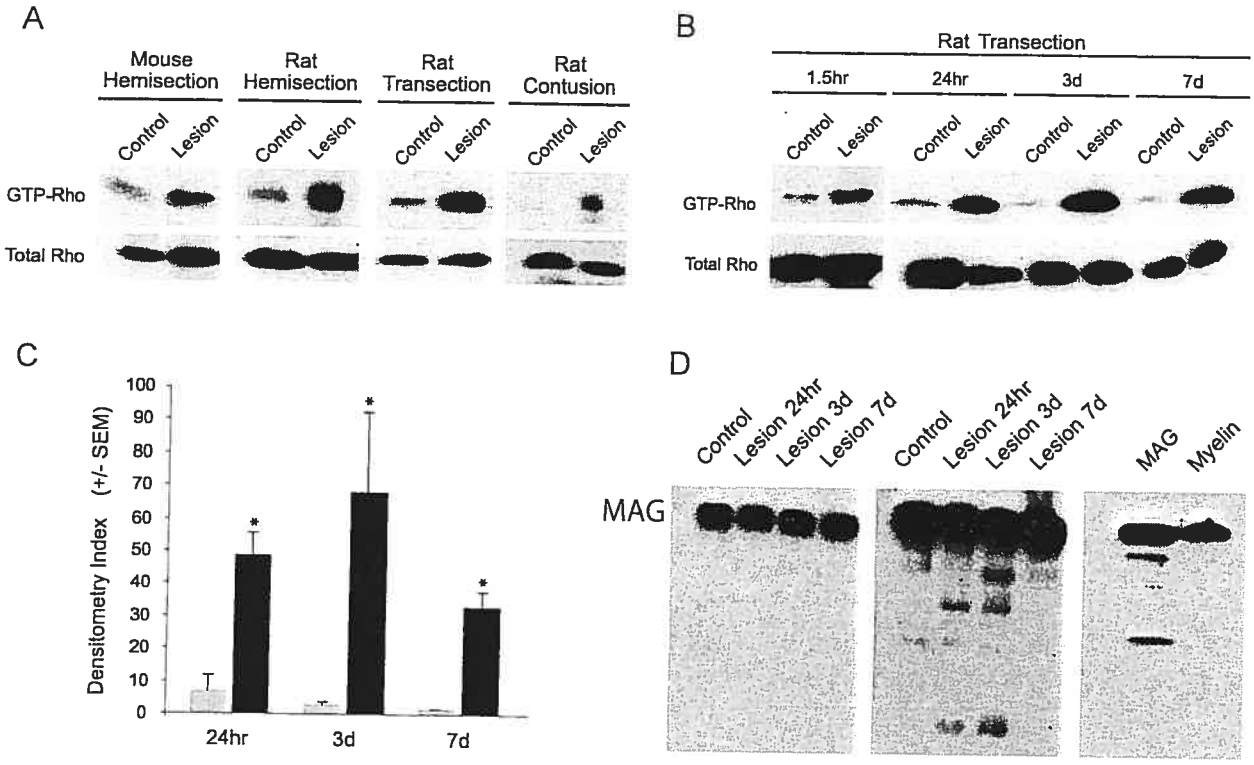
A
Mouse Hemisection
Rat Hemisection
Rat Transection
Rat Contusion
Control
Lesion
GTP-Rho
Total Rho
B
Rat Transection
1.5hr
24hr
3d
7d
Control
Lesion
GTP-Rho
Total Rho
C
Densitometry Index (+/- SEM)
100
90
80
70
60
50
40
30
20
10
0
24hr
3d
7d
D
Control
Lesion 24hr
Lesion 3d
Lesion 7d
MAG
Myelin

Figure 2

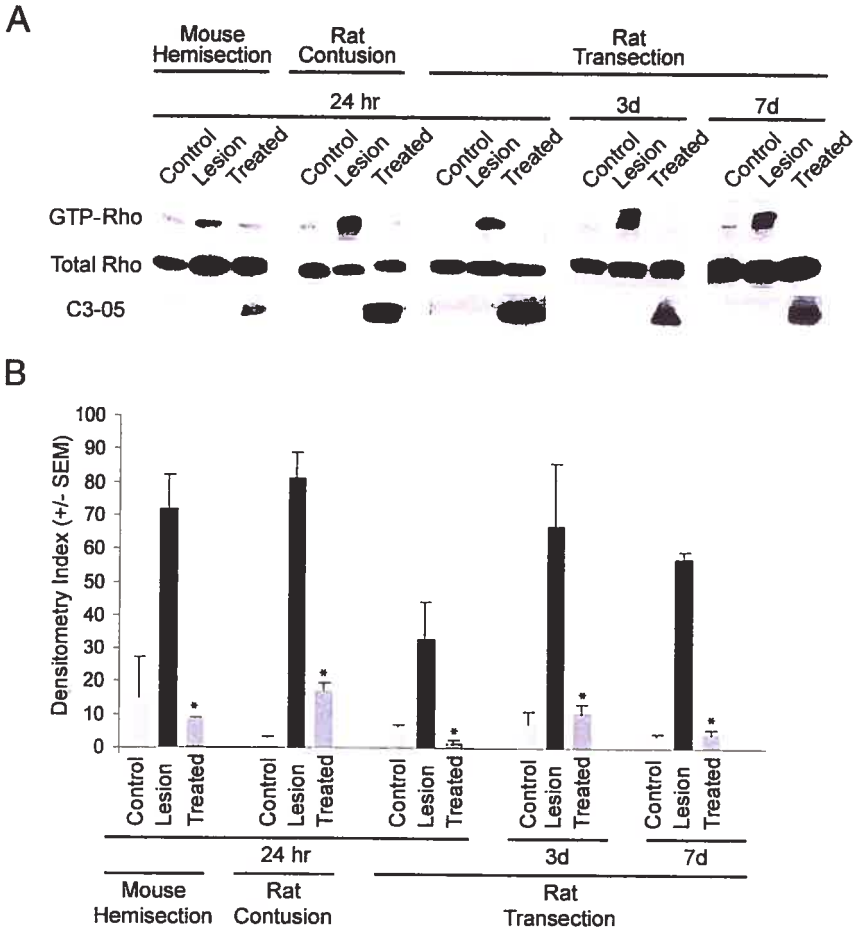
A
Mouse Hemisection
Rat Contusion
Rat Transection
24 hr
3d
7d
Control
Lesion
Treated
GTP-Rho
Total Rho
C3-05
B
Densitometry Index (+/- SEM)
100
90
80
70
60
50
40
30
20
10
0
24 hr
3d
7d
Mouse Hemisection
Rat Contusion
Rat Transection
C
NeuN
C3
GFAP
C3
MAB328
C3

Figure 3

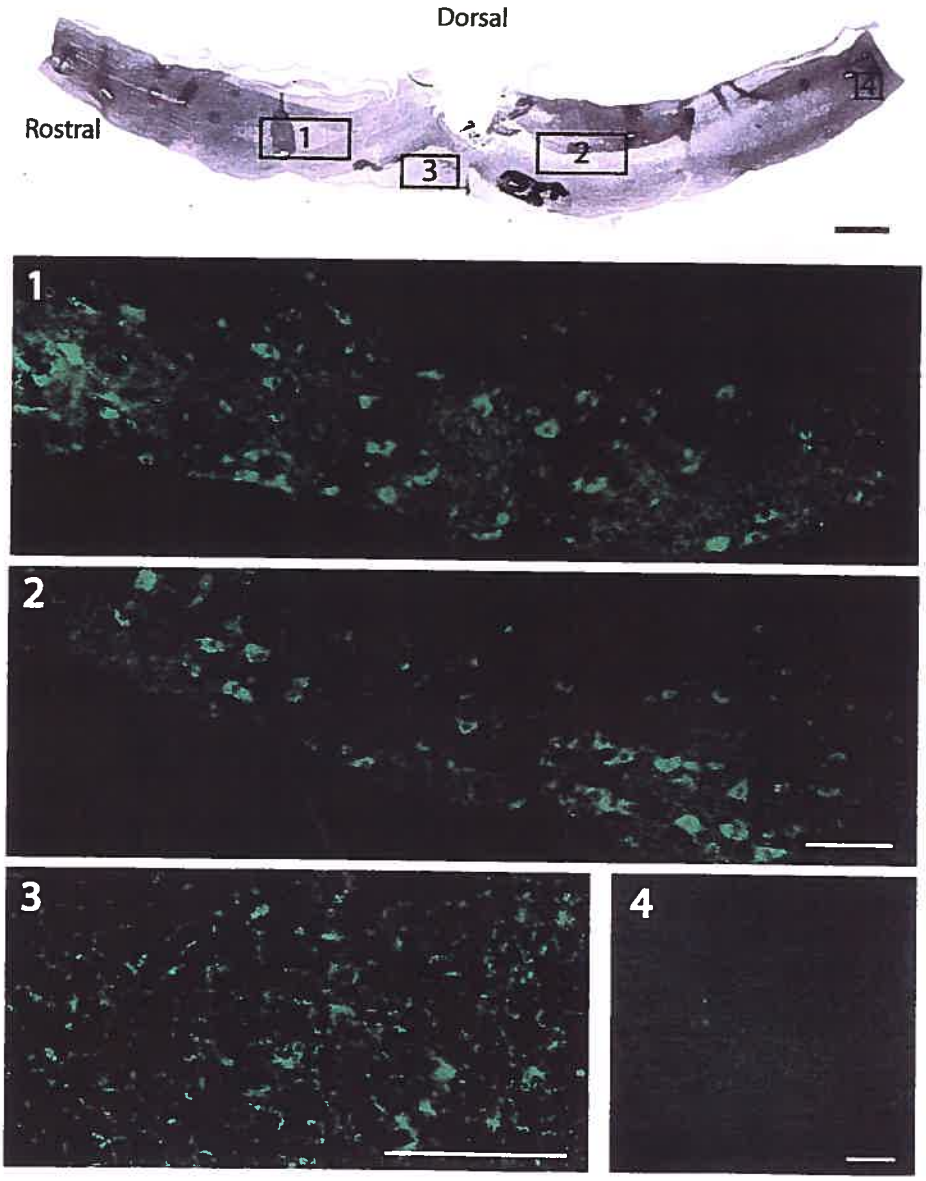

Figure 4

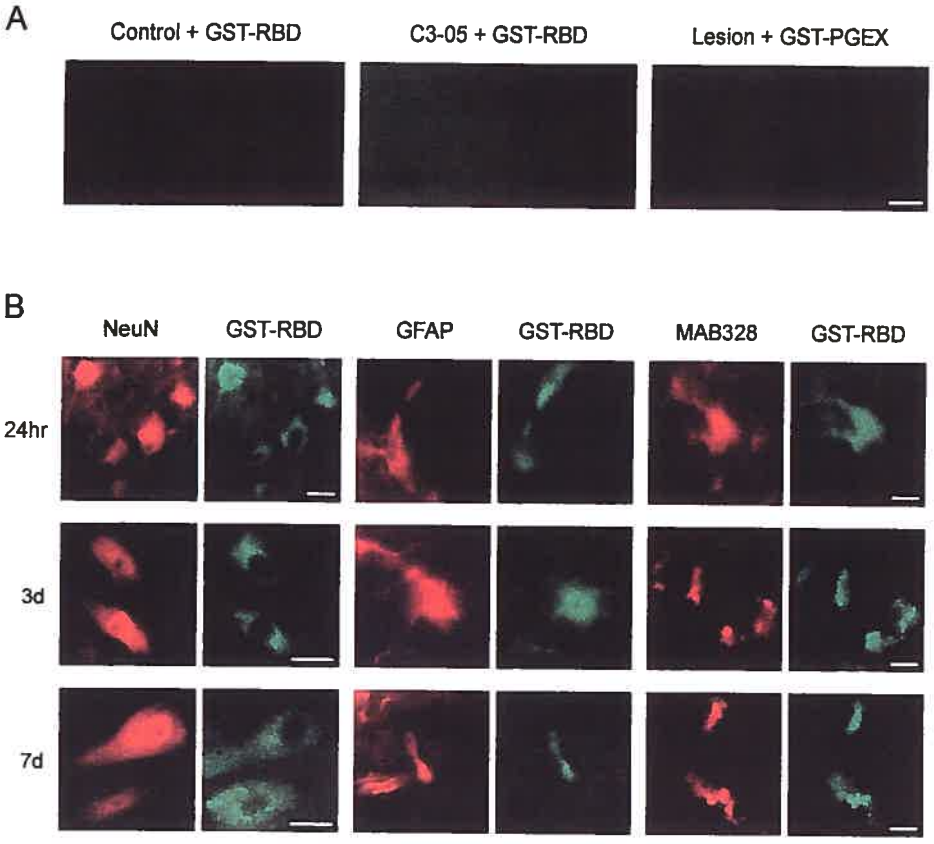
A
Control + GST-RBD
C3-05 + GST-RBD
Lesion + GST-PGEX
B
NeuN
GST-RBD
GFAP
GST-RBD
MAB328
GST-RBD
24hr
3d
7d

Figure 5

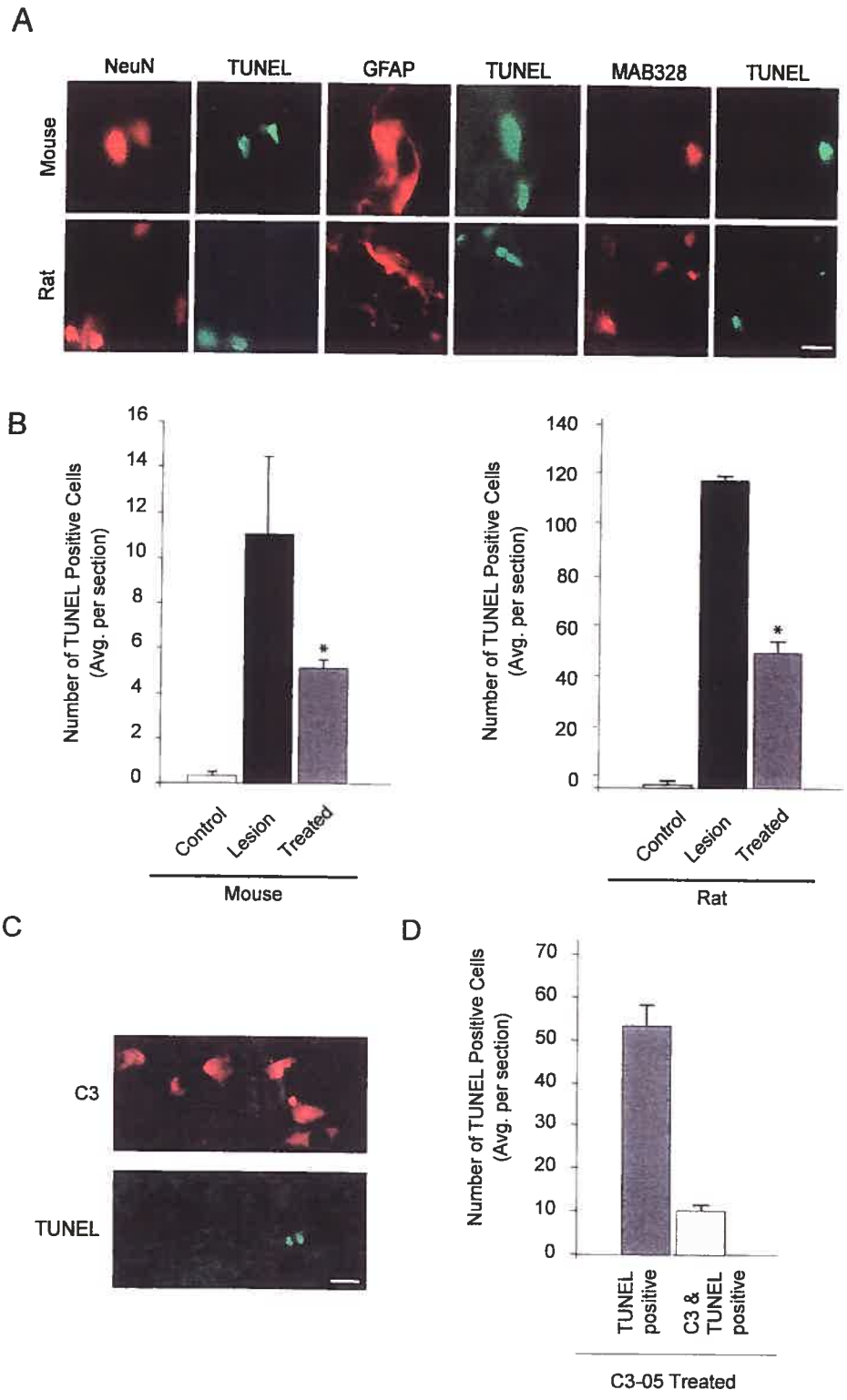
A
NeuN
TUNEL
GFAP
TUNEL
MAB328
TUNEL
Mouse
Rat
B
Number of TUNEL Positive Cells (Avg. per section)
16
14
12
10
8
6
4
2
0
*
Control
Lesion
Treated
Mouse
Number of TUNEL Positive Cells (Avg. per section)
140
120
100
80
60
40
20
0
*
Control
Lesion
Treated
Rat
C
C3
TUNEL
D
Number of TUNEL Positive Cells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

Figure 6

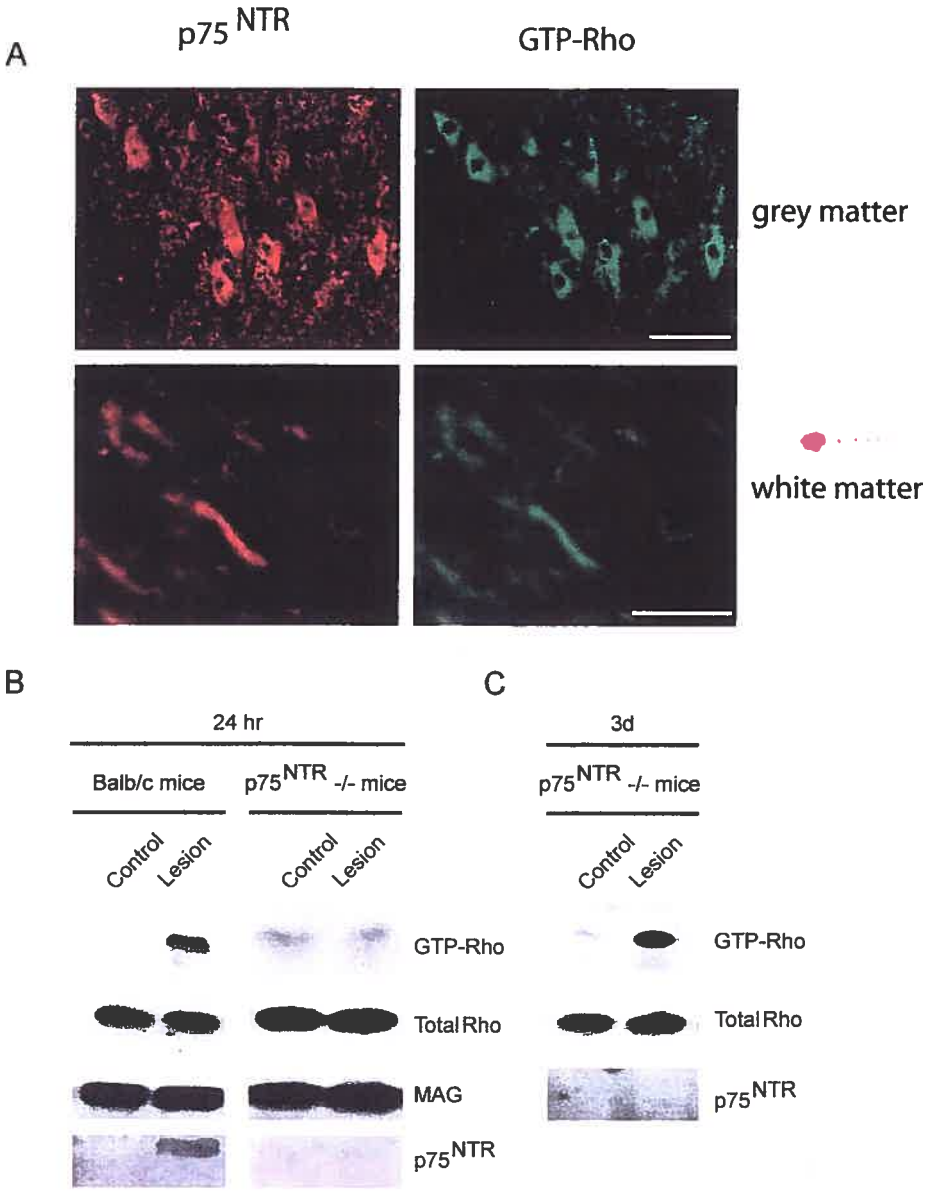
A
p75 NTR
GTP-Rho
grey matter
white matter
B
24 hr
Balb/c mice
p75NTR -/- mice
Control
Lesion
Control
Lesion
GTP-Rho
Total Rho
MAG
p75NTR
C
3d
p75NTR -/- mice
Control
Lesion
GTP-Rho
Total Rho
p75NTR

Figure 7

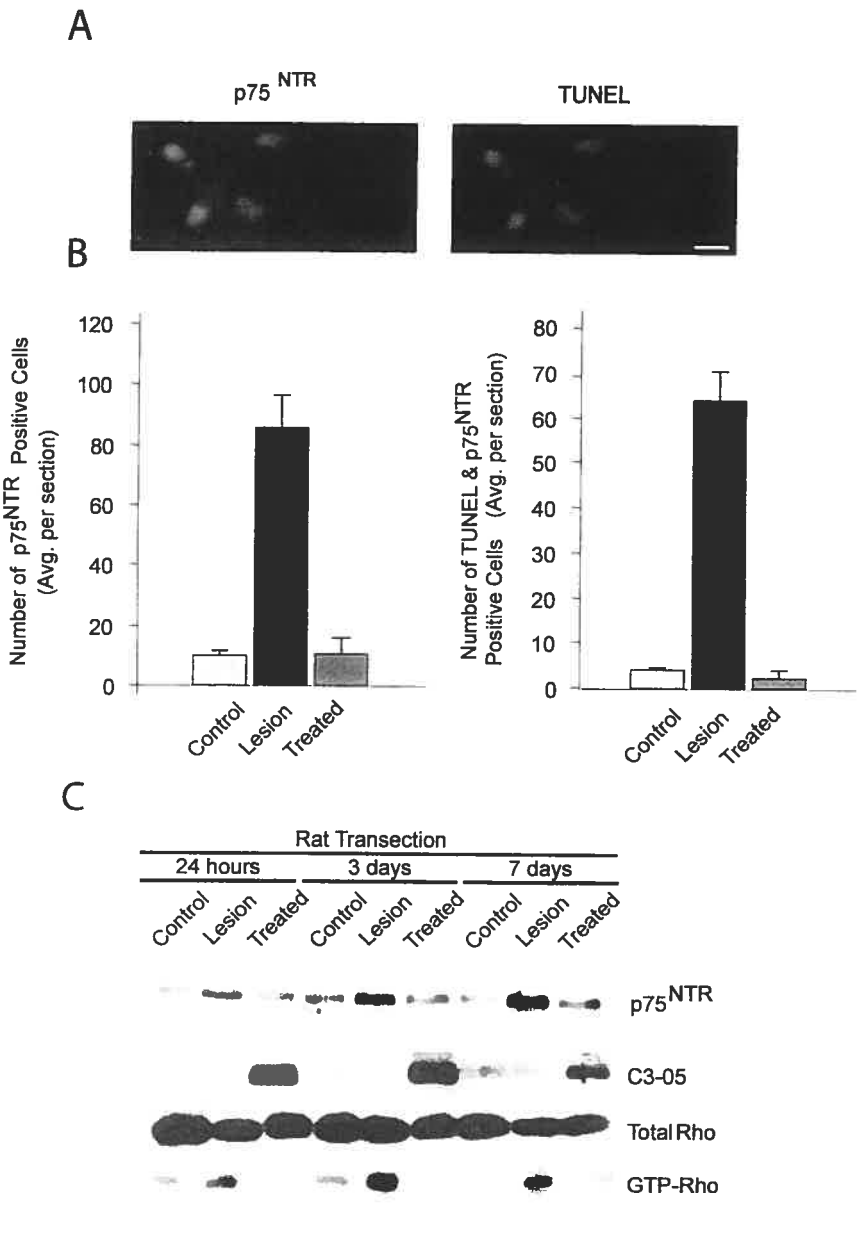
A
p75 NTR
TUNEL
B
Number of p75NTR Positive Cells (Avg. per section)
120
100
80
60
40
20
0
Control
Lesion
Treated
Number of TUNEL & p75NTR Positive Cells (Avg. per section)
80
70
60
50
40
30
20
10
0
Control
Lesion
Treated
C
Rat Transection
24 hours
3 days
7 days
Control
Lesion
Treated
Control
Lesion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

Figure 8

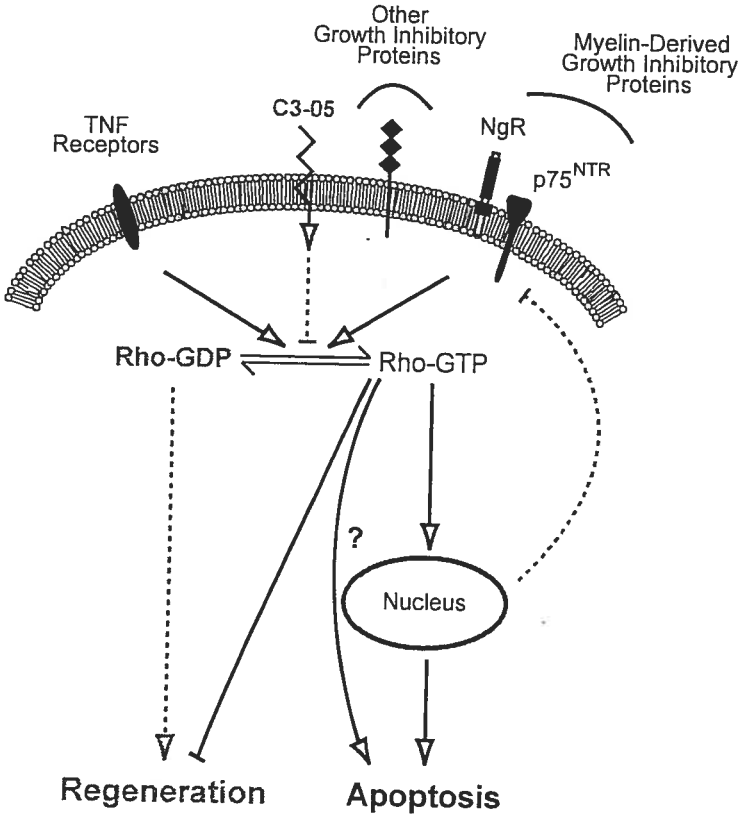
Other
Growth Inhibitory
Proteins
Myelin-Derived
Growth Inhibitory
Proteins
TNF
Receptors
C3-05
NgR
p75NTR
Rho-GDP
Rho-GTP
?
Nucleus
Regeneration
Apoptosis

Figure 9

Chapitre 3

3 Deuxième article

C.I. Dubreuil, Kathleen Deschamps, M. J. Winton, L.B. Loy, P. Morley and L. McKerracher. **Distribution of active Rho correlates with the presence of inflammatory proteins after spinal cord injury.** Soumis a *MCN (molecular and cellular neuroscience)*

Distribution of active Rho correlates with the presence of inflammatory proteins after spinal cord injury

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3.1 Abstract

After spinal cord injury (SCI), Rho is activated at the lesion site as compared to control animals. Here we examine the spatial temporal distribution patterns of GTP bound Rho throughout the thoracic rat spinal cord after injury. Active Rho is restricted to the lesion site and rostral-caudal activation peaked 24 hours after complete transection, and 3 days, after contusion injury. In both lesions, GTP-Rho is detected throughout the tested regions of the cord. Seven days after injury, active Rho was only detected in the lesion area, independent of lesion type. We found that the spread of Rho activation throughout the spinal cord was dependant on the severity of the injury indicating that Rho activation is a key response to SCI as lack of motor function can be correlated to levels of GTP-Rho. We also found that TNF-α and its receptor TNFR1 are unregulated throughout injured spinal cord following a similar pattern as with active Rho at peak distribution time. $P75^{NTR}$ levels were also found to be slightly but significantly upregulated at the far most rostral and caudal spinal cord. The inflammatory response is therefore potentially the most likely Rho activator distally from the lesion.

3.2 Introduction

In neurons, GTP bound active Rho causes growth cone collapse and cell rounding (Jalink et al., 1994; Tigyi et al., 1996). Rho is a key signaling molecule in mediating the cellular response to myelin derived growth inhibitory proteins (for review see [Schwab, 2004 #132;McKerracher, 2002 #106;He, 2004 #151;David, 2003 #118]). When Rho is inactivated, neurons can extend neurites in the presence of these inhibitors (Winton et al., 2002). *In vivo*, inactivation of Rho leads to an increase in regenerating fibers (Dergham et al., 2002) and a decrease in cell death [Dubreuil, 2003 #152], together leading to increase in functional recovery after SCI (Dergham et al., 2002).

Multiple *in vitro* and *in vivo* evidence implicate activated Rho in apoptosis. Inactivation of Rho has been shown to decrease caspase levels leading to apoptosis (ref). Thrombin, a serine protease has also been shown to induce Rho dependant apoptosis in cultured neurons and astrocytes, and treatment with Rho antagonist C3 rescued the cell death (Donovan et al., 1997; Citron et al., 2000). *In vivo*, inactivation of Rho after SCI leads to a 50 % reduction in the number of apoptotic cells found at the lesion site in both mice and rats (Dubreuil et al., 2003). RhoB has been shown to play a role in apoptosis after stroke as treatment with actin depolomerizing factors affecting Rho GTPase leads to the inhibition of caspase 3 activation (Trapp et al., 2001). Moreover, inactivation of Rho with Rho specific antagonist C3, decreases infarct seize in middle cerebral artery (MCA) stroke model, indicating a role for activated Rho in neuronal apoptosis after stroke (Laufs et al., 2000).

E-NOS, an enzyme that produces nitric oxide (NO) in endothelial cells, is a marker for blood flow and is used as an ischemic marker. E-NOS expression has beneficial effects in ischemic stroke as NO induces vasodilatation and increases blood flow (Endres et al., 2004; Sessa, 2004). E-Nos expression can also protect from stroke as it can inhibit oxidative damage and inflammation thereby inhibiting apoptosis (Endres et al., 2004). Interestingly e-NOS levels are negatively regulated by activated GTP bound Rho. Inactivation of Rho leads to increased e-NOS levels by prolonging eNOS mRNA half-life and after ischemic stroke Rho inactivation contributes to decreasing the infarct volume through e-NOS (Laufs et al., 2000).

SCI induces a strong inflammatory response (Popovich et al., 1997; Hausmann, 2003; Popovich and Jones, 2003) characterized by the infiltration of immune cells such as macrophages and lymphocytes as well as the activation of resident astrocytes and microglia. The inflammatory response has been shown to have both beneficial and detrimental effects after injury. The infiltrating cells clear away debris and can secrete factors that activate macrophage and microglia. Macrophages and microglia secrete cytokines such as TNF, IL - and 6 and induce the production of chemokines (ex. MIP1) as well as some growth factors (ex. BDNF and PDGF) (Moser and Willimann, 2004). TNF-α is a pro-inflammatory cytokine that is produced by activated macrophages, astrocytes and microglia after injury (Bartholdi and Schwab, 1997; Klusman and Schwab, 1997; Schnell et al., 1999b; Schnell et al., 1999a; Bareyre and Schwab, 2003). TNF signalling has been shown to have neuroprotective and detrimental as it can lead to microglial activation and neuronal and glial apoptosis after SCI (Popovich et al., 1997;

Sidoti-de Fraisse et al., 1998; Fontaine et al., 2002; Hausmann, 2003; Popovich and Jones, 2003). TNF signals to 2 receptors TNFR1 and TNFR2. Signalling through TNFR1 has been shown to be detrimental whereas TNFR2 is protective (Fontaine et al., 2002; MacEwan, 2002a, b). TNF, a potent Rho activator, may signal to activate Rho through TNFR1, as both signal in lipid rafts (Vinson et al., 2003; Lotocki et al., 2004), causing neuronal and glial apoptosis after SCI.

We have previously shown that after SCI, Rho is significantly activated at the lesion site as compared to uninjured controls (Dubreuil et al., 2003). Here we use different injury models to study the distribution patterns of GTP-Rho after SCI. We report that the spread of Rho activation across the spinal cord is dependant on time and severity of injury. The rostral-caudal distribution of active Rho also varied according to the type of lesion, where peak activation occurs earlier in complete transection than in contusion injury. We show for the first time that ischemic assault activates Rho. However, ischemic injury does not account for the majority of long distance Rho activation in the spinal cord. We also show that TNF levels are increased throughout the rostral spinal cord coinciding with spread of activated Rho.

3.3 Materials and Methods

3.3.1 Surgical Procedures

3.3.3.1 Spinal cord injuries

Rats were anesthetized under 2-3% isoflurane. Spinal cord injury was performed at thoracic level T10-T11, in adult female Sprague Dawley rats (180-200g) (n=32). Controls were unoperated animals (n=9). Dorsal over-hemisections were done at a depth of 1.5 mm with calibrated scissors. For contusion experiments, the NYU impactor device was used with 10 g weight drop at 25 mm. 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). From 24 hours to 7 days after SCI (n=6/ time point; at 24 h n=8), animals received an overdose of chloral hydrate anesthetic, were perfused with saline, and the spinal cords were dissected out and 5 mm segments spanning the thoracic region (including the lesion site) were frozen on dry ice and stored at –80 °C.

3.3.3.2 Global ischemia

For the global ischemia model of stroke, male Wistar rats weighing 205-215 g were prepared using the 4-vessel occlusion (4-VO) model (Pulsinelli et al., 1982). Both vertebral arteries were electrocauterized and reversible ligatures were placed around both common carotids and around the musculature. The wounds were closed, and animals were allowed free access to water, but not food, overnight. Animals were reperfused by removing the carotid clamps and the collateral snare. Animals were sacrificed 3 days later, and the hippocampi were removed and frozen on dry ice.

3.3.2 Pull down assays

Purification of GST-Rho Binding Domain (GST-RBD) and pull down assays were performed as previously described (Dubreuil et al., 2003). For *in situ* pull down assays, rat spinal cord cryosections (12-14 μm thickness) were post fixed with 4% PFA and incubated with the clarified bacterial lysate, prepared from bacteria expressing GST-RBD or GST alone, overnight at 4°C. The sections were then washed, blocked for 1 hr at room temperature and incubated with anti-GST antibody (Cell signalling, New England Biolabs, Mississauga, Canada) overnight at 4°C. Sections were washed and conjugated to FITC secondary antibodies (Jackson ImmunoResearch, Mississauga, Canada).

3.3.3 Immunoprecipitation and immunoblotting

For immunoprecipitations, tissue homogenates (400-600 μg of protein in a volume of 400-600 μl) were incubated over night at 4°C (rocking) with e-NOS antibody (BD Transduction Labs). Immune complexes were retained on protein A sepharose beads (1-2hrs, rocking, 4°C). The bound beads were then washed in lysis buffer and eluded with sample buffer. For all blots, 20 micrograms of protein from tissue homogenates was loaded into each lane, equal protein loading was confirmed with βTubulin (DSHB, Iowa, IA, USA) blots. Western blots were probed with $P75^{NTR}$ (Promega, Madison, Wyoming), NgR (ADI, San Antonio, Texas), p-c-jun (Cell signalling, New England Biolabs, Mississauga, Canada), TNFR-1 (Santa Cruz, Santa Cruz, California) and e-NOS (BD Transduction Labs) antibodies. Bands were visualized with peroxidase-linked secondary antibodies (Promega, Madison, Wyoming) and an HRP based chemiluminescence

reaction (Pierce, Rockford, Illinois). 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).

3.3.4 TNF ELISA

Rat TNF-α concentrations were determined using Quantikine immunoassay (R&D systems, Minneapolis, MN, USA). 5mm of spinal cord was homiginized in 100 μl of NP-40 lysis buffer and clarified by 15 minute centrifugation at 13, 000 rpm. Homoginates were stored at –80°C until use. ELISA was preformed as indicated in manufacturer's instructions, 50 μl of sample or control was loaded into each well.

3.4 Results

3.4.1 Distribution of Rho-GTP after SCI

In previous studies, we show that after SCI, Rho is activated at the lesion sites as early as 1.5 hrs post lesion and was sustained for at least 7 days (Dubreuil et al., 2003). To assess the distribution of active Rho after SCI, we examined GTP-Rho levels throughout a 5.5cm span of the rat spinal cord after complete transection and contusion injuries. After brief saline perfusion the spinal cords were removed, and sectioned into 5mm segments for analysis (Figure 1). Unoperated control rats showed very low levels or no Rho activation throughout the tested region of the spinal cord (Figure 2 and 3, control). We found that 1.5 hours after complete transection, Rho activation was limited to the lesion site and at 5 hours post injury, GTP-Rho spreads mostly rostrally across the spinal cord. The distribution of Rho activation spread after 24 hours where GTP-Rho was detected both rostrally and caudally from the lesion site. By 7 days, Rho activation was only detected in the area surrounding lesion area (Figure 3).

In contusion injury, the rostral-caudal spread of active Rho, was detected after 3 days as compared to 24 hours after complete transection. After 7 days Rho activation was restricted to the lesion area (Figure 3). In all injured animals tested, Rho activation levels at the lesion site were consistently greater or equal to levels seen throughout tested segments. In the case of only 3 animals (n = 32), no active Rho was detected at the lesion site, although GTP-Rho could be detected in other segments.

3.4.2 The severity of injury determines the level of Rho activation

We next examined if severity of the injury affected the Rho activation patterns. We use an *in situ* pulldown assay to detect active Rho and compared the extent of Rho activation in animals that underwent hemisection or very deep transection (over ¾ of the cord). After hemisection, Rho activation was only detected in the area surrounding the lesion site. In contrast, after deep transection, GTP-Rho was detected throughout the 1.5 cm section, at levels where no activation was detected after hemisection (Figure 4a). Next we studied the effects of severity by examining loss of hindlimb function. Rho activation was assessed by pulldown assay, 24 hours after SCI, from contused animals that lost all normal hind-limb function and contused animals that showed no paralysis. Paralysed animals had high levels of Rho activation at the lesion sites and GTP-Rho was detected throughout the examined regions of the spinal cord (Figure 4b, top left panel) at levels comparable to those found in the contused animals from separate experiments (Figure 3, 24 hour time point). Animals that were not paralysed showed no distribution of GTP-Rho (Figure 4b). In theses animals, GTP-Rho levels were similar to those seen in controls rats (Figure 2,3 control). Therefore, Rho activation is dependant of the severity of the injury and can also correlate to motor function loss.

3.4.3 Ischemic stroke activates Rho

Blood flow is disrupted after SCI leading to ischemic damage to the tissue surrounding the lesion site. We hypothesized that ischemia due to the severe disruption of blood flow after CT injury could lead to Rho activation throughout the thoracic rat spinal cord. Although Rho had been shown to play a role in apoptosis after stroke, it is not know if

ischemic injury can directly activate Rho. We studied Rho activation after 4 vessel occlusion ischemic stroke model by GTP-Rho pull down assay and found that Rho was significantly activated by ischemic assault as compared to controls (Figure 5).

Next, we examined if the thoracic rat spinal cord showed signs of ischemia or ischemic related damage after SCI by examining the protein expression of 2 ischemic markers: e-NOS and p-c-jun. We only find significant changes e-NOS expression levels at the lesion site, although eNOS levels are slightly increased rostrally and caudally to the lesion as compared to controls (Figure 6a). Phospho c-jun levels are also increased in response to ischemic injury (ref), and can be used as a marker for ischemia. Although we detect increased p-c-jun levels at the lesion site, we find no changes in p-c-jun levels rostrally and caudally to the lesion, where expression levels are similar to those seen in controls (Figure 6b). Although e-NOS expression indicates a very slight role for ischemia distal to the lesion site, it is not the contributing Rho activating factor at such distances from the lesion site.

3.4.4 Distribution of myelin signalling components distal to the lesion after SCI

To determine the long distance mechanism of Rho activation distal to the lesion, we examined changes in the distribution of myelin derived growth inhibitory receptors. Consistent with the literature, we find no significant changes in NgR expression after SCI (Figure 7a). We have previously shown that $p75^{NTR}$ was slightly upregulated at the lesion site after SCI. Here we find that 24 hours after injury, $p75^{NTR}$ is also slightly but significantly upregulated at the lesion site as well as in the far most rostral and caudal segments tested (Figure 7b). We have previously shown that $p75^{NTR}$ expression can be

regulated by GTP-Rho after SCI. We hypothesize that at these distances from the lesion site, Rho is regulating $p75^{NTR}$ expression. Therefore $p75^{NTR}$ expression is not the cause but a consequence of Rho activation.

3.4.5 TNF signalling accounts for the spread of active Rho

We next tested if TNF-α mediated neuroinflammation was present distally to the lesion area after SCI leading to high Rho activation that we observed. Rat spinal cords were removed after brief saline perfusion 4 and 24 hours after CT injury, and TNF-α levels were measured by TNF ELISA. We found significant increases in TNF-α levels at the lesion site but not rostally or caudally at both time points tested (Figure 8a). Next, we examined TNF-α levels in injured tissue of animals that were not saline perfused, as perfusion may wash out TNF present in the CSF or blood serum. We find that TNF-α levels are significantly increased throughout the rostral spinal cord 4 and 24 hours after injury (Figure 8b). Interestingly, no changes in TNF levels were observed in the CSF or in blood serum as compared to controls. In all CSF or blood serum samples tested control or injured, TNF levels were undetectable (value of 0 pg/ml) or lower than the ELISA kit sensitivity (< 3 pg/ml). TNF receptors have previously been shown to be upregulated after over a 14 mm segment, including at the lesion site after SCI (Yan et al., 2003). We examined distribution of TNFR1 expression throughout the effected regions of the rat spinal cord and find very high levels at the lesion site and caudal to the lesion. TNFR1 is also upregulated rostral to the lesion as compared to control, although not as extensively as in the lesion site (Figure 8c). TNF-α, a potent Rho activator (Neumann et al., 2002),

may signal through TNFR1 to activate Rho, as both Rho and TNFR1 are recruited to membrane rafts to signal (Vinson et al., 2003; Lotocki et al., 2004).

3.5 Discussion

Here we show that the distribution of active Rho throughout the spinal cord after lesion dependant on time and severity of the injury. Little active Rho is detected early after injury, spreads rostrally and caudally between 24 hrs-3 days, and at 7 days is restricted to the area immediately surrounding the lesion site. We found that the severity of injury, affected Rho activation, where the more severe the lesion, the more Rho was activated at the lesion site and across the tested regions of the spinal cord. We also found that ischemic injury can activate Rho and that the inflammatory response but not growth inhibitory signalling play a role in the peak distribution of activated Rho.

The spread of rostral-caudal GTP Rho distribution throughout the spinal cord differs depending on the type of injury: 24 hours after complete transection vs 3 days after contusion. Complete transactions (CT) being a more severe injury might have a faster BBB breakdown and electrical circuitry disruption than in the contusion model. Both lesion models also show differences in cavitation after injuty, wehere transection models show a more limited spread of secondary injury. The CT model may produce a more rapid immune response and more hemmorage than the contusion injury. Also, in mice, it BBB disruption occurs 3 days after compression lesions as compared to immediately after transection injuries (Pan and Kastin, 2001a). Together these may account for the kinetic differences in Rho activation observed after different injuries.

3.5.1 Time course for Rho activating factors after SCI

After injury, we found that Rho activation spread in a rostral-caudal time dependant manner. We observe active Rho at the lesion site 1.5 hours after lesion in all lesions tested indicating that Rho activation is an early, but sustained, response to injury. Rostral-caudal spread peaks at 24 hours to 3 days after SCI and is only detected in the area surrounding the lesion after 7 days. Many factors present in the area of the lesion site are known Rho activators. Such factors include myelin derived growth inhibitory proteins from the degrading white matter (Tang et al., 2001), inhibitory guidance molecules such as netrins [Shifman, 2000 #162;Ellezam, 2001 #157], ephrins [Willson, 2002 #163;Wahl, 2000 #45] and semaphorins (De Winter et al., 2002; He et al., 2002; Swiercz et al., 2002), CSPGs produced by reactive glia [Borisoff, 2003 #156;Monnier, 2003 #153;Sivasankaran, 2004 #149;Jones, 2003 #154], glutamate [Nesic, 2002 #160;Vera-Portocarrero, 2002 #161;Kim, 2004 #158;Jeon, 2002 #159] and TNF-α secreated from astrocytes and macrophages (Wang et al., 1996; Lee et al., 2000; Neumann et al., 2002). Other factors include LPA and thrombin present after SCI (Donovan et al., 1997; Citron et al., 2000) and other inflammatory cytokines produced by reactive glia and immune response [Hausmann, 2003 #165;Bareyre, 2003 #164]. Rho dependant apoptosis of both neurons and glia is also present around lesion site [Casha, 2001 #8;Beattie, 2002 #90;Donovan, 1997 #91;Dubreuil, 2003 #152;Crowe, 1997 #6;Katoh, 1996 #7]. Such factors are produced at different times after SCI, explaning the early and sustained Rho activation observed in the lesion area.

3.5.2 Mechanism for spread of Rho activation after SCI

Surprisingly Rho activation spread far beyond the lesion area. In fact, GTP-Rho was consistently detected in the whole thoracic region of the rat spinal cord, a total distance of 5.5 cm. Most if not all Rho activating factors present after SCI, as listed above, are restricted to the lesion area and spread no further than approximately a total of 2 cm (including the lesion site). After SCI, certain Rho activating factors present can be secreted or released into the CSF could also have a role in long distance Rho activation. Another possibility is cytoskeletal rearrangements controlled by GTP-Rho in response to electrical activity and neurotransmitter release changes after injury.

We find that the major contributing factor to long distance Rho activation 24 hours after SCI is the inflammatory response. It has previously been shown that MIP-1, a chemokine, mRNA levels are increased and show the same spatio-temporal distribution as activated Rho after SCI (Bartholdi and Schwab, 1997). We show increased levels of TNF-α and TNFR1 (Figure 8) throughout the injured cord. Although we find high levels of TNF present at the lesion site 4 and 24 hours after SCI, no TNF was detected rostral or caudal to the injury in perfused animals. This data shows TNF levels present in the spinal cord tissue, indicating source of TNF is endogenous. To test if we were washing out the TNF, we did the same experiment on unperfused animals. In this case we found significant increases in TNF in the rostral spinal cord. Surprisingly no TNF was detected in the CSF, as was previously described (Wang et al., 1996) or in the blood serum after SCI. It is possible that the amounts of TNF present in the serum are too dilute to be detected by ELISA. TNF can also enter injured tissue through specific transporters. It has been shown that TNF uptake through its transporters is increased throughout the spinal cord after

injury, independently of the site of the BBB breakdown (Pan et al., 1999; Pan and Kastin, 2001a, b). Increases in the transporters levels have also been reported (Pan and Kastin, 2001b). Therefore, the TNF we detected is most likely due to the increased transport of a peripheral TNF source leading to Rho activation. Since we find increased TNFR-1 expression, it may be possible that less TNF is needed to activate Rho. TNF had been shown to have both beneficial and detrimental effects after SCI. It is most likely that the massive amounts of TNF present at the lesion are detrimental. As TNF levels found distal to the lesion are lower than those found at the lesion site, it might have beneficial effects at such distances from the injury.

Beneficial effects may also result from increased e-NOS levels (Endres et al., 2004). Our e-NOS data also suggest beneficial effects. e-NOS mediates protection from ischemic injury by improving cerebral blood flow and increasing endothelium dependant vasodilatation (Endres et al., 2004). Since SCI causes decreases in blood flow early after injury the high e-NOS levels 24 hours after lesion may be beneficial by increasing blood flow after injury. This would indicate an endogenous healing response to SCI.

3.6 Figure legends

Figure 1

Model of spinal cord dissection and sectioning used to study the distribution of GTP-Rho levels throughout the rat spinal cord of injured and control animals. All spinal cords were removed after saline perfusion, and sectioned into 5mm segments for further analysis.

Figure 2

Distribution of GTP-Rho after complete transection is time dependant. GTP-Rho is shown on the left side and total Rho is shown on the right side. Distribution is shown from 1.5 hours after injury to 7 days post lesion. Rostral-caudal spread of Rho activation throughout the thoracic spinal cord peaks 24 hours after injury.

Figure 3

Distribution of GTP-Rho after contusion injury is time dependant. GTP-Rho is shown on the left side and total Rho is shown on the right side. Distribution is shown from 1.5 hours after injury to 7 days post lesion. Rostral-caudal spread of Rho activation peaks 3 days after injury.

Figure 4

Distribution of active Rho throughout the cord is dependant on the severity of the injury. (A) *in situ* pull down assay showing GTP-Rho on spinal cord sections. Left: hemisection model showing no activation of Rho at the end of the section (1) whereas cells with

active Rho are seen close to the lesion site (2). Right: very deep transection showing active Rho at the end of the section (1) as well as close to the lesion site (2). Nissel stained cords: scale bare, 1mm; RBD (in green): scale bare, X μm. (B) Pull down assay on tissue homogenates showing high levels of GTP-Rho at the lesion site and throughout the tested segments in a paralyzed animal (top panle) versus low levels of GTP-Rho at the lesion site and throughout the tested segments, in an injured animal that had not lost motor skill. Total Rho is shown in the bottom panel.

Figure 5

Ischemic injury activates Rho. (A) Pull down assay and (B) quantification of GTP-Rho activation in the hippocampus 3 days after stroke. Active GTP-bound Rho (top panel) and total Rho levels are shown (bottom panel). +/- SEM; $p < 0.05$

Figure 6

Changes in ischemic markers are only seen in the lesion site of the injured spinal cord. (A) e-NOS immunopercipitation and quantification of e-NOS levels after SCI, significant increases in e-NOS levels are only detected in the lesion site (n=9) as compared to control (n=5), +/- SEM; $p < 0.05$. (B) P-c-jun levels after SCI (n=4) (top panel) and tubulin levels show equal protein (bottom panel).

Figure 7

$p75^{NTR}$ but not NgR is upregulated rostrally and causally to the lesion site after SCI. (A) Quantification of NgR expression in the spinal cord after injury ; +/- SEM; n=4. (B) Quantification of $p75^{NTR}$ expression after SCI +/- SEM; n=13; $p<0.05$ as compared to control n=8.

Figure 8

Increased TNF-α levels in the thoracic cord after SCI. (A) rat TNF-α levels (pg/ml) as determined by ELISA, in segments of spinal cord of perfused animals, 4 hours (n=7) and 24 hours (n=7) injury post as compared to control (n=4, +/- SEM; $p<0.05$. (B) rat TNF-α levels (pg/ml) determined by ELISA, in un-perfused animals, 4 hours (n=4) and 24 hours (n=11) post injury as compared to controls (n=8), +/- SEM; $p<0.05$. (C) western blot of TNFR-1 after SCI (top panel), and tubulin (bottom panel).

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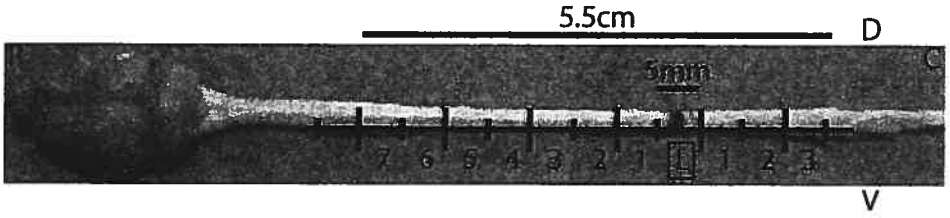

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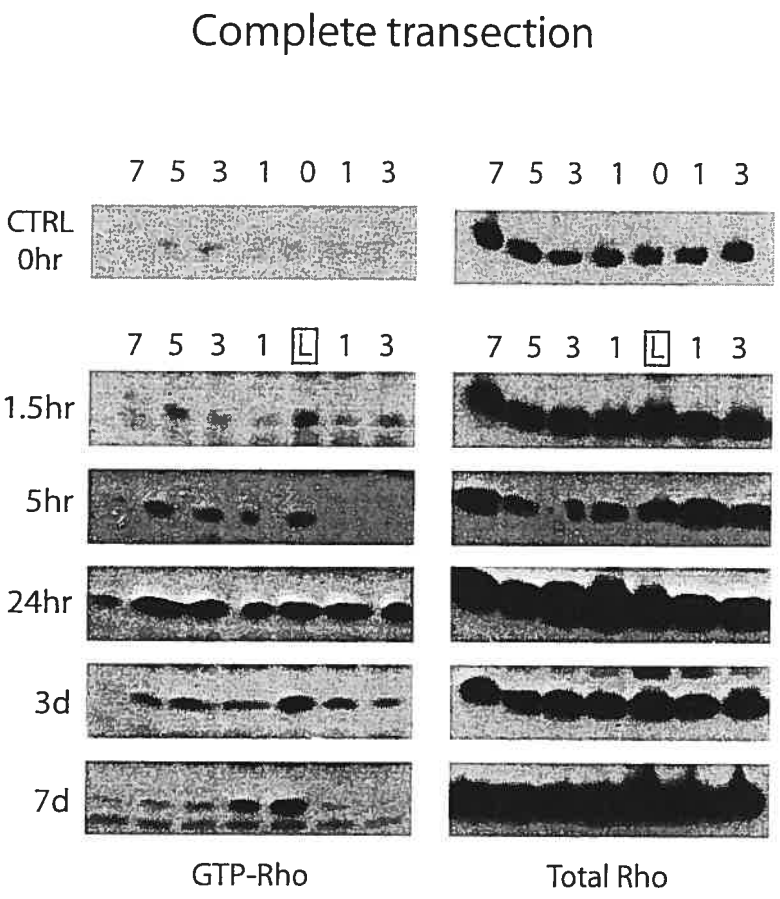
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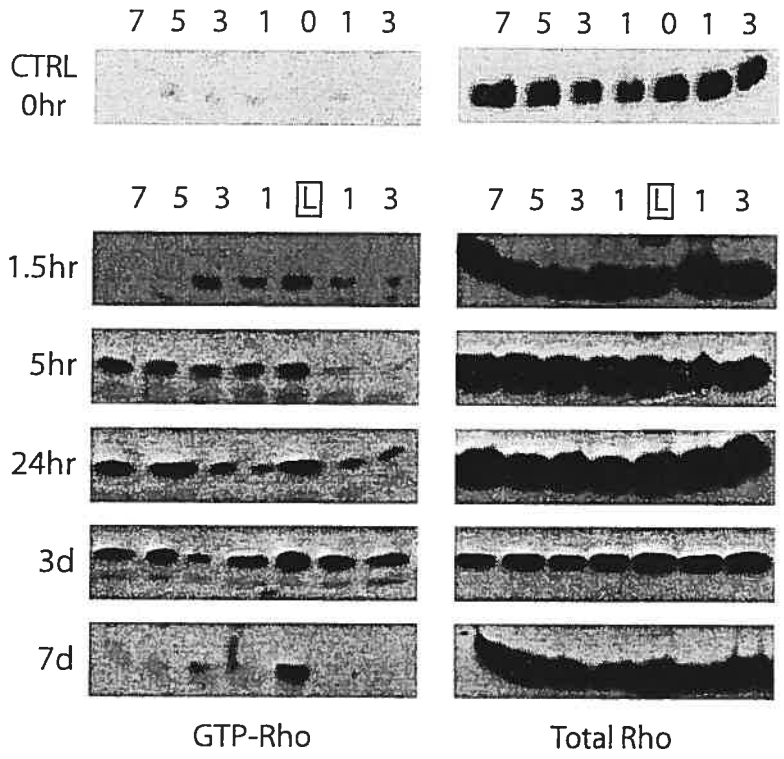
5hr
24hr
3d
7d
GTP-Rho
Total Rho

Figure 2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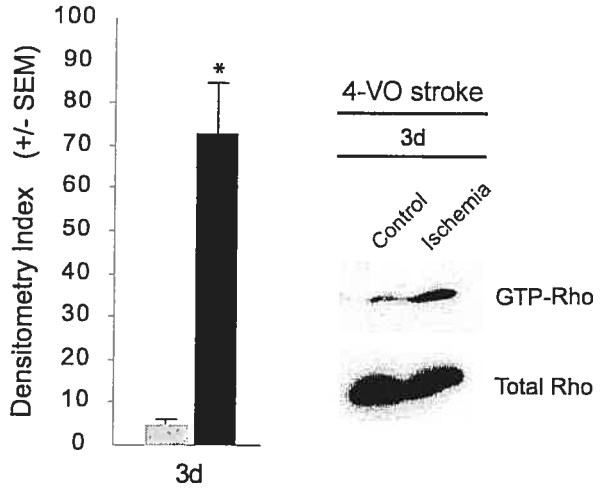
7d
GTP-Rho
Total Rho

Figure 3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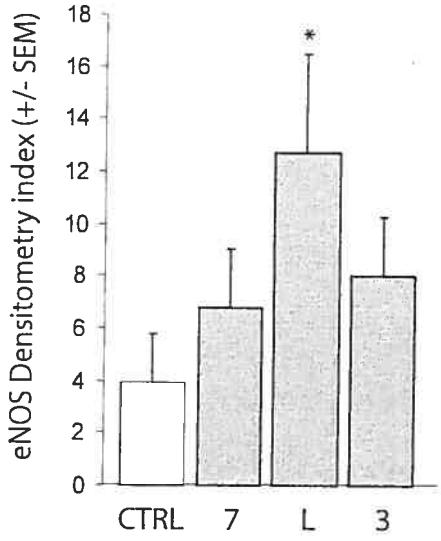
Control
Ischemia
GTP-Rho
Total Rho

Figure 5

A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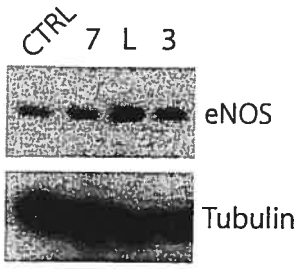
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eNOS
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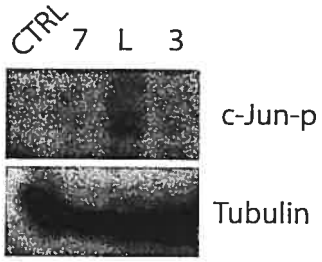
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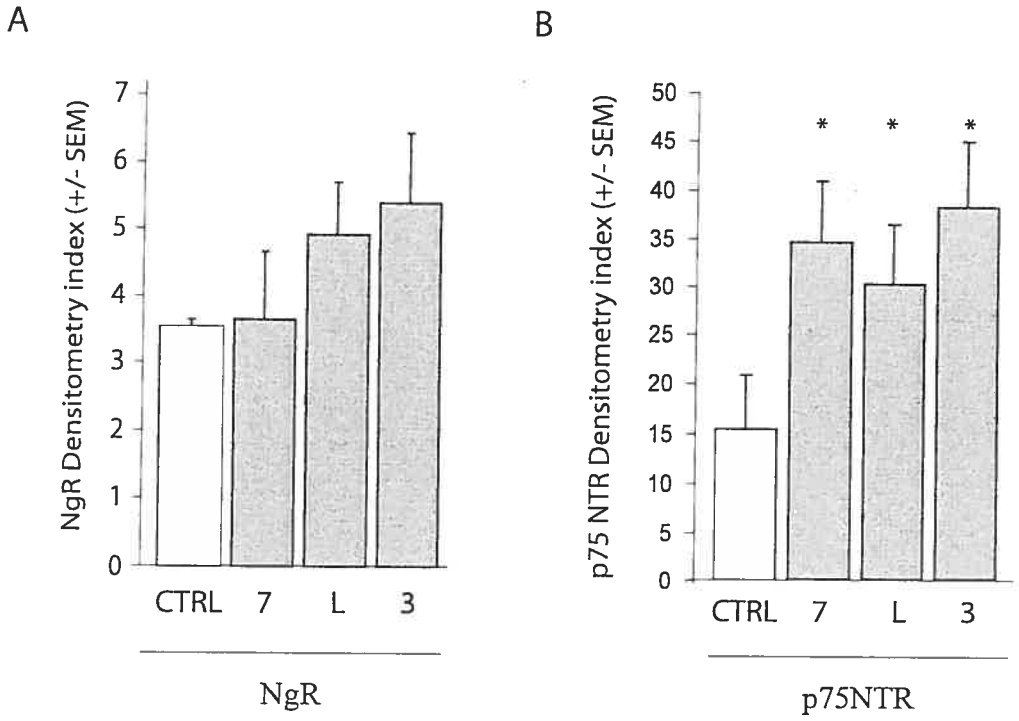
Figure 6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NgR Densitometry index (+/- SEM)
p75 NTR Densitometry index (+/- SEM)
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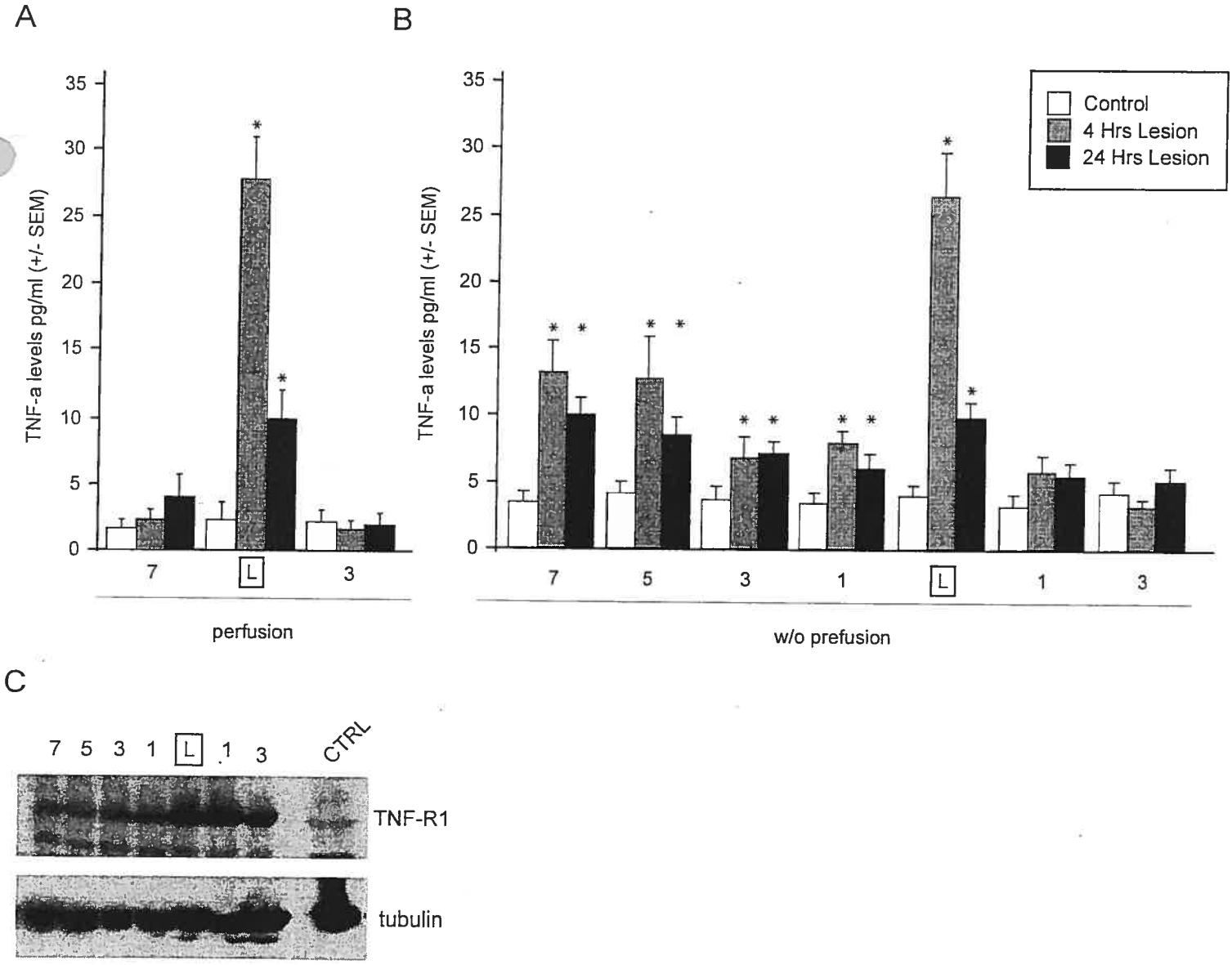
NgR
p75NTR

Figure 7


A
B
TNF-a levels pg/ml (+/- SEM)
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20
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L
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perfusion
Control
4 Hrs Lesion
24 Hrs Lesion
7
5
3
1
L
1
3
w/o prefusion
C
7 5 3 1 L 1 3 CTRL
TNF-R1
tubulin

Figure 8

Chapitre 4

4 Troisième article

Catherine I. Dubreuil, Niklas Marklund, Kathleen Deschamps, Tracy K. McIntosh and Lisa McKerracher. **Activation of Rho after traumatic brain injury and seizure in rats** soumis à Experimental Neurology

Activation of Rho after traumatic brain injury and seizure in rats

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4.1 Abstract

Traumatic brain injury (TBI) is characterized by a progressive cell loss and a lack of axonal regeneration. In the central nervous system (CNS), the Rho signaling pathway regulates the neuronal response to growth inhibitory proteins and regeneration of damaged axons, and Rho activation is also correlated with an increased susceptibility to apoptosis. To evaluate whether traumatic brain injury (TBI) results in changes in Rho activation in vulnerable regions of the brain, GTP-RhoA pull down assays were performed on rat cortical and hippocampal tissue homogenates obtained from 24 hours –3 days following lateral fluid percussion brain injury (FPI). Following FPI, a significantly increased RhoA activation was observed from 24 hours to 3 days post-injury in the cortex, and by 3 days in the hippocampus ipsilateral to the injury. Since we also detected activated RhoA in the cortex and hippocampus contralateral to the injury, without concomitant changes in total RhoA levels, suggesting that immediate post-traumatic events such as seizures may activate Rho, we examined RhoA activation in the brains of rats with kainic acid-induced seizures. Severe seizures resulted in bilateral RhoA activation in the cortex and hippocampus. Together, these results indicate that RhoA is activated in vulnerable brain regions following traumatic and epileptic insults to the CNS.

Keywords: Rho, TBI, Kainic Acid, seizure, cortex, hippocampus

4.2 Introduction

Traumatic brain injury (TBI) in man is a devastating disease that commonly causes persisting emotional and mental disturbances, loss of motor skills and cognitive deficits. In addition, post-traumatic epilepsy is commonly observed in survivors of TBI, particularly in young adults (Bruns and Hauser, 2003). The pathology associated with TBI is complex, but can be divided into (1) "primary" and (2) "secondary" injury. Primary injury is associated with the initial mechanical insult, resulting in immediate and often irreversible damage to neuronal cell bodies, dendrites, axons, glial cells and brain vasculature. The primary injury also results in tissue deformation and compression, leading to seizures, respiratory depression, apnea, ischemic and hypoxic damage resulting in cellular injury (Bramlett and Dietrich, 2004; Gaetz, 2004). The secondary injury is caused by an incompletely understood and complex cascade of physiological and biochemical factors continuing for hours to days post-injury that results in progressive tissue damage (Bramlett and Dietrich, 2004; Gaetz, 2004; Thompson et al., 2005). Ultimately, tissue necrosis and progressive neuronal cell death and occur within and outside of immediately damaged areas.

The lateral fluid percussion model of brain injury (FPI) reproduces many aspects of human TBI (Royo et al., 2003; Thompson et al., 2005), including epileptic type seizures immediately following the injury, changes in cerebral blood flow, gliosis and widespread axonal injury (Thompson et al., 2005). FPI induces both a focal and diffuse brain injury by a pressurized pulse of saline striking the intact dura, and produces a reproducible injury with well-characterized behavioural deficits (McIntosh et al., 1989; Fujimoto et al.,

2004). One hallmark of the morphological changes that occur following TBI is necrotic and apoptotic cell death in selectively vulnerable areas including the cortex and hippocampus (Conti et al., 1998; Royo et al., 2003; Raghupathi, 2004). Important contributing factors to the observed cell death include glutamate release (Faden et al., 1989), and the immune response which results in the release of pro-inflammatory cytokines such as TNF-α (Fan et al., 1996; Marciano et al., 2002; Vitarbo et al., 2004). The cellular mechanisms whereby these changes induce cell death are not completely understood.

Growing evidence suggests a role for the intracellular GTPase Rho in the neuronal response to traumatic injury to the CNS. After traumatic injury of the spinal cord, RhoA is highly activated, and inactivation by treatment with Rho antagonists reduces the number of apoptotic cells (Dubreuil et al., 2003) and improves functional recovery (Dergham et al., 2002). Similarly, inactivation of Rho after ischemic stroke in rats reduces infarct size (Laufs et al., 2000a). These studies suggest the possibility that abnormal activation of Rho may also play a role in tissue damage following TBI. Moreover, factors that contribute to early cell death following TBI, such as TNF-α and glutamate can activate Rho (Choi and Rothman, 1990; Neumann et al., 2002). Recently, it was found that RhoA and RhoB are upregulated in human brains after TBI, with an increase in expression localized to reactive glia and swollen neurites (Brabeck et al., 2004). Here we show that Rho is significantly activated in the cortex and hippocampus after TBI. We also detected activated Rho contralateral to the lesion, without concomitant changes in total RhoA levels. We provide evidence that seizure activity in both the cortex

and hippocampus contributes to increased levels of activated RhoA. Together, these results indicate that Rho is activated in vulnerable brain regions following insults to the CNS

4.3 Materials and Methods

4.3.1 Animal care

All animals were housed with food and water *ad libitum* in a 12-hour light/dark cycle in pairs for 1 week prior to surgical procedures. All procedures were approved by the Institutional Animal Care and Use Committee in accordance with National Research Council Guidelines for TBI experiments (University of Pennsylvania), and approved by the Comité de Déontologie following Canadian Council for Animal Care guidelines (Université de Montréal) for seizure experiments.

4.3.2 Traumatic Brain Injury

Male Sprague-Dawley rats (weight 340-398 g) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and surgically prepared for lateral fluid percussion brain injury (FPI) of moderate severity as originally described (McIntosh et al., 1989). Briefly, the animals were placed in a stereotaxic frame and the scalp and temporal muscle were reflected. A 5-mm craniectomy was performed over the left parietal cortex, midway between lambda and bregma, leaving the dura mater intact. A hollow modified Luer-Lok fitting was positioned over the craniectomy and held in place with dental cement (DentSply®, Dentsply International Inc., York, PA, USA) and filled with sterile saline. A stainless-steel screw was placed anterior to the coronal suture to further anchor the luer-loc and dental cement to the skull bone. Ninety minutes after induction of anesthesia, animals were attached to the FP device, a saline-filled cylinder, via the Luer-Lok fitting, and brain injury of moderate severity (2.4-2,6 atm.) was produced by a rapid (22 ms) delivery of a pressurized pulse of

saline striking the intact dura deforming the underlying brain tissue as originally described (McIntosh et al., 1989). Injury-induced mortality of 20 % of animals, consistent with previous reports using FPI of moderate severity (Saatman et al., 1997) was observed. Apnea and seizure times were recorded; if the apnea time exceeded 60s, animals were excluded from the study. After the injury, the Luer-Lok was removed, and the skin was sutured. Sham (n=8) animals were anesthetized and surgically prepared as described above but were not subjected to brain injury. A total of 31 animals were used in FPI experiments, with a total of 5 controls (unoperated animals), 8 sham and 18 brain injured rats, animal numbers for each specific experiment and time point are detailed in the figure legends. While anesthetized, animal body and cerebral temperature were maintained with a thermostatically controlled heating pad set at 37 °C until the animals were able to ambulate. Brain injury produced an apnea immediately post-injury (mean ± SD; 28 ± 12s, range 12-60 s). In addition, most animals displayed a brief tonic-clonic seizure immediately post-injury (seizure duration of 13 ± 13 s, range 0-41s).

To obtain tissue homogenates, all animals were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) at indicated time points and were perfused through the heart with cold saline at 4°C and decapitated. Each brain was quickly removed from the cranium, and a 3 mm coronal slice was cut at the occipito-parietal level. The cortex underlying the craniectomy at the maximal site of injury and the hippocampus from both hemispheres (ipsilateral and contralateral to the lesion) were dissected on a chilled glass plate over dry ice as previously described (Soares et al., 1992; Marklund et al., 2005). These regions were chosen since they display marked tissue damage following FPI (Hicks et al., 1996;

Sato et al., 2001). The brain tissue were snap-frozen in isopentane (2-methylbutane) at -65°C and stored at -80°C until analyzed. Rho activation states were analyzed on brain homogenates as previously described (Dubreuil et al., 2003).

4.3.3 Global Kainic acid induced seizures

To induce seizure in adult male Sprague-Dawley rats (225-250 g, n=17) kainic acid (KA) was administered intraperitonialy (ip) (10 mg/kg in 0.9% saline). All seizure activity was stopped by the anti-convulsive agent Diazepam (30 mg/kg, ip) after desired seizure grade was attained. Seizures grades were divided as 1-2 (mild), 3-4 (moderate), 5-6 (severe), as previously described (Zhang et al., 1997), and animal numbers were n=3, n=5 and n=4 respectively. Mild (stage 1-2) was characterized by staring spells, crouching and freezing behaviors. These behaviors typically occurred within 5-15 minute of KA administration. Animals with stage 2 seizures exhibited numerous wet dog shakes (WDS) within 30-45 minutes. Moderate (stage 3- 4) was characterized by hyperactivity (incessant walking and head nodding) and rearing (rearing and slight forelimb clonic jerks) within 45-60 minutes of KA administration. Severe (stages 5-6) was identified as intense rearing, falling and jumping phases, with severe forelimb and upper body clonic jerks with white foaming at the mouth while standing on their hind limbs, animals would then fall in the cage. In stage 6, animals were rolling, circling, or were intensely agitated, although no jumping behavior was observed. These behaviors occurred 60-90 minutes after KA administration (Zhang et al., 1997). Sham animals received vehicle alone (0.9% saline) (n=2) or vehicle plus diazepam (n=3). Animals were sacrificed 24 hours after KA injection, and the bilateral cortex and hippocampi were rapidly removed. To enable

comparison between TBI and KA-induced seizures, the same tissue regions (bilateral cortex and hippocampus) of similar size were dissected in both experiments.

4.3.4 Pull Down assays

Pull down assays were performed as previously described (Dubreuil et al., 2003). Purification of GST-Rho Binding Domain (GST-RBD) was performed as previously described (Ren and Schwartz, 2000). Frozen tissue was homogenized in modified RIPA buffer and homoginates were incubated with GST-RBD coupled beads (20-30 μg/sample). GTP- bound RhoA and total RhoA present in tissue homogenates were detected by Western blot using a monoclonal RhoA antibody (Santa Cruz, Santa Cruz, California). Although isoforms of Rho exist (Rho A, B and C), RhoA is expressed at higher levels than RhoB and RhoC (Lehmann et al., 1999). Therefore, we have focused on RhoA activation patterns. Blots were scanned into Adobe photoshop 6.0 for densitometry using an Epson perfection 1200U scanner, 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 after background subtraction, and the densitometry value is in arbitrary units. Statistical tests were performed using In Stat (Graph Pad, San Diego, California), for all experiments unpaired t-tests were used to compare either control or sham vs. brain injured animals.

4.4 Results

4.4.1 RhoA is activated in the cortex after TBI

To examine RhoA activation states after fluid percussion injury (FPI), we measured levels of GTP bound active RhoA in tissue homogenates from control, sham and brain-injured animals by GTP Rho pull down assay. Low levels of GTP bound-RhoA were detected in cortex of control and sham animals (Figure 1). We found that 24 after TBI, RhoA was significantly activated in the injured (ipsilateral) cortex, as compared to both control and sham animals (Figure 1a,b). We also found that RhoA was significantly activated in the contralateral cortex of brain-injured animals as compared to controls (Figure 1a,b). We noted high variability in the RhoA activation patterns amongst all brain injured animals in both ipsilateral and contralateral cortex (Figure 1). Of the 10 animals analyzed at 24 hrs post-injury, predominant RhoA activation in the ipsilateral cortex was observed in 5 animals, equal RhoA activation in ipsilateral and contralateral cortex was observed in 2 animals, and predominate RhoA activation in the contralateral cortex was observed in 3 animals. There were no significant differences between the ipsi- and contralateral cortex of brain-injured animals.

To determine whether RhoA activation levels were sustained after TBI, we examined GTP-RhoA levels in cortical tissue homogenates 3 days and 7 days after FPI. Rho was significantly activated in the ipsilateral cortex 3 days post injury (Figure 2 a,b) but the increase was not significant at 7 days post-injury (Figure 2b) when compared to sham animals. Again, similar to the 24 hour post injury time point, there was a high variability in the degree of RhoA activation. Together, the time course shows that RhoA activation

is increased in the cortex of brain-injured animals by 24 hours, and peaks by 3 days, and with some RhoA activation 7 days after TBI, as compared to sham animals.

4.4.2 RhoA activation patterns in the hippocampus after TBI

In addition to the cortex, FPI also causes bilateral damage to the hippocampus (Conti et al., 1998). To determine whether RhoA is activated in the hippocampus after TBI, we examined GTP-RhoA 24 hours and 3 days post injury. At 24 hours post injury, RhoA activation in the hippocampus did not significantly differ between the sham and brain injured groups (Figure 3a,c). By 3 days, active RhoA was significantly increased as compared to sham-operated animals (Figure 3b,c). Again, high levels of active RhoA were detected in contralateral hippocampus after TBI. At both time points tested no significant changes in total RhoA levels were detected (Figure 3d).

4.4.3 RhoA activation after KA induced seizures

To better understand the possible mechanisms underlying the observed contralateral RhoA activation after TBI, we investigated the effect of seizures on RhoA activation. Seizure activity commonly occurs after TBI, and we hypothesized that seizure activity after FPI may have influenced RhoA activation patterns in the brain contralateral to the lesion. To induce seizure in rats without TBI, we used kainic acid (KA) to induce seizures. KA was injected to induce different grades of seizure ranging from mild to severe (Zhang et al., 1997) and the cortex and hippocampus were removed and examined 24 hours after seizure induction. RhoA was not activated by mild seizures (stages 1-2) in either left or right cortex, nor in the hippocampus (Figure 4). However, RhoA was

activated in the cortex bilaterally after moderate seizures (stages 3-4) (Figure 4a). In animals with moderate seizure activity, no RhoA activation was detected in the hippocampus (Figure 4b). After severe global seizures (stages 5-6), RhoA was activated bilaterally in both the cortex and the hippocampus (Figure 4). Therefore, severe seizure activity may contribute to RhoA activation after TBI .

4.5 Discussion

In the present study we examined the activation patterns of RhoA in the cortex and hippocampus after TBI. We found that RhoA was significantly activated in the cortex by 24 hours and 3 days after TBI, and in the hippocampus 3 days post injury. The different temporal activation of RhoA in cortex and the hippocampus reflects the known patterns of apoptosis after TBI (Conti et al., 1998; Newcomb et al., 1999; Clark et al., 2000). Significant apoptosis is observed in the cortex by 24 hours after injury with maximal apoptotic death occurring in the CA3 region of the hippocampus by 48 hours post-injury. The parallel time course between RhoA activation patterns and apoptotic cell death after TBI are in agreement with the known contribution of Rho activation to cell death in spinal cord trauma or cerebral ischemia (Laufs et al., 2000a; Dubreuil et al., 2003).

Recently, Brabeck et al, (2004) showed increased levels of RhoA and B in human brain tissue after TBI. Immunocytochemistry of tissue sections was used to demonstrate increased Rho immunoreactivity in glial cells, inflammatory cells, and swollen neurite fibres (Brabeck et al., 2004). We show here that the global activation state of Rho increases in response to TBI, a finding in agreement with other studies that have detected activated Rho in neurons and glia after spinal cord injury (Dubreuil et al., 2003; Madura et al., 2004). In contrast to Brabeck et al., (2004), we did not detect changes in overall RhoA protein levels after TBI possibly because increases in individual cells were counterbalanced by ongoing death of other cells. We studied total tissue homogenates by biochemical methods, and differential responses in individual cells would not be detected. Therefore, our results together with Brabeck et al (2004) suggest that there are

changes in global Rho activation after TBI, and Rho protein expression increases in individual surviving cells.

4.5.1 Potential mechanisms for Rho activation after TBI

Myelin contains growth inhibitory proteins (MAG, Nogo and OMgp) that block axon regeneration. These myelin inhibitors signal to a common receptor complex: the Nogo-66 receptor (NgR), $p75^{NTR}$ and Lingo-1. Moreover, a new member of the TNF receptor family, TROY, plays an important role in growth inhibitory signaling through Nogo receptor in cells that do not express the $p75^{NTR}$. Myelin derived growth inhibitory proteins are known to activate Rho (Niederost et al., 2002; Winton et al., 2002; Fournier et al., 2003), and TROY has also been shown to signal to and activate Rho (Park et al., 2005; Shao et al., 2005). Recently, the levels of Nogo were observed to increase in the cortex from 24h- 7 days following FPI (Marklund et al., submitted 2005) and Nogo inhibitors have been shown to improve behavioral outcome following experimental TBI in rats (Lenzlinger et al, in press, Neuroscience 2005; Marklund et al, submitted 2005). In addition, there is evidence that signaling to Rho plays a role in apoptosis after CNS trauma (Dubreuil et al., 2003). Thus growth inhibitors, released from damaged white matter, may activate RhoA contributing to the lack of regeneration and to tissue damage after TBI as there is widespread axonal injury observed in human and experimental TBI (Maxwell et al., 1997; Graham et al., 2000).

After TBI, the inflammatory response may be an important mechanism of secondary damage, contributing to cell death and necrosis (Lenzlinger et al., 2001; Morganti-

Kossmann et al., 2002). The initial immune reaction occurs in first few hours after TBI, and is characterized by the activation of microglial cells, rapidly followed by the recruitment of hematogenous neutrophils and macrophages to the site of injury (Soares et al., 1995; Royo et al., 1999; Lenzlinger et al., 2001) (Stahel et al., 2000; Kinoshita et al., 2002a).The invasion of macrophages further augments the inflammatory cascade by the induction of proinflammatory cytokines, such as including TNF-α and Il-1 (Fan et al., 1996; Kinoshita et al., 2002b; Morganti-Kossmann et al., 2002) both shown to activate Rho (Neumann et al., 2002). TNF-α signals by binding to membrane receptors, and the receptor TNFR1 is implicated in initiating cell death after TBI (Lotocki et al., 2004). TNF-α, a potent Rho activator (Neumann et al., 2002), may cause cellular damage through TNFR1 activation of Rho, as both Rho and TNFR1 are recruited to lipid rafts to signal (Vinson et al., 2003).

After TBI, cerebral edema and cell swelling are common, and it is possible that it may contribute to RhoA activation in neurotrauma as cell swelling has been shown to occur in a $p75^{NTR}$ dependant manner (Peterson and Bogenmann, 2003). Thus, TBI related edema may affect RhoA activation patterns through $p75^{NTR}$. TBI results in diverse types of tissue damage, and a growing body of literature suggests that active Rho contributes to tissue damage (Laufs et al., 2000b; Trapp et al., 2001; Dergham et al., 2002; Dubreuil et al., 2003). Rho activation in vulnerable brain regions following seizure and TBI may contribute to the lack of axonal regeneration, progressive cell loss, and some of the histological hallmarks of neurotrauma.

4.5.2 Factors leading to contralateral Rho activation after TBI

In the present study, we have shown that, in addition to activation of RhoA in the brain ipsilateral to the brain injury, RhoA is significantly activated in the cortex and hippocampus contralateral to the injury. Certain markers show contralateral expression after TBI such as changes in binding potential for certain glutamate receptor subtypes (Sihver et al., 2001; Thompson et al., 2005) and immediate early genes such as c-fos (Yang et al., 1994; Raghupathi et al., 1995). It was reported that active Rho signals to the c-fos transcription factor (Benitah et al., 2004), so perhaps Rho activation and c-fos expression in the contralateral cortex may reflect a common signaling mechanism. In addition, levels of proinflammatory cytokines including TNF-α increase bilaterally after TBI (Fan et al., 1996; Vitarbo et al., 2004). In human TBI patients, TNF-α is increased in the cerebral spinal fluid by 24 hours after TBI (Shohami et al., 1997; Morganti-Kossmann et al., 2001; Hayakata et al., 2004). Therefore, proinflammatory cytokines present in contralateral brain may contribute to the contralateral RhoA activation we observed after TBI.

Rho GTPases play a role in synaptic remodeling, neuronal activity and plasticity in the CNS (Luo, 2002; O'Kane et al., 2003, 2004), Since generalized seizures commonly occur following TBI, we wished to determine whether seizures, observed following TBI, may play a role in Rho activation and contribute to the contralateral Rho activation. To test this hypothesis, we used a kainic acid-induces seizure model (Zhang et al., 1997) and showed that RhoA is activated by moderate-to-severe seizure activity. These results indicate that the cortex is more sensitive than hippocampus to RhoA activation following

seizure activity. Our results indicate that activation of RhoA following TBI may be related to post-traumatic seizure activity. In conclusion, contralateral activation of Rho may result from several mechanisms that include glutamate release, inflammation and seizures.

4.5.3 Role for Rho signaling in seizures

Our data are the first to demonstrate that RhoA is activated in the cortex and hippocampus after seizure activity. Seizure activity has been shown to cause apoptotic cell death of cortical and hippocampal cells in a $p75^{NTR}$ dependant manner, where over 80% of TUNEL positive apoptotic cells expressed $p75^{NTR}$ (Roux et al., 1999). More recently, $p75^{NTR}$ were shown in apoptotic neurons in the hippocampus after KA induced seizure (Yi et al., 2003). In the present study, we show that RhoA is activated in the cortex and hippocampus by 24 hours after severe seizures. Moreover, increases in $p75^{NTR}$ protein expression are detected 24 hours, 3 days and 7days in the hippocampus and 3 days in the cortex after seizures (Roux et al., 1999). Since $p75^{NTR}$ dependant Rho activation leading to apoptosis has previously been shown after CNS injury, Rho may regulate cell death in a $p75^{NTR}$ dependant manner after seizure. The proteins c-fos, MMP-9 and p38 MAPK have also been shown to be involved in neuronal cell death in the hippocampus after seizure (Jourquin et al., 2003; Kim et al., 2004). Interestingly, Rho has been shown to signal to or to regulate all 3 factors (Wong et al., 2001; Benitah et al., 2004), further indicating an important role for Rho dependant apoptosis after epileptic type seizures. It will be important to determine if Rho activation is a contributing factor to the development of post-traumatic epilepsy.

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4.7 Figure Legends

Figure 1. RhoA activation in the cortex after TBI

A. RhoA was examined in tissue homogenates from control (CTRL), sham and brain-injured rat ipsilateral (Ipsi) and contralateral (Contra) cortex 24 hours after FPI. GTP-RhoA was examined by pull down assay and detected by Western Blot using an anti-RhoA antibody. Total RhoA levels are shown in bottom panel. B. Densitometry of GTP-RhoA band +/- SEM; control, n=5; sham, n=3; TBI ipsilateral and TBI contralateral, n=10; Significance, $p< 0.05$, TBI samples were compared to controls. C. Densitometry of RhoA levels in homogenates of cortical tissue 24 hours after TBI. No significant changes between the groups were observed.

Figure 2. RhoA activation in the cortex persists up to 7 days

A. RhoA is significantly activated in the cortex 3 days after TBI both ipsilateral (Ipsi) and contralateral (Contra) to the injury. Western Blots show GTP-RhoA (top panel) and Total RhoA (bottom panel) levels in the cortex 3 days after TBI. B. Quantification by densitometry of GTP-RhoA bands 3 days after TBI; Control, n=5; sham, n=2; TBI ipsilateral and TBI contralateral, n=3; and 7 days after TBI ; control, n=5; sham, n=3; TBI ipsilateral and TBI contralateral, n=5; +/- SEM; Significance, $p< 0.05$, TBI samples were compared to controls. C. Densitometry of total RhoA levels in cortex tissue homogenates 3 and 7 days after TBI. No significant changes between the groups were observed.

Figure 3. RhoA activation in the hippocampus following TBI

A. Western blots showing GTP-RhoA and Total RhoA levels in the hippocampus 24 hours and B. 3 days after TBI. C. RhoA is significantly activated after TBI as compared to sham operated animals, in both ipsilateral (Ipsi) and contralateral (Contra) hippocampus 3 days after FPI. Quantification of GTP-RhoA levels in the hippocampus 24 hours after TBI; sham, n=3; TBI ipsilateral and TBI contralateral, n=10, and 3 days after TBI; sham, n=2; TBI ipsilateral and TBI contralateral, n=3; +/- SEM; Significance, $p< 0.05$, TBI samples were compared to controls.

Figure 4. Global KA induced seizures activate RhoA

KA was injected in adult male rats to induce different seizure grades: mild (grade 1-2), n=3; moderate (grade 3-4), n=5; and severe (grade 5-6), n=4; saline vehicle, n=2; diazepam, n=3. (A, B) Twenty-four hours after the onset of seizures, bilateral cortical (A) RhoA activation was observed after moderate seizures (grade 3-4) in the cortex, Cortical (A) and hippocampal (B) RhoA activation was observed after severe seizure (stages 5-6) in both the right (R) and left (L) hemispheres.

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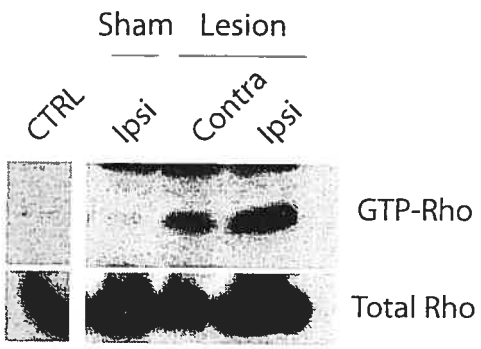
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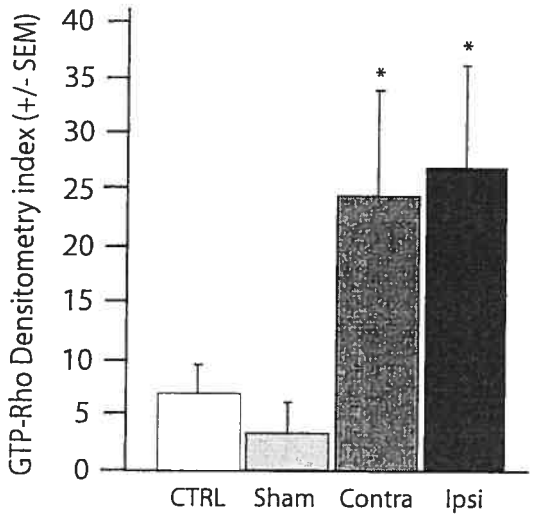
Lesion
CTRL
Ipsi
Contra
Ipsi
GTP-Rho
Total Rho

B


GTP-Rho Densitometry index (+/- SEM)
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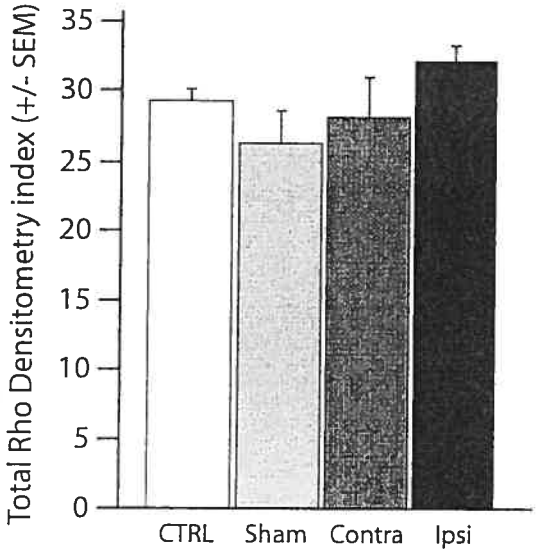
35
30
25
20
15
10
5
0
*
*
CTRL
Sham
Contra
Ipsi

C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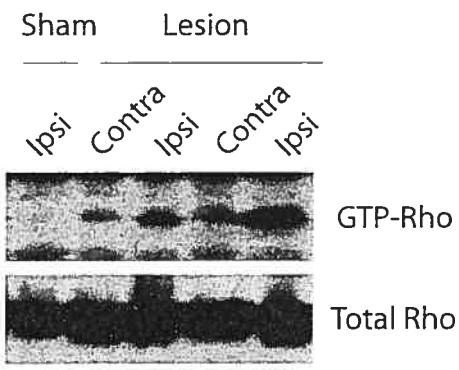
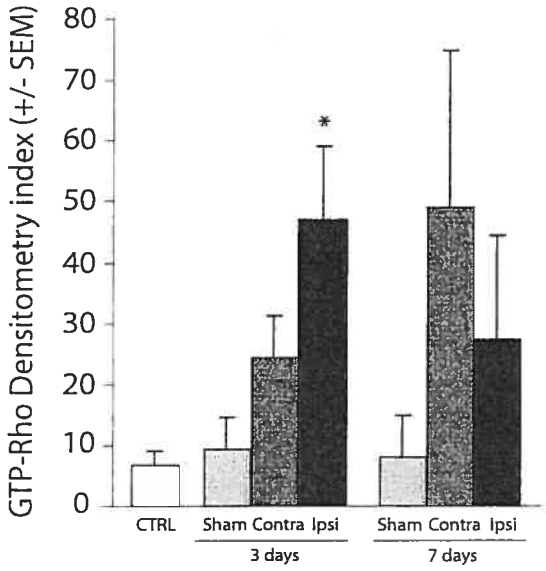
0
CTRL
Sham
Contra
Ipsi

Figure 1

A


Sham
Lesion
Ipsi
Contra
Ipsi
Contra
Ipsi
GTP-Rho
Total Rho

B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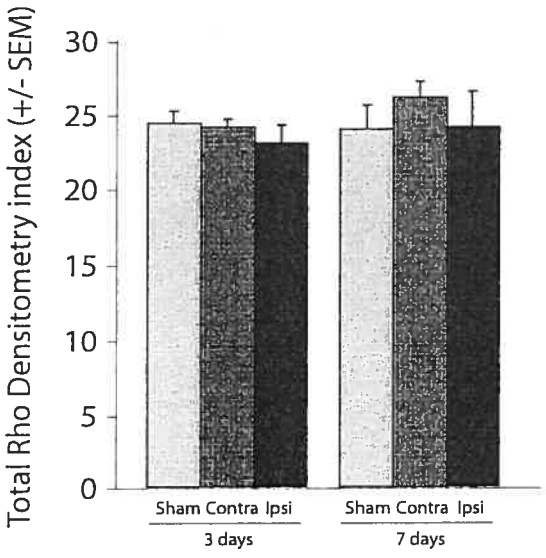
70
60
50
40
30
20
10
0
*
CTRL
Sham Contra Ipsi
3 days
Sham Contra Ipsi
7 days

C


Total Rho Densitometry index (+/- SEM)
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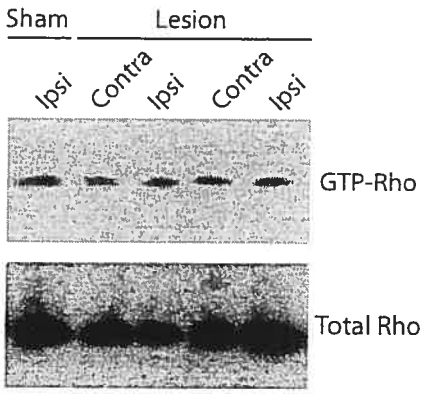
20
15
10
5
0
Sham Contra Ipsi
3 days
Sham Contra Ipsi
7 days

Figure 2

A



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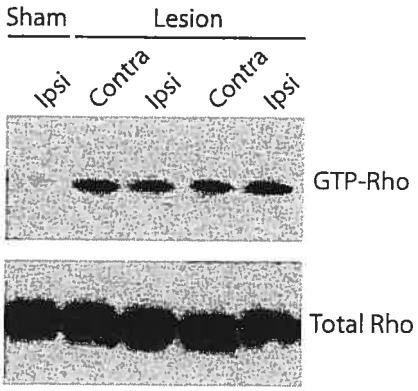

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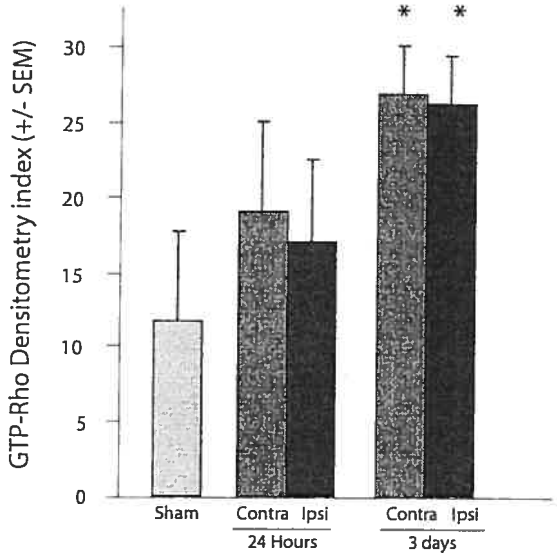

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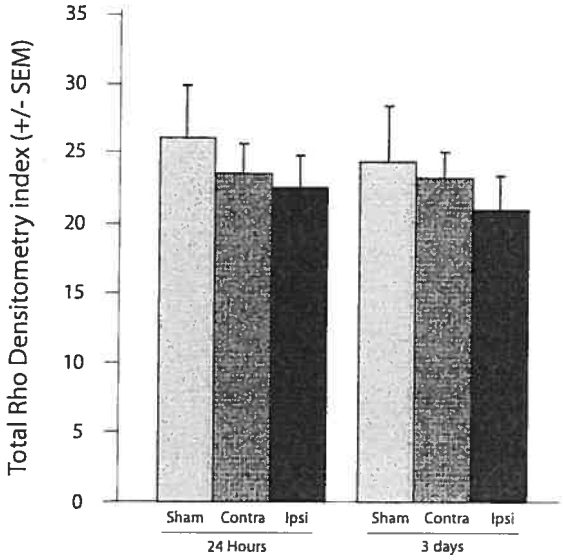

Figure 3

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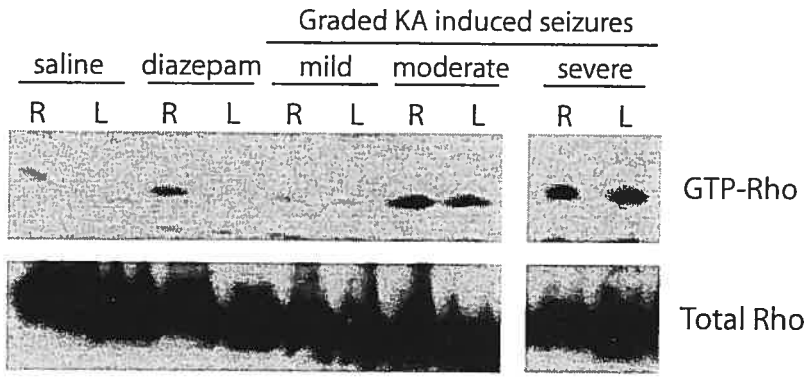
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diazepam
mild
moderate
severe
R L R L R L R L R L
GTP-Rho
Total Rho

B

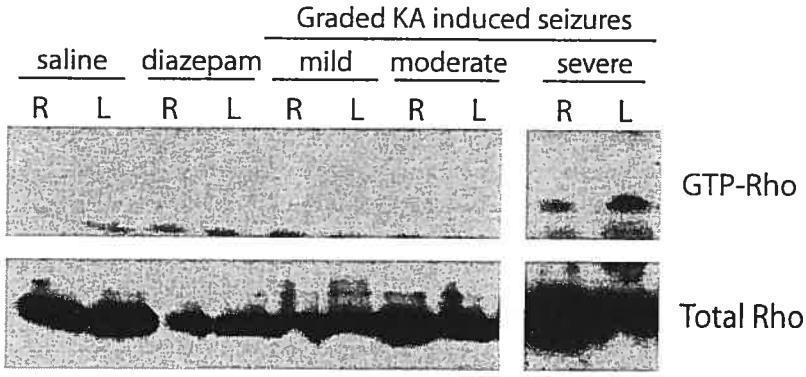
Graded KA induced seizures
saline
diazepam
mild
moderate
severe
R L R L R L R L R L
GTP-Rho
Total Rho

Figure 4

Chapitre 5

5 Discussion Générale

5.1 Cibler Rho suite aux traumatismes du SNC

Les lésions traumatiques du SNC tel que les lésions de la moelle épinière, les lésions traumatiques du cerveau et les attaques ischémiques induisent des pertes de fonction motrices et cognitives. Présentement il y a au moins 41 000 personnes qui soufrent des séquelles des lésions de la moelle au Canada et 400 000 aux États-Unis, et il est estimé que plus de 1100 et 7800 nouveaux cas seront rapportés à chaque année au Canada et aux États-Unis respectivement. Majoritairement ces lésions affectent les hommes âgés entre 15 et 34 ans et sont causées par des traumatismes violents, des chutes, des accidents sportifs ainsi que des accidents de véhicules moteurs. Le papyrus chirurgical de Edwin Smith provenant de l'époque de l'Egypte ancienne, indiquait qu'il n'y avait aucun traitement pour les personnes soufrant de pertes de fonctions motrices dues aux lésions de la colonne vertébrale. En Amérique, il y a 1.5 millions de nouveaux cas déclarés de TBI par an et il est estimé que 5.3 millions d'individus soufrent de pertes de fonction et cognitives dues à ce type de lésion. Les attaques ischémiques sont la 4ème plus haute cause de mortalité au Canada où 16 000 canadiens y succombent par année. Il y a plus de 40 000 attaques ischémiques par an et au-delà de 300 000 personnes qui vivent avec les effets de ces traumatismes. Présentement il n'existe aucun traitement sur le marché, malgré qu'il y en a plusieurs en phases expérimentales, qui promouvoit la régénération des axones lésés et/ou la récupération des pertes de fonctions motrices suite à ces types de lésion.

Plusieurs facteurs qui sont libérés suite aux traumatismes du SNC, tel que le TNF, la thrombine, le glutamate, activent Rho et induisent l'apoptose des neurones. L'activation

de Rho peut aussi induire la production de MMP (matrix metalloproteases) et des protéines impliquées dans le stress oxydatif (Lozano et al., 2003). Le TNF est un puissant activateur de Rho qui est sécrété et libéré suite aux lésions de la moelle et suite au TBI. De plus, le TNF peut induire la mort apoptotique neuronale suite à ces blessures (Fan et al., 1996; Wang et al., 1996; Lee et al., 2000; Neumann et al., 2002a). La thrombine est une sérine protéase qui est libérée suite aux lésions du SNC (Xi et al., 2003). Cette protéase induit la mort apoptotique des neurones et des astrocytes de façon dépendante de Rho et l'inactivation de Rho par la C3 diminue le nombre de cellules apoptotiques induit par la thrombine (Donovan et al., 1997; Citron et al., 2000). Le glutamate, un acide animé excitateur est aussi relâché suite à ces lésions et joue un rôle important dans la mort secondaire des cellules suite aux traumatismes du SNC (Choi and Rothman, 1990; Choi, 1992; Michaelis, 1998; Nesic et al., 2002; Vera-Portocarrero et al., 2002). Les protéines inhibitrices de croissance de la myéline, MAG, Nogo (Nogo 66 et animo Nogo), OMgp, sémaphorine 3, et de la cicatrice gliale (CSPGs) activent aussi la GTPase Rho (He and Koprivica, 2004; McKerracher and David, 2004). L'activation de Rho par ces inhibiteurs induit l'effondrement du cône de croissance et l'inhibition de la croissance axonale des neurones en contact avec ces protéines. Suite aux lésions de la moelle épinière la formation de la cicatrice gliale est une barrière importante à la régénération puisqu'elle forme une barrière physique et chimique à la régénération. Les protéines inhibitrices de la myéline sont relâchées suite au dommage de la gaine de la myéline induite par la lésion et participent à l'inhibition de la croissance des axones blessés des neurones qui survivent *in vivo*. Donc, différents événements neuropathologiques suite aux traumatismes du SNC peuvent expliquer l'activation de Rho dans les cellules nerveuses et gliales.

Nos études ont montré que Rho est activée suite aux lésions traumatiques du SNC, plus spécifiquement nous avons montré que Rho est activée dans les neurones, les astrocytes et les oligodendrocytes suite aux lésions de la moelle épinière (Dubreuil et al., 2003), dans le cortex et l'hippocampe suite au TBI, ainsi que suite à une ischémie cérébrale et suite aux convulsions épileptiques. L'inactivation de Rho induit non seulement la régénération des axones lésés et l'augmentation de la récupération fonctionnelle motrice mais cause une diminution du nombre de cellules apoptotiques dans la moelle épinière et dans la rétine (CGR). Ces études montrent que l'inactivation de Rho est bénéfique car elle induit non seulement la régénération des axones mais aussi la réduction du nombre de cellules apoptotiques.

Récemment plusieurs groupes ont inhibé l'environnent inhibiteur de croissance produit par les protéines inhibitrices de croissance de la myéline et de la cicatrice gliale pour augmenter le taux de régénération axonale suite aux lésions de la moelle épinière (Davies et al., 2004; Grimpe and Silver, 2004; Kim et al., 2004; Li et al., 2004; Song et al., 2004; Li et al., 2005; Zheng et al., 2005). En effet, l'ablation de Nogo, MAG, $p75^{NTR}$, NgR et des CSPGs induit à des degrés différents de la régénération axonale suite aux lésions de la moelle épinière. L'inhibition de la signalisation par les inhibiteurs de la myéline (les protéines ou les récepteurs) reste contradictoire, mais en général leur inhibition ou ablation induisent de faibles degrés de régénération axonale suite aux lésions de la moelle. L'inhibition des CSPGs augmente le nombre d'axones qui régénèrent ainsi que la récupération fonctionnelle motrice des animaux. Rho semble donc être une bonne cible

thérapeutique puisque chacune de ces protéines inhibe la croissance axonale en activant Rho. Ceci est appuyé par nos résultats de régénération dans le nerf optique et la moelle épinière.

La production de niveaux robuste de régénération et récupération dans le SNC va nécessiter plus que bloquer les inhibiteurs de croissance présents suite aux lésions. Une étude récente par Fisher (Fischer et al., 2004a) et al. montre que l'inactivation de la signalisation des protéines inhibitrices de la myéline (avec un mutant dominat négatif de Nogo) induit seulement la régénération des axones lorsque les cellules sont préalablement mis en mode de croissance par une lésion du cristallin, ce qui cause l'invasion et l'activation de macrophages. Ces résultats indiquent que l'environnement inhibiteur n'est pas le seul facteur responsable de l'inhibition de la croissance axonale dans le SNC. D'autres études ont aussi utilisé l'approche de traitements combinés, contrant l'environnement inhibiteur et simultanément mettre les cellules en mode de croissance, pour induire de hauts niveaux de régénération. La combinaison d'AMPc, qui inhibe les effets des protéines inhibitrices de la myéline, et d'implantation de cellules de Schwann cause la survie et la régénération des neurones, la myélinisation des axones de la moelle épinière et la récupération fonctionnelle des animaux (Lu et al., 2004; Pearse et al., 2004). La combinaison de chondroitinase ABC, inhibe les CSPGs inhibiteur de la cicatrice gliale, et d'implantation de cellules de Schwann induit aussi la régénération des axones de la moelle épinière à travers les cellules de Schwann (Chau et al., 2004). Une étude plus récente a aussi utilisé des 'olfactory ensheathing glia' en plus de la combinaison de chondroitinase ABC et l'implantation de cellules de Schwann pour

induire la régénération axonale de fibres sérotoninergiques, la myélinisation de ces axones et la récupération motrice de ces animaux suite aux lésions de la moelle épinière (Fouad et al., 2005). Par contre ces effets n'affectent pas toutes les populations neuronales de la moelle épinière. Ensemble ces résultats indiquent que la combinaison de l'inhibition de l'environnement inhibiteur et simultanément mettre les cellules en mode de croissance est très bénéfique malgré qu'elle ne semble pas être efficace dans toutes les populations neuronales de la moelle épinière.

L'inactivation de Rho induit des effets prometteurs *in vivo*, mais il semblerait que ce ne soit pas nécessairement suffisant pour faire complètement repousser les axones lésés. Une étude récente de Fisher et al, montre que l'inactivation de Rho en combinaison avec une lésion du cristallin induit des taux de régénération les plus élevés dans le nerf optique que préalablement rapporté dans ce modèle (Fischer et al., 2004b). La C3 inactive Rho A, B et C et il se peut que les isoformes aient des effets différents suites aux lésions du SNC. Il faut aussi émettre de la caution en ciblant Rho, puisque cette GTPase participe à la régulation de plusieurs voies de signalisation qui pourrait avoir des effets bénéfiques suite à ces traumatismes tel que la motilité des cellules immunitaires et des astrocytes au site de la lésion (Jones et al., 2000; Ridley, 2001a, b). Les astrocytes réactifs produisent cytokines et des facteurs de croissance qui peuvent avoir des effets bénéfiques aux neurones et donc l'inhibition de leur migration aux sites de lésion peut avoir des effets néfastes. Rho joue aussi un rôle dans la phagocytose, par ses effets sur l'actine, et son inhibition pourrait donc empêcher la phagocytose des débris cellulaires au site de la lésion. Donc, l'inactivation de Rho induit des effets bébifiques sur la régénération

axonale et la survie *in vivo,* ce qui indique que Rho, est une bonne cible thérapeutique dans le SNC.

5.2 Mécanismes apoptotiques potentiels en aval de Rho

Plusieurs études ont démontré le rôle de Rho dans l'induction de l'apoptose suite aux lésions de la moelle épinière et à une ischémie cérébrale. Par contre les voies de signalisation apoptotiques activée par la GTPase est très peu connue. Les seuls indices de protéines en aval de Rho provenant d'expériences *in vitro* dans des cellules non neuronales. Jusqu'à présent ces voies ne sont pas élucidées *in vivo.* Rho active (*in vitro*) des facteurs de transcription impliqués dans la synthèse de protéines pro-apoptotique (Aznar and Lacal, 2001) tels que NFκβ et c-jun (Benitah et al., 2004). L'inactivation de Rho suite aux lésions de la moelle épinière pourrait donc bloquer l'apoptose en agissant sur ces protéines. PKN et ROCK, deux effecteurs de Rho sont impliquées dans l'apoptose et dans l'activation de c-jun et NFκβ.

5.2.1 PKN et l'apoptose

PKN est un effecteur de Rho qui peut induire l'apoptose et activer le facteur de transcription c-jun. PKN est une sérine thréonine kinase de 120 KDa qui est exprimée dans le cerveau (Kawamata et al., 1998) et qui lie la forme active de RhoA (Amano et al., 1996b; Reid et al., 1996). PKN induit la réorganisation du cytosquelette par Rho et peut lier l'α-actine, la vimentine, le GFAP et MLC (Matsuzawa et al., 1997; Mukai et al., 1997; Mukai, 2003) . PKN a aussi un rôle dans l'apoptose. PKN est un substrat de la caspase 3 : cette dernière clive PNK et génère des fragments de kinases activés (de 55

Kda) (Takahashi et al., 1998). Des fragments de 55 KDa (résultant du clivage de PKN par la caspase-3) se retrouvent dans des extraits d'hippocampes suite à une ischémie ainsi que dans des rétines de rats (modèle ischémie-reperfusion) (Sumioka et al., 2000). Ces fragments sont aussi générés par l'induction de l'apoptose par FAS. De plus, il a été montré que le produit du clivage de PKN dans les étapes précoces de l'apoptose, inhibe la phosphorylation (Ser 473 et Thr 308) et l'activation de la protéine anti-apoptotique PKB. PNK peut aussi activer c-jun un médiateur important de l'apoptose par l'intermédiaire de p38γ (ERK6) qui va activer c-jun (par l'activation de jAP1, MEF2) (Marinissen et al., 2001). La production de fragments de kinases activés (Takahashi et al., 1998), résultant du clivage de PKN par la caspase-3 suite à l'induction de l'apoptose, pourrait produire un effet de feedback où ces fragments actifs pourrait alors continuellement activer c-jun. Ceci aurait alors comme effet d'augmenter l'activation de c-jun et possiblement la production d'autres gènes pro-apoptotiques, poussant la cellule à s'engager de façon définitive à l'apoptose. Ensemble, ces résultats démontrent un rôle pour PKN dans la régulation de la mort cellulaire.

Rho peut activer NFκβ par son effecteur PKN. L'activation de NFκβ par les récepteurs de TNF semble se faire par l'intermédiaire des protéines TRAFs. A cet effet, il a été montré que PKN s'associe à la protéine TRAF2 (tumor necrosis factor α receptor associated factor 2) ce qui induit par la suite l'activation de NFκβ par TNFR1. PKN contient la séquence de liaison pour TRAF et ce dernier co-immunoprécipite ensemble. Cette interaction est abolie dans des mutants qui présente dans la séquence de liaison pour TRAF de PKN, et l'inhibition de PKN par des ARN interférant inhibe l'activation

normale de NFκβ par TRAF2 (Gotoh et al., 2004). PKN interagit aussi avec les autres TRAFs 1,2,3,5 et 6. Ensemble ces résultats indiquent de PKN peut activer NFκβ par son intéraction avec les protéines TRAFs.

5.2.2 ROCK et l'apoptose

ROCK joue aussi un rôle dans la régulation de l'apoptose. ROCK joue un rôle important dans le 'membrane blebbing' lors de l'apoptose et peut aussi activer c-jun et NFκβ. De plus, ROCK peut aussi induire l'activation de la caspase 8 par ses effets sur MLC pour induire l'apoptose médiée par le TNF (Jin et al., 2001; Petrache et al., 2001; Petrache et al., 2003). L'activation de la voie Rho-ROCK par MLCK et MLC-p induit alors la translocation de TNFR1 à la membrane et l'activation de la caspase 8 qui active de façon subséquente la caspase 3. Il a récemment été montré que Rho peut aussi activer c-jun par l'activation de son effecteur ROCK. Le groupe de Gutkind, montre que Rho active c-jun en activant par une voie de signalisation où ROCK active JNK, par l'intermédiaire de la protéine SEK, pour activer c-jun (par l'activation de Jun et ATF2) (Marinissen et al., 2004). ROCK est aussi un substrat de la caspase 3, ce qui produit des fragments de kinases actives. Ceci pourrait produire un effet de feedback menant à l'activation de c-jun comme le mécanisme proposé pour PKN.

ROCK active aussi la voie de NFκβ : ROCK phosphoryle IkB, l'inhibiteur de NFκβ ce qui cause sa dégradation subséquente et induit la translocation au noyau et l'activation de NFκβ (Aznar and Lacal, 2001; Benitah et al., 2004). L'inactivation de ROCK avec des inhibiteurs connus n'a pas été bien caractérisée. Il serait intéressant d'utiliser ces inhibiteurs de ROCK *in vivo* pour étudier le rôle de la kinase dans la mort cellulaire

cellulaire apoptotique suite aux lésions de la moelle épinière et aux autres traumatismes du SNC tel que le TBI, l'ischémie et l'épilepsie.

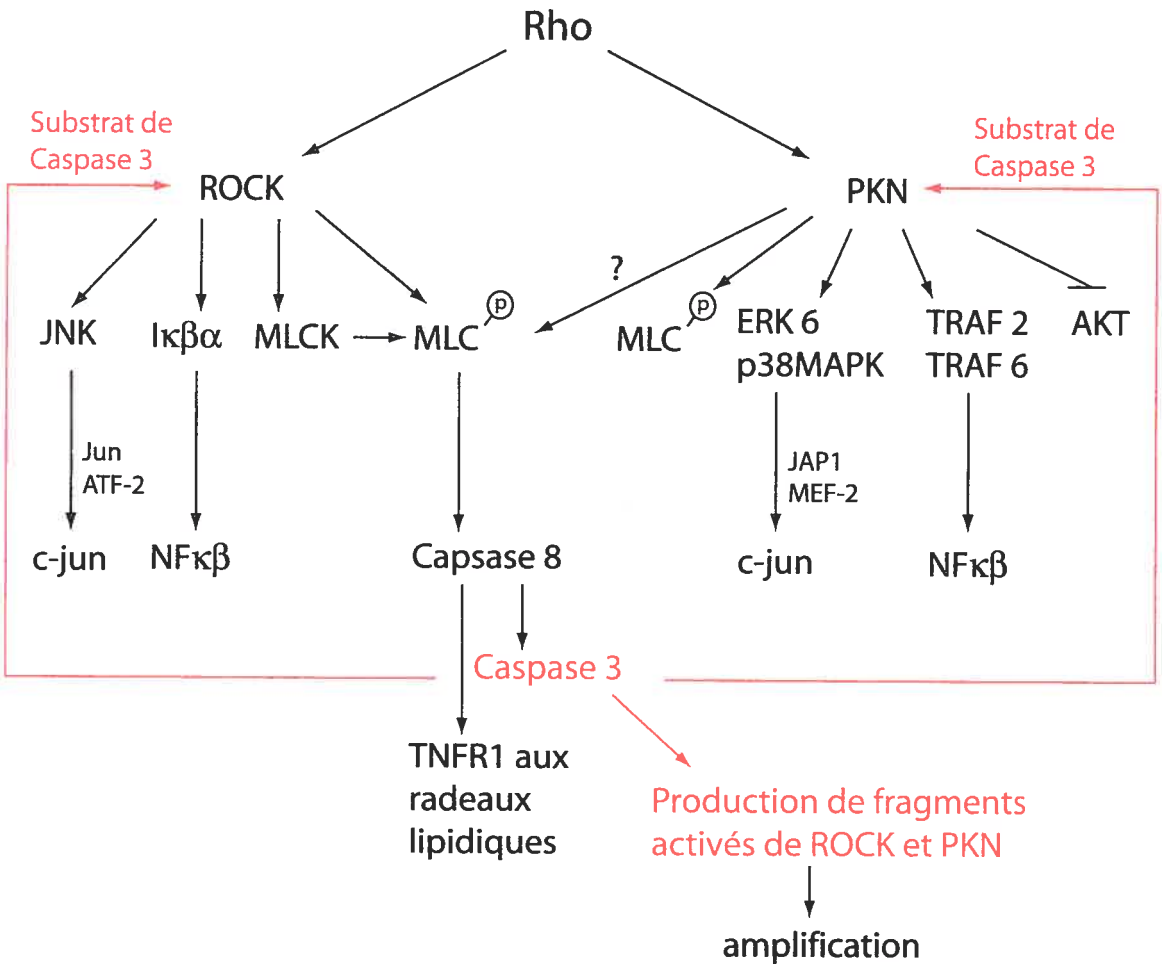

Figure 1 Voies de signalisation apototiques engendrées par Rho
Voir texte pour détails

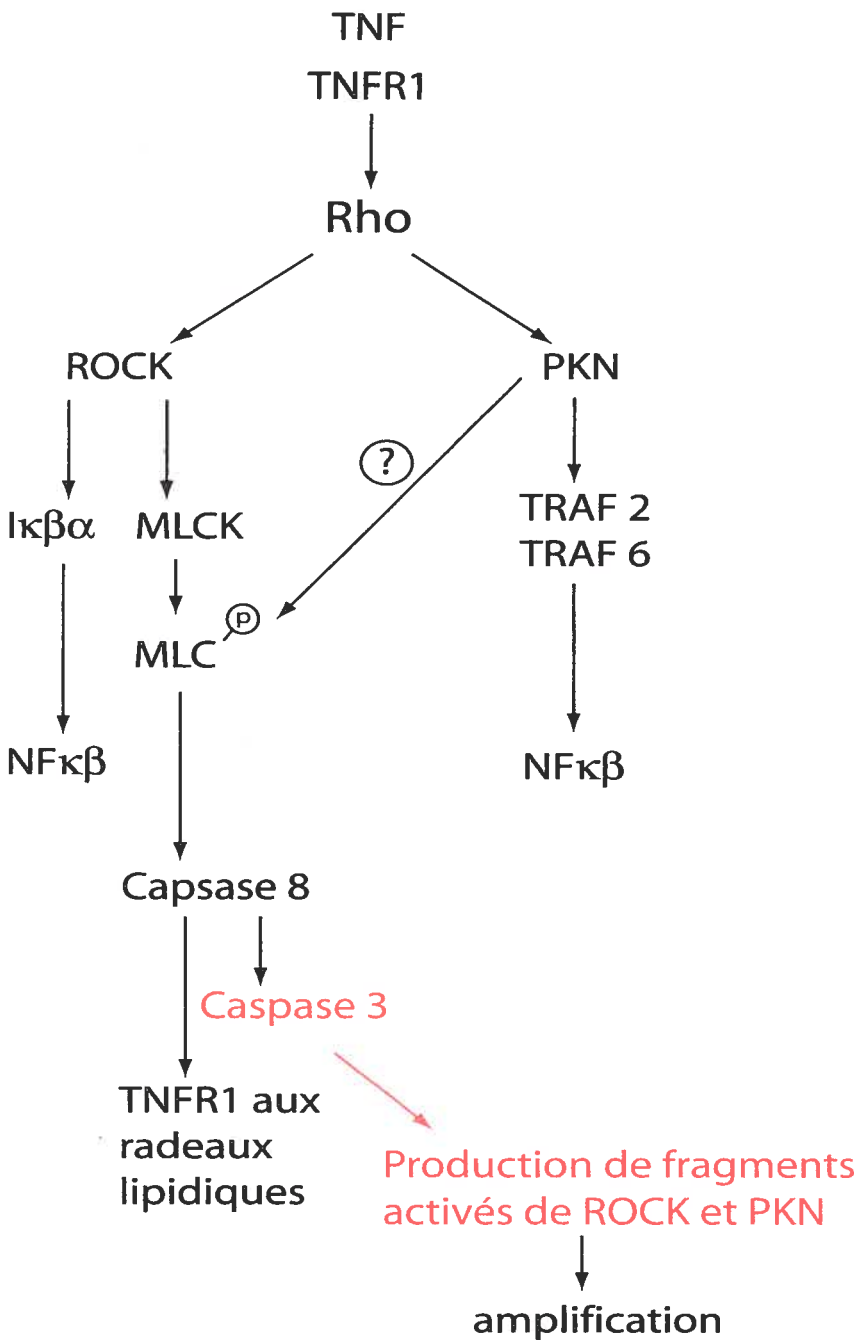

Figure 2 Signalisation apoptotique induite par Rho et TNF
Voies de signalisation possibles induisant la mort cellulaire apoptotique par Rho et TNF, voir texte pour plus de détails

5.2.3 Mécanismes apoptotiques induit par $p75^{NTR}$ et Rho

$P75^{NTR}$ est un récepteur de la famille de récepteurs TNF qui peut lier 1.les neurotrophines, formes pro- et clivées, 2. former des complexes de récepteurs avec les récepteurs Trk, pour induire la signalisation des neurotrophines, 3. former des complexes de récepteurs avec sortilin pour la signalisation des pro-neurotrophines, 4. former des complexes de récepteurs avec les récepteurs NgR et LINGO-1 pour médier les effets des protéines inhibitrices de la myéline. Le récepteur $p75^{NTR}$ est un médiateur connu de l'apoptose dans les neurones (Barrett, 2000; Kaplan and Miller, 2000) et les oligodedrocytes (Casha et al., 2001; Beattie et al., 2002) et peut induire l'apoptose des neurones lésés. Nous avons montré que l'inactivation de Rho diminue le nombre de cellules apoptotiques et affecte les niveaux d'expression de $p75^{NTR}$ suite à une blessure de la moelle épinière (Dubreuil et al., 2003), suggérant que dans ce modèle, l'apoptose est en partie régulé par l'activation de Rho par $p75^{NTR}$. De plus, il a été montré que les niveaux d'expression de $p75^{NTR}$ augmentent suite à une ischémie cérébrale (Bagum et al., 2001; Jover et al., 2002) et que dans ce système l'inactivation de Rho a un rôle protecteur (Laufs et al., 2000). Nous suggérons donc que l'apoptose suite à une ischémie cérébrale puisse aussi être en partie régulé par l'activation de Rho par $p75^{NTR}$ comme dans le cas pour les lésions de la moelle épinière.

$P75^{NTR}$ induit majoritairement la mort cellulaire par l'activation de JNK et c-jun (Barrett, 2000; Roux and Barker, 2002; Bhakar et al., 2003; Becker et al., 2004; Gentry et al., 2004; Linggi et al., 2005). L'activation de JNK active BAX et induit la relâche du cytochrome c suivi de l'activation des caspases 9 et 3. Suite à une lésion de la moelle

épinière et dans des neurones (DRG) lésés il y a une augmentation marquée de l'expression de c-jun (Broude et al., 1999). La phosphorylation prolongée de c-jun active aussi l'apoptose suite à une ischémie cérébrale (Herdegen et al., 1998; Herdegen and Leah, 1998). Nous proposons alors un mécanisme par lequel l'activation de Rho par $p75^{NTR}$, augmente l'expression et l'activité de c-jun ce qui mène à la transcription de molécules pro-apoptotiques. Ceci suggère que Rho pourrait réguler l'apoptose médiée par c-jun par deux voies de signalisation distinctes : celles de PKN et celle de ROCK.

Le récepteur $p75^{NTR}$ peut réguler le niveau d'activation de NFκβ (Barrett, 2000; Roux and Barker, 2002; Gentry et al., 2004), qui joue un rôle important dans la régulation de la survie cellulaire (Aznar and Lacal, 2001; Benitah et al., 2004; Meffert and Baltimore, 2005). L'activation de NFκβ *in vivo* suite à une lésion de la moelle épinière peut induire la mort des cellules dans la moelle (Bethea et al., 1998; Xu et al., 1998). L'activation de NFκβ par $p75^{NTR}$ se fait seulement par l'association avec TRAF 6 (Bhakar et al., 1999). PKN s'associe à TRAF6 ce qui indique que l'activation de Rho par $p75^{NTR}$ pourrait activer NFκβ par l'association de TRAF6 avec PKN. L'activation de $p75^{NTR}$ induirait donc l'association de TRAF6-PNK au récepteur et l'activation de Rho par $p75^{NTR}$ activerait alors PNK par conséquent NFκβ. Ensemble ces résultats indiquent que $p75^{NTR}$ est un important médiateur de l'apoptose et que la mort apoptotique induit par $p75^{NTR}$ et Rho pourrait se faire par l'intermédiaire des effecteurs PNK et ROCK qui par des voies de signalisation distinctes jouent sur l'activation de c-jun et NFκβ.

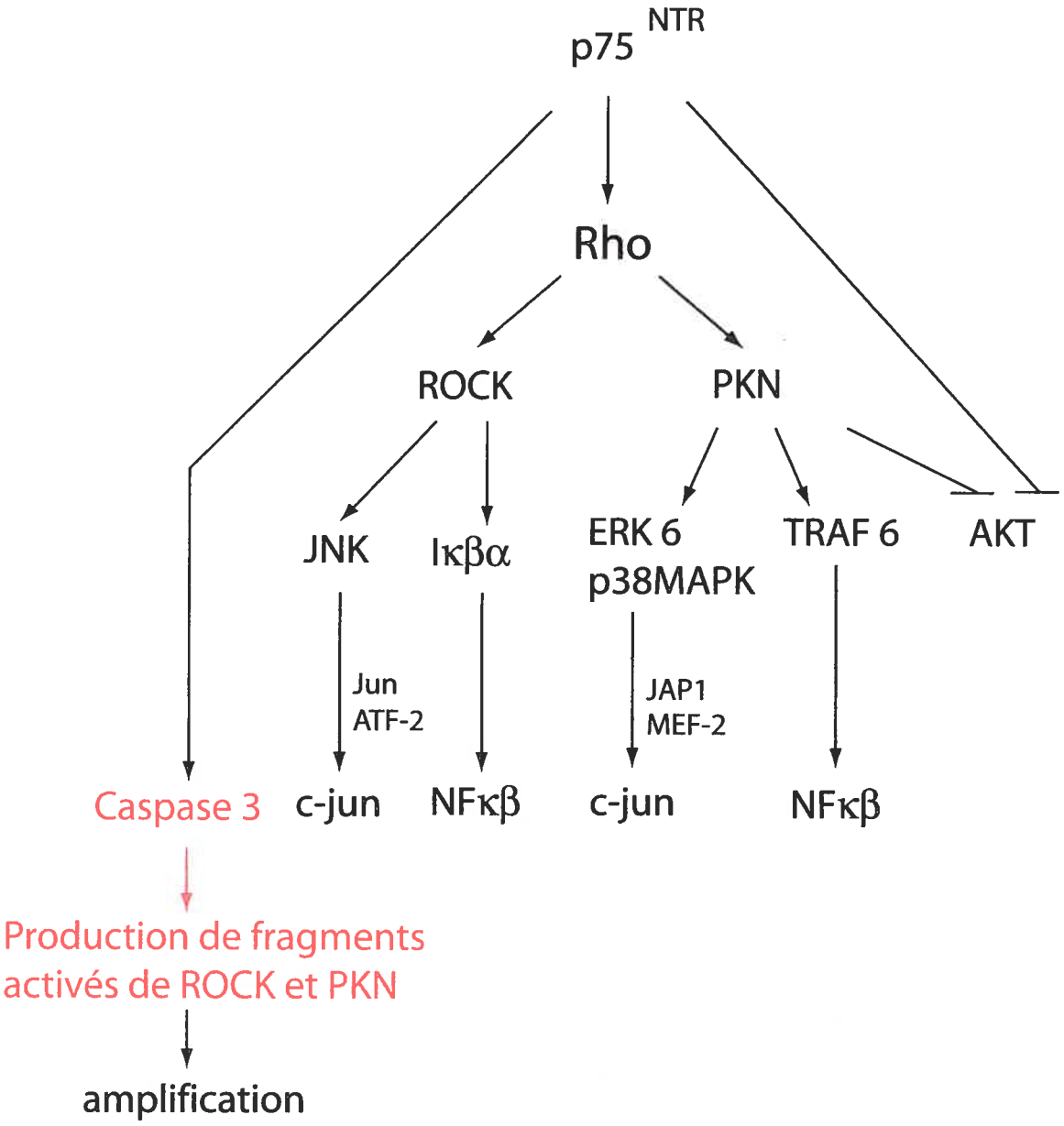

Figure 3 Signalisation apoptotique induite par Rho et p75^{NTR}
Voies de signalisation possibles induisant la mort cellulaire apoptotique par Rho et p75, voir texte pour plus de détails

5.2.4 MMP, ADAMs et Rho

En dernier lieu, de façon plus généralisée Rho pourrait aussi influencer l'apoptose par ses effets sur les MMPs et les ADAMs. Les MMPs (matrix metalloproteinases) sont des endopeptidases qui ciblent et dégradent la matrice extracellulaire et plusieurs autres protéines (ex. cytokines). Les ADAMs (a disintegrin and metalloprotease) sont aussi des protéases qui contiennent des domaines metalloprotease qui clivent des récepteurs transmembranaires et d'autres protéines telles que les cytokines (Moss and Lambert, 2002; Seals and Courtneidge, 2003; Moss and Bartsch, 2004). L'expression des MMPs, induite par des cytokines tel que TNF et IL-1, est produite par les proto-oncogènes c-fos et c-jun (Leppert et al., 2001). Non seulement Rho peut activer c-fos et c-jun (lacal 04), mais Rho peut aussi induire l'expression d'au moins deux MMP : MMP-2 (Matsumoto et al., 2001) et MMP-9 (Abecassis et al., 2003). Les MMPs sont inhibées par les TIMPs (tissue inhibitors of metalloproteinases) (Leib et al., 2001). Le clivage de la portion extracellulaire des récepteurs peut induire leur activation. À date, il existe aucun lien entre la signalisation de Rho et la régulation des ADAMs. Puisque les TIMP1 et 3 inhibent respectivement les MMPs 9 et 2 qui sont sous la régulation de Rho il est possible que Rho puisse aussi affecter les ADAMs 10 et 17.

Les ADAMs 9, 10 et 17 sont impliquées dans le clivage de l'APP par la α-sécrétase, et il a même été proposé que ces dernières soient des formes de α-sécrétase. Le traitement avec les drogues NSAIDs (anti-inflammatoires non stéroïdiens) et les statins (réducteurs de cholestérol) réduisent la production de beta amyloide, qui est neurotoxique (qui provient du clivage de l'APP par les βet γ sécétases; et qui est impliqué dans la

pathogenèse de la maladie d'Alzheimer), tout en augmentant les niveaux de la forme sAPP α, qui provient du clivage de APP par la α-sécrétase, et est neuroprotecteur (Allinson et al., 2003). Les NSAIDs et les statins causent l'augmentation de l'expression de ADAM-17 et ADAM-10 respectivement et ces deux drogues ont aussi comme effet d'inactiver Rho (Zhou et al., 2003; Turner et al., 2005). Il semblerait alors que l'activation de Rho serait inversement proportionnelle au niveau d'expression des ADAMs 9-10 et 17. Par contre, il se peut que la régulation des MMP et des ADAMs par Rho diffère en conditions neuropathologiques ou suite à un traumatisme.

Nous avons montré que l'activation de Rho par $p75^{NTR}$ et par le TNF *in vivo* induit la mort apoptotique des neurones. Il se pourrait que la mort neuronale induite par ces récepteurs résulte d'une ou de plusieurs des voies apoptotiques médiée par Rho et ses effecteurs PKN et ROCK.

5.5 Conclusion générale

La petite GTPase Rho joue un rôle important dans la modulation de la croissance axonale, la régénération et la régulation de l'apoptose. Nous avons en premier lieu montré que Rho est activée par les protéines inhibitrices de la myéline et que Rho est une molécule clé dans l'inhibition de croissance induite par ces protéines (Winton et al., 2002). Dans un second temps, nous avons étudier les états d'activation de Rho ainsi que ses fonctions *in vivo* en réponse aux traumatismes du SNC. Nous avons pu montrer que Rho est activée suite à une lésion de la moelle épinière (transections et compressions-contusions) dans les neurones et les cellules gliales, ainsi que dans le cortex et l'hippocampe suite à une lésion traumatique au cerveau (TBI). En plus du TBI, Rho est activée dans l'hippocampe suite à une ischémie cérébrale ainsi que suite à des convulsions épileptiques. Ces résultats indiquent que l'activation de Rho est une réponse aux traumatismes du système nerveux. De plus, nous avons par la suite élucidé les voies de signalisation induite par l'activation de Rho. Nous avons dans un premier temps montré que l'activation de Rho induit la mort cellulaire dans la moelle épinière de manière dépendante de $p75^{NTR}$. Nous avons aussi montré que la réponse inflammatoire, plus spécifiquement le TNF joue un rôle important dans l'activation de Rho. Dans un dernier temps, nous avons voulu élucider plus en détails les mécanismes apoptotiques impliquant Rho. A cet effet nous avons montré que l'activation de Rho par la combinaison de la myéline et du TNF induit la mort neuronale de neurones *in vitro* et *in vivo* des neurones cholinergiques du septum. Ces résultats montrent un rôle important pour Rho dans la médiation de l'apoptose induit par la neuroinflammation.

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ANNEXES

ANNEXE 1

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

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.

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

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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ANNEXE 2

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

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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 disassembly of actin stress fibers and focal adhesions complexes in fibroblasts. These studies demonstrate that the new C3-like proteins are effective in delivering biologically active C3 into different cell types, thereby, inactivating Rho

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)[1], 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 signalling. One solution has been to create a fusion protein that increases the efficiency of C3 delivery across the 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 (9). This fusion protein is only effective in cells that contain DT receptors, therefore excluding most rodent cells (9). A fusion protein between the bacterial toxin C2 and C3-transferase, C2IN-C3, also increases the ability of C3 to cross the cell membrane by

receptor-mediated endocytosis (10). However, C2IN-C3 cannot 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 translocation (19). 4) Peptides of 7 amino acids in length or longer that contain basic, arginine-rich sequences can act as effective transport peptides (20). 5) The hydrophobic regions of several membrane transport sequences (MTS) can translocate across the cellular membrane and accumulate in the nucleus (21). 6) Recently, a short amphipathic peptide carrier, Pep-1, was also shown to be able to translocate across cellular

membranes (22). We have made and tested four different classes of transport peptides: Tat (C3-02, C3-03); Antennapedia homeodomain (C3- 04); a proline-rich fusogenic peptide sequence (C3-05); 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.

EXPERIMENTAL PROCEDURES

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 antibodies were purchased from Jackson ImmunoResearch Laboratories (Mississauga, Ontario, Canada). All protease inhibitors were purchased from Sigma (Oakville, Ontario, Canada).

Preparation of cDNAs encoding new C3-like chimeric proteins —

cDNA encoding C3-transferase in the plasmid pGEX-2T was obtained from Natalie Lamarche (Department of Anatomy and Cell Biology, McGill University). To add 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 EcoRV restriction site in pSTBlue-1 and further sub-cloned into pGEX-4T/C3, using the EcoRI restriction sites. C3-04 was constructed with oligonucleotide sequences corresponding to the third helix of Antennapedia; 5'AATTCCGCCAGA TCAAGATTTGGTTCCAGAATCGTCGCATGAAGTGGAAGAAGG 3' and 5'GGCGGTCTAGTTCTAAACCAAGCTCTTAGCAGCGTAGT TCACCTTCTTCCAGC 3'. These oligonucleotides were annealed and ligated into pGEX-4T/C3 at the EcoRI and SalI restriction sites. A vector containing the full length 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' GGCCGCCTGCAGTCT TCTTTTTCTTCTTTGTTTCCTTCTG 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.

Preparation of Recombinant Proteins —

Recombinant C3 and C3-like chimeric proteins were purified by affinity chromatography. Bacteria were grown in L-broth (10 g/L Bacto-Tryptone, 5 g/L yeast extract, 10 g/L NaCl) with 50 μg/ml ampicillin (Roche, Québec, Canada), in a shaking incubator for 1.5 hrs at 37°C and 300 rpm. Isopropyl β-dithiogalactopyranoside (IPTG), (Gibco, Burlington, Ontario, Canada) was added to a final concentration of 0.5 mM, to 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-aminobenzamidine agarose beads (Sigma, Oakville, Ontario, Canada) were added and left mixing for 45 minutes at 4°C. The protein was centrifuged to remove the beads and then concentrated using a centriprep-10 concentrator (Millipore, Ontario, Canada). The concentrated protein was desalted by PD-10 column containing Sephadex G-25M (Amersham Pharmacia, Québec, Canada) and 10, 0.5 ml aliquots were collected. The appropriate aliquots were pooled, sterilized by filtration, and stored at – 80°C. Concentration of proteins was determined by protein assay (DC assay, Bio-Rad, Mississauga, Ontario, Canada). Purity of the sample was determined by SDS-PAGE, and confirmed by western blot with anti-C3 antibody (eg. Fig.1).

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 experiments with unmodified C3, NGF primed PC-12 cells were trypsinized, washed with scrape loading buffer (114 mM KCL, 15 mM NaCl, 5.5 mM $MgCl_2$, and 10 mM Tris-HCL) and scraped with a rubber policeman in the presence, or absence of C3. For all experiments, at least four separate experiments were performed in duplicate. Since myelin is phase dense, cells plated on myelin substrates were immunostained with β-III tubulin antibody (Sigma, Oakville, Ontario, Canada, 1:500), and detected with mouse HRP secondary antibody and revealed by 3,3'-diaminobenzidine. For each well, twelve images 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 phalloidin-TRITC (Sigma, Oakville, Ontario, Canada), phalloidin-rhodamin (Molecular Probes, Eugene, OR), or probed with an anti-vinculin antibody, 1:400, (Sigma, Oakville, Ontario, Canada). For all treatments at least three separate experiments were performed in duplicate. A minimum of six images per well, showing approximately 20 cells, was collected for detailed analysis. The micrographs shown in Figs 6 and 7A-B show individual cells representative of the total cell population.

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.

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 slides as described above. The cells were treated with 10 μg/ml of C3-05, or C3 added directly to the media and incubated at 37°C for 24 hours. The cells were fixed with 4% PFA and probed with an anti-C3 antibody followed by FITC staining.

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).

Pull down assays to detect Rho-GTP —

The activity assays were preformed as previously described (25,26). PC-12 cells were grown on poly-l-lysine, or myelin coated 6 well culture dishes. After the cells settled (3-6 hours at 37°C), the media was aspirated and fresh media containing the test C3-like chimeric proteins was added to the cultures. NIH 3T3 cells were plated in 6 well culture dishes and incubated at 37°C for 3-6 hours. After the cells settled, the media was aspirated and fresh media containing the test C3-like chimeric proteins was added to the cultures. At indicated times (Fig. 8B), the cells were washed with ice cold Tris buffered saline (TBS) 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.

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 that even with the scrape-loading technique, high concentrations of C3 were required to stimulate neurite outgrowth on myelin (Fig. 2). When C3 was added to the culture medium of pre-plated cells, C3 had no significant effect (Fig. 2).

To test the ability of the new C3-like chimeric proteins to promote neurite outgrowth, we performed dose response experiments with PC-12 cells plated on myelin substrates (Fig.

3). In these experiments, the C3-like chimeric proteins were added directly to the culture medium after pre-plating cells. This procedure was carefully preformed to avoid 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.

ADP-ribosylation of Rho causes it to migrate with a larger apparent molecular weight on SDS gels (7,27). To study the ability of C3-05 to ADP-ribosylate Rho, we examined the electrophoretic mobility of RhoA by Western blot of cell lysates treated for 24 hours with 25 μg/ml of scrape-loaded C3, or 10 μg/ml of C3-05 added directly to the media (Fig. 4,

top). Both scrape loaded C3 and C3-05 caused a similar molecular weight shift, confirming the ability of C3-05 to ADP-ribosylate Rho (Fig. 4, top). As a control for the specificity of this molecular weight shift effect, we stripped and 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 (Fig. 5B-C). These results further confirm the permeability of C3-05.

The activation of Rho in fibroblasts stimulates the assembly of actin stress fibers and focal adhesions. Microinjection of C3 into fibroblast cells results in the loss of stress fibers (1). To test if C3-05 is effective in crossing the cell membrane and inactivating

Rho in fibroblasts, we treated CHO cells and NIH 3T3 cells with varying concentrations of C3-05, and stained the cells with phalloidin to visualize actin stress fibers (Fig. 6). When CHO and NIH 3T3 cells were plated in serum containing medium in the absence of C3-05, well-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 can reverse the formation of actin stress fibers induced by LPA.

Focal adhesions are sites of attachment between cells and their extracellular matrices that are regulated by Rho (1). To examine the effects of C3-05 on focal adhesions, we stimulated serum starved NIH 3T3 cells with 100 nM LPA, treated them with 0.0025 μg/ml of C3-05 and visualized focal adhesion complexes with an anti-vinculin antibody (Fig. 7B). Serum starvation of NIH 3T3 cells prevented formation of focal adhesions. LPA stimulation to activate Rho promoted an increased formation of focal adhesions compared 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 inactive 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 results show that myelin causes Rho activation resulting in inhibition of neurite outgrowth, and that this inhibition can be completely reversed by treatment with C3-05.

In NIH 3T3 cells Rho activation is correlated with the formation of actin stress fibers (Fig.4). Pull down assays of homogenates prepared from NIH 3T3 cells grown in the presence of serum showed high Rho activation levels, in agreement with the spread morphology of these cells (Fig 8C). Treatment with varying concentrations of C3-05 at 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).

DISCUSSION

Here we report the construction of five new C3-like chimeric proteins, all of which posses the ability to translocate across the plasma membrane to ADP-ribosylate and inactivate Rho. By a bioassay in which PC-12 were cells plated on growth inhibitory myelin substrates, we have shown that, to varying extents, all five C3-like chimeric proteins promoted neurite outgrowth. Based on our experiments with unmodified C3, 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).

The third helix of the Antennapedia homeodomain can cross cell membranes by both energy and receptor independent mechanisms (16). Antp is a basic peptide that interacts with the charged phospholipids on the outer side of the cell membrane, causing destabilization of the lipid bilayer and the formation of inverted micelles. The formation of this hydrophobic structure allows the Antp and protein cargo to travel freely across the membrane, releasing the transported protein inside the cell once the hydrophobic pocket opens (16,17,29). One drawback to this family of transport peptides is that they lose their 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 cytoplasm would increase the ability of C3-05 to inactive Rho, a cytoplasmic protein.

Highly basic, arginine-rich peptides are another class of transport peptides. A simple string of seven, or more arginines covalently linked to a fluorescein moiety at the N-terminal was able to cross the cell membrane when analyzed by flow cytometry (21). These transport peptides were more effective than both Tat and Antennapedia when compared directly (20). C3-06 contains a basic transport sequence, which corresponding to the reverse Tat sequence. As previously reported (20), we also found it to be a more effective 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-independent mechanisms, and therefore should not be trapped within endocytotic compartments. In addition, C2IN-C3 is not independently cell permeable because the C2II, the binding component of C2 toxin, must be present to induce the uptake of the C2IN-C3 fusion protein by endocytosis. Furthermore, 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 used in this paper are under 50 amino acids, which may enhance uptake, as the total size of the protein is not dramatically increased.

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 improve the extent of regeneration in the central nervous system following spinal cord injury.

The pathological progression of cancer involves abnormal cell growth, resulting in the formation of tumors, and increased cell motility, causing invasive properties and metastasis. Recent studies provide evidence that Rho A, B and C, all substrates for C3, play a role in both tumor development and metastatic progression by regulating the growth and motility of cells (34-40). For example, over expression of Rho is implicated in tumor formation of neck squamous cell cancer, aggressive ductal adenocarcinoma of the pancreas 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 (40). 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 translocating peptide sequences to the carboxyl terminal of C3 does not interfere with its ability to ADP-ribosylate Rho and cause neurite outgrowth on inhibitory substrates in neuronal cells, or the reduction of actin stress fibers in fibroblasts. The transport sequences do not affect the activity of C3, as C3-like chimeric proteins were still active in the lysates of cells, determined by pull down assay, several days after treatment. By increasing the efficiency of C3 entry into cells, smaller volumes and lower concentrations of C3 can be 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.

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[1]The abbreviations used are C3, C3-ADP-ribolytransferase; CSPG, chondroitin sulfate proteoglycan; PCR, polymerase chain reaction; Tat, human immunodeficiency virus transactivator protein; Antp, Antennapedia homeodomain; DTT, Dithiotheitol; PMSF, Phenylmethyl-sulfonyl fluoride; IPTG, isopropyltho-β-D-galactoside; PBS, phosphate buffered saline; TBS, Tris buffered saline; TBS-T, Tris buffered saline and 0.1% Tween-20; HIV, human immunodeficiency virus; DT, Diptheria toxin; DMEM, Dulbecco`s Modified Eagle Medium; NGF, nerve growth factor; BDNF, brain-derived growth factor; aa, amino acid; LPA, lysophosphatidic acid.

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 ($p \leq 0.05$).

Fig. 3. C3-like chimeric proteins efficiently promote neurite outgrowth on myelin substrates. PC-12 cells were plated on myelin substrates in the absence (black bars), or presence of C3-like proteins (C3-02, white bars; C3-03, light gray bars; C3-04, gray bars; C3-05, dark gray bars; C3-06, charcoal bars) at increasing concentrations, between 0.0025 μg/ml and 50 μg/ml, for 24 hours. A, the percent of cells that extended neurites (neurite outgrowth); B, the length of the longest neurite per cell (neurite length). Test

proteins were added to the culture media of pre-plated cells. The data represents the average of for four or 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 (6). 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 C3 antibody. C, D, PC-12 cells plated on myelin substrates, were incubated with 10 μg/ml C3; or D, 10μg/ml C3-05 for 24 hours, and immunostained with a polyclonal anti-C3 antibody. Scale bar, 50 μm.

Fig.6. C3-05 reverses stress fiber formation in CHO and NIH 3T3 cells. Micrographs of phalloidin stained CHO and NIH 3T3 cells treated with increasing concentrations of C3-05 (0.0025 μg/ml to 25μg/ml) for 24 hours. The chimeric C3-like protein was added directly 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 induces 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 Rho (top) and total Rho (bottom) were detected by western blot with a Rho A antibody. B, measurement of Rho inactivation over time after treatment with C3-05 at different concentrations. C,GTP-bound Rho (top) and total Rho (bottom) of NIH 3T3 cells were

detected by western blot with an anti-RhoA antibody. D, measurement of Rho inactivation 24 after treatment of NIH 3T3 cells with C3-05 at different concentrations.

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.

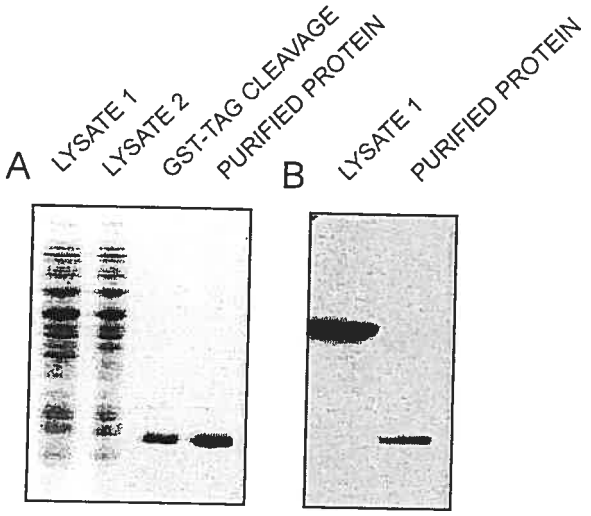
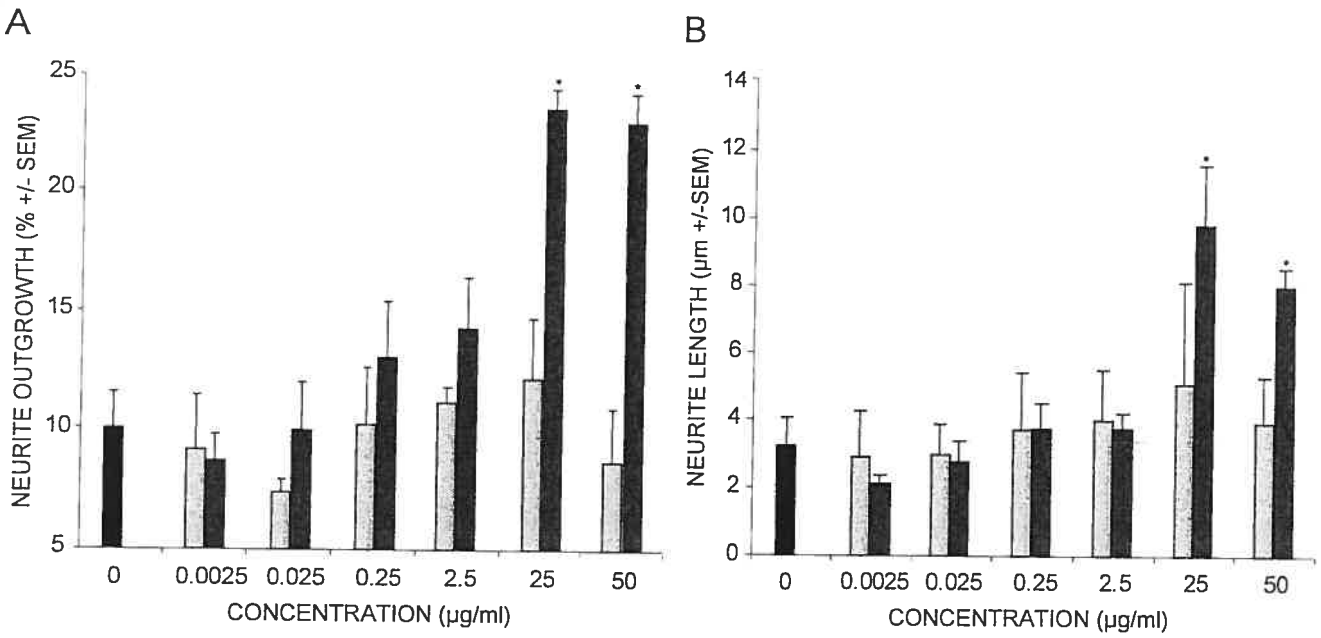

A
LYSATE 1
LYSATE 2
GST-TAG CLEAVAGE
PURIFIED PROTEIN
B
LYSATE 1
PURIFIED PROTEIN

Figure 1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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)

Figure 2

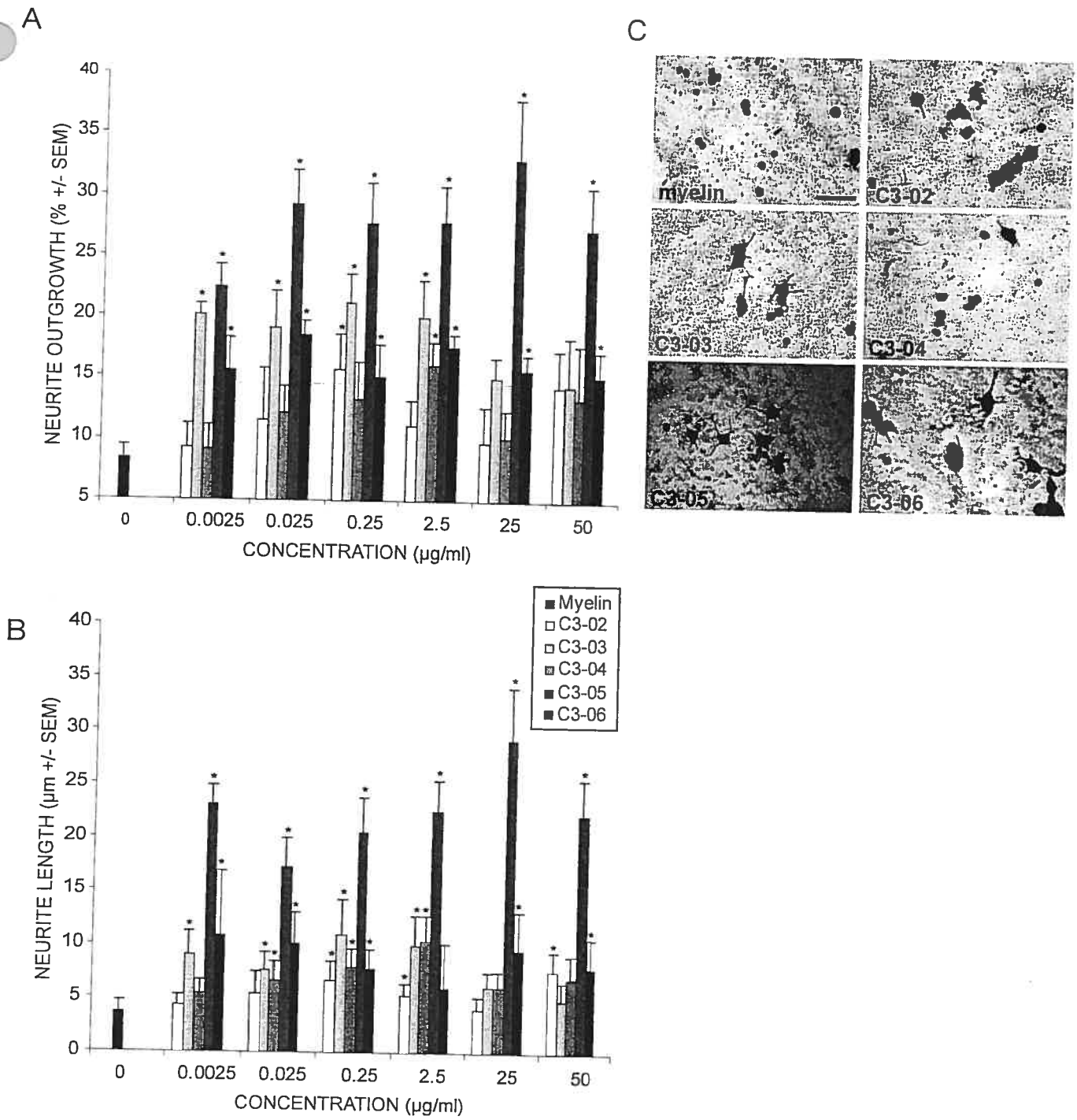

A
NEURITE OUTGROWTH (% +/- SEM)
40
35
30
25
20
15
10
5
0
0.0025
0.025
0.25
2.5
25
50
CONCENTRATION (µg/ml)
B
NEURITE LENGTH (µm +/- SEM)
Myelin
C3-02
C3-03
C3-04
C3-05
C3-06
CONCENTRATION (µg/ml)
C
myelin
C3-02
C3-03
C3-04
C3-05
C3-06

Figure 3

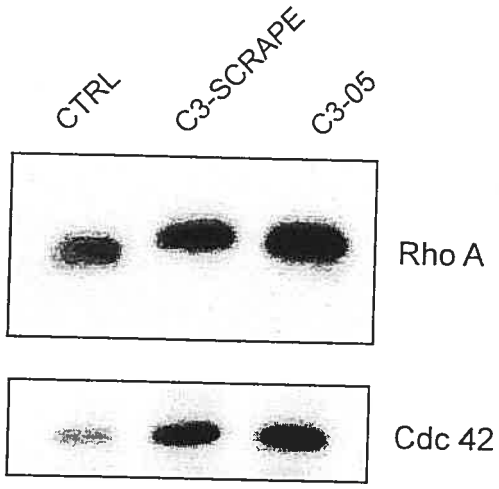


Figure 4

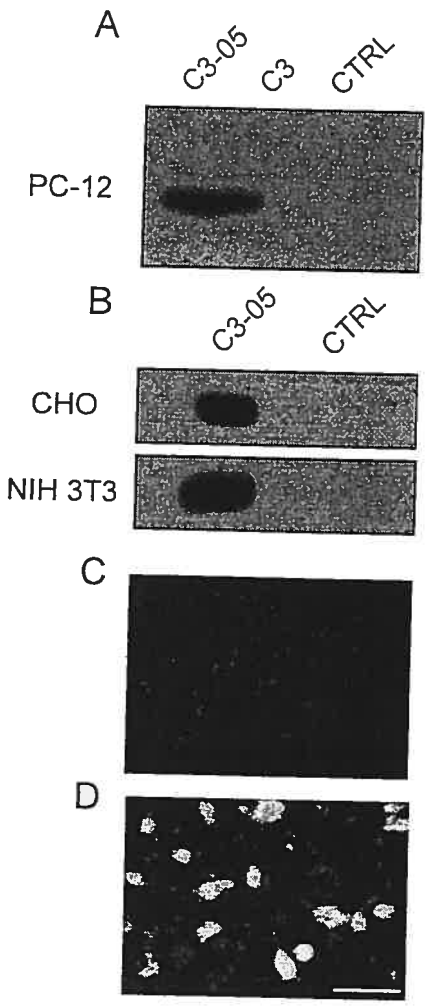


Figure 5

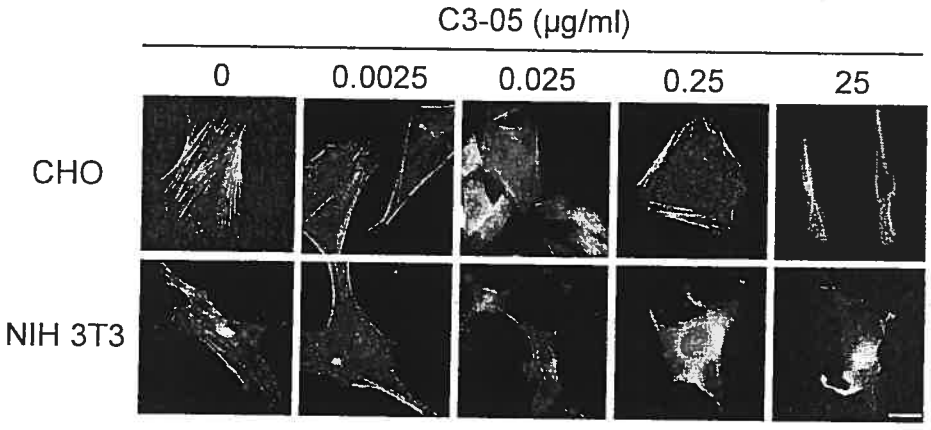
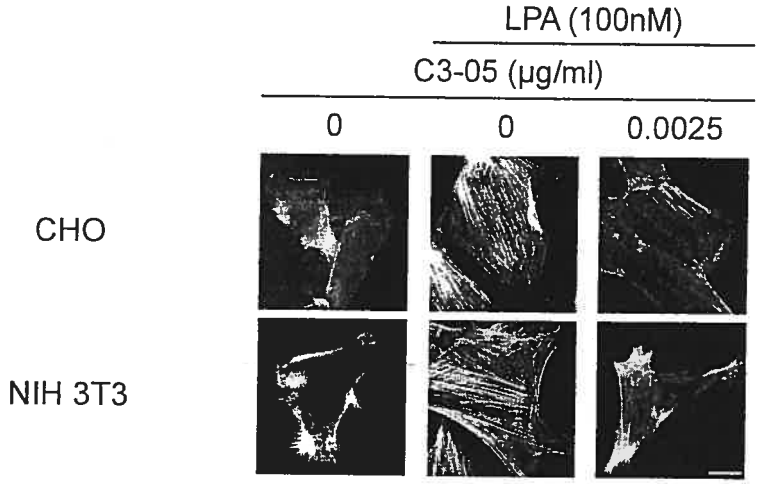


Figure 6

A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C3-05 (μg/ml)
0
0
0.0025
CHO
NIH 3T3

B

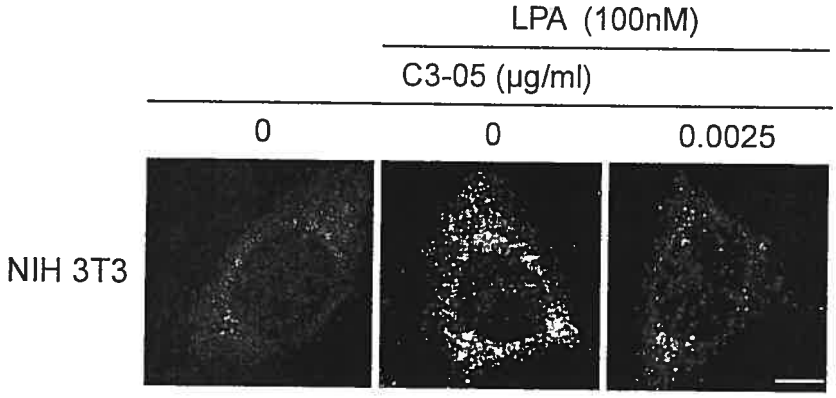
LPA (100nM)
C3-05 (μg/ml)
0
0
0.0025
NIH 3T3

Figure 7

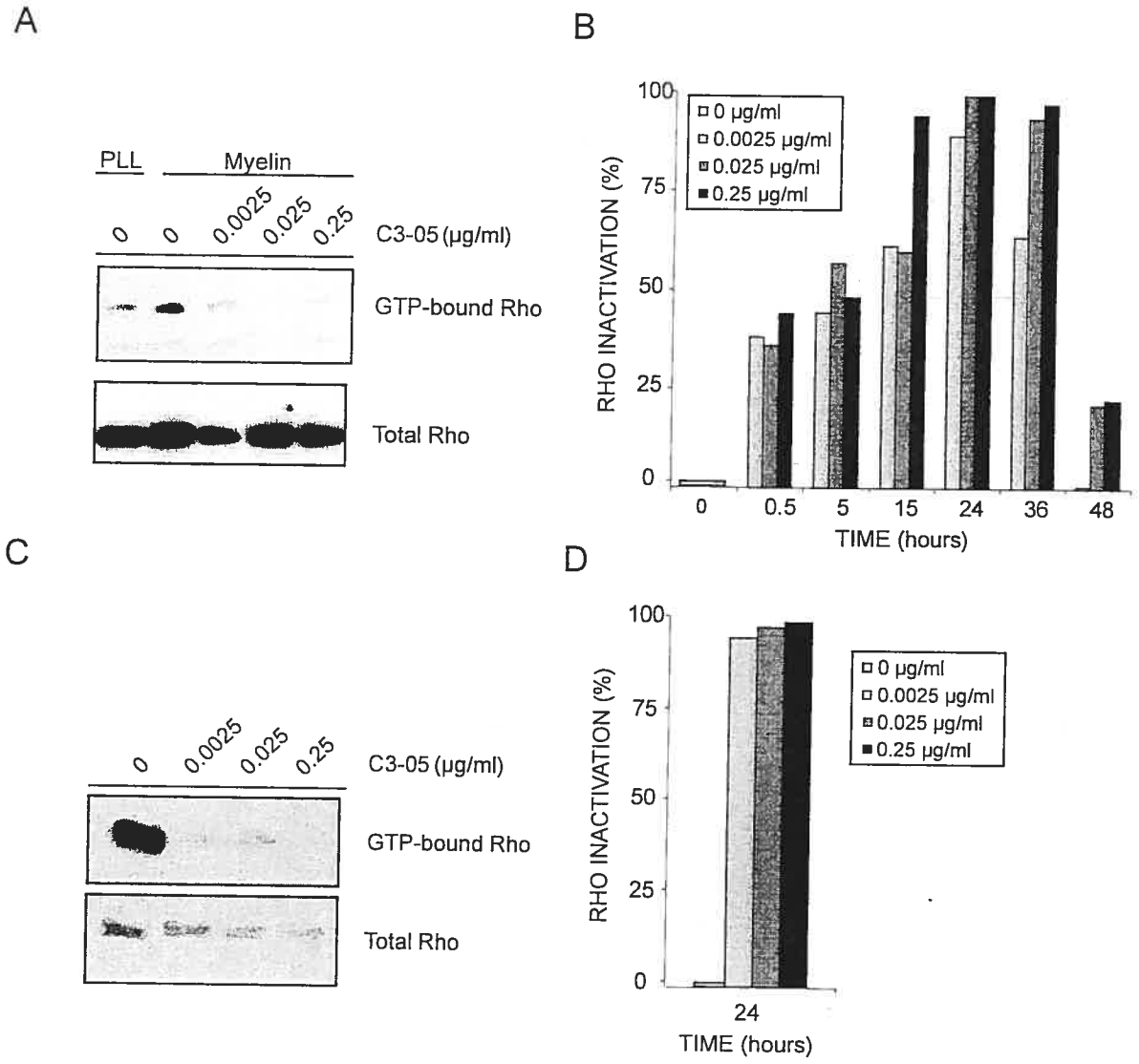
A
PLL
Myelin
0
0
0.0025
0.025
0.25
C3-05 (µg/ml)
GTP-bound Rho
Total Rho
B
RHO INACTIVATION (%)
100
75
50
25
0
0 µg/ml
0.0025 µg/ml
0.025 µg/ml
0.25 µg/ml
0
0.5
5
15
24
36
48
TIME (hours)
C
0
0.0025
0.025
0.25
C3-05 (µg/ml)
GTP-bound Rho
Total Rho
D
RHO INACTIVATION (%)
100
75
50
25
0
0 µg/ml
0.0025 µg/ml
0.025 µg/ml
0.25 µg/ml
24
TIME (hours)

Figure 8

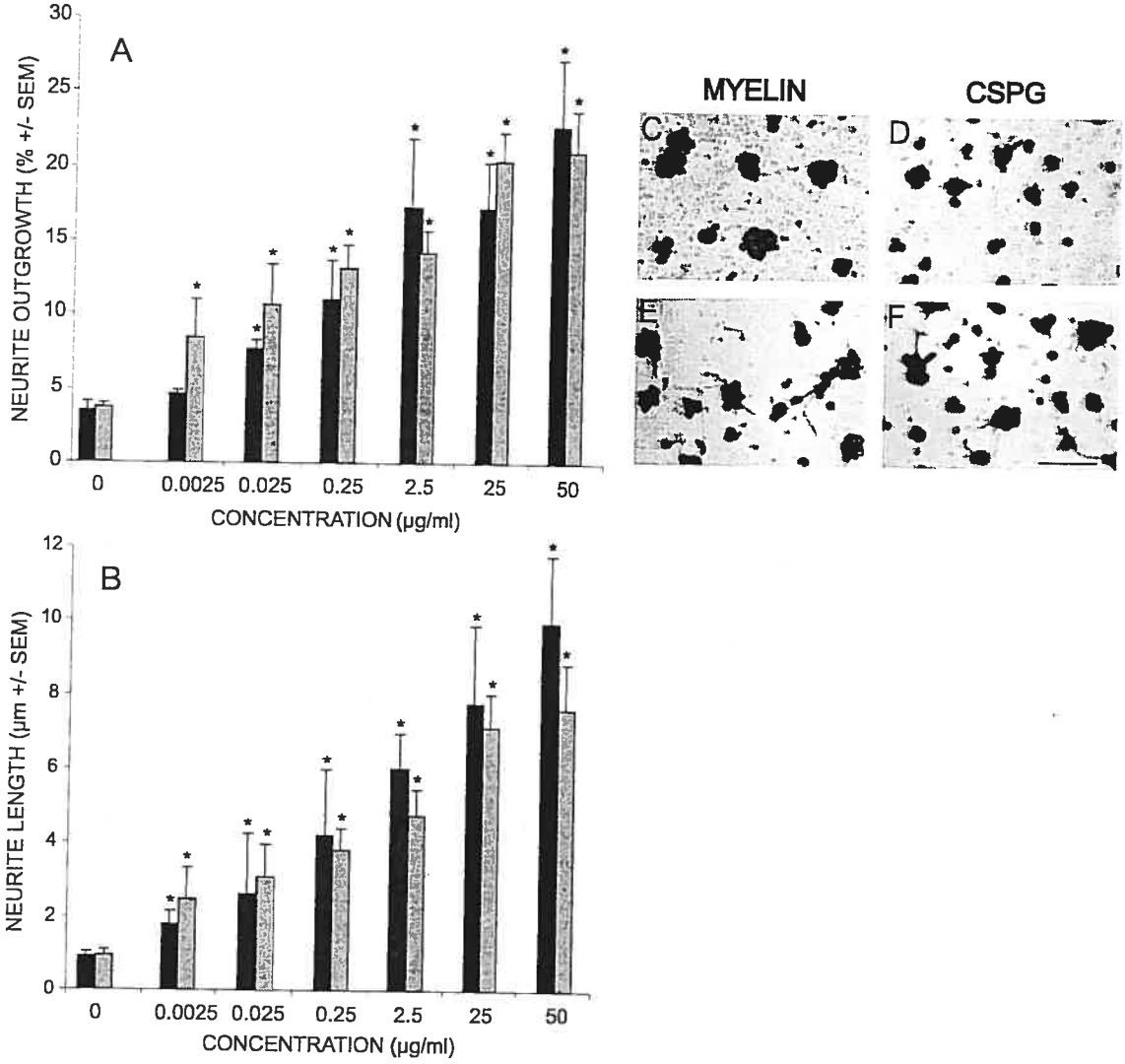
A
NEURITE OUTGROWTH (% +/- SEM)
30
25
20
15
10
5
0
0
0.0025
0.025
0.25
2.5
25
50
CONCENTRATION (µg/ml)
MYELIN
CSPG
C
D
E
F
B
NEURITE LENGTH (µm +/- SEM)
12
10
8
6
4
2
0
0
0.0025
0.025
0.25
2.5
25
50
CONCENTRATION (µg/ml)

Figure 9

ANNEXE 3

Myelin inhibitory proteins contribute to apoptosis in central nervous system inflammation

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Myelin-derived growth inhibitory proteins block regeneration in the CNS by signaling through Rho GTPase. After CNS injury, apoptotic neurons have high levels of activated Rho, however, it is not understood if inhibitory proteins contribute to apoptosis. Here we show that myelin-derived growth inhibitory increase neuronal apoptosis in the presence of tumor necrosis factor-alpha (TNF-α). This increase in apoptosis was blocked by treatment with antagonists to Rho (C3-07), Nogo receptor (NEP1-40), or AMPA receptors (CNQX), all of which reduce Rho activation levels. To test *in vivo* if myelin inhibitors contribute to neuronal cell death in CNS inflammation, myelin, lypopolysaccharide (LPS), TNF-α, or a combination of LPS/myelin, or TNF-α/myelin were microinjected into the medial septal nucleus of adult rats. Combined microinjection of LPS/myelin, or TNF-α/myelin resulted in a significant increase in the death of cholinergic neurons. Our results show that myelin inhibitors contribute to neuron degeneration in neuroinflammatory disease.

Growth inhibitory proteins present in central nervous system (CNS) myelin block axon regeneration after CNS trauma. The three major myelin inhibitory proteins, myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte-myelin glycoprotein (OMgp), all bind a common neuronal receptor, the Nogo-66 receptor (NgR) [1,2]. Ligand binding to NgR activates Rho GTPase [3-5], and blockage of NgR or Rho inactivation promotes regeneration [6-8]. Evidence from our lab [3,9,10] and others [1,2] suggests that both NgR-dependent and NgR-independent growth inhibitory proteins share a common signaling pathway, with Rho GTPase being the key intracellular signaling molecule. Studies involving myelin, Nogo and MAG have demonstrated that in the presence of growth inhibitory proteins neurons posses high levels of Rho-GTP[3-5]. Recently, Dubreuil et al. [9] provided evidence that Rho is abnormally activated in both neuronal and glial cells after spinal cord injury (SCI). Moreover, the inactivation of Rho after SCI is cell protective, suggesting that abnormal Rho activation after neuronal trauma contributes to cell death [9]. Myelin is known to activate Rho [3,9], however, it is not understood if myelin-derived growth inhibitory proteins contribute to Rho mediated apoptosis.

In addition to growth inhibitory proteins, pro-inflammatory cytokines contribute to cell death and failed regeneration in the damaged CNS. After traumatic injury, reactive astrocytes and microglia secrete tumor necrosis factor-alpha (TNF-α) [11] a potent pro-inflammatory cytokine. In addition to inhibiting the elongation and branching of neurites, in a Rho dependent manner, [12] TNF-α also induces the apoptotic cell death of neurons [13]. 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 neuronal apoptosis in culture involve treatment with the broad-

spectrum protein kinase inhibitor staurosporine (STS) [14], treatment with TNF-α [13], or the withdrawal of growth factors, such as nerve growth factor (NGF), from growth factor-dependent cultures [15].

To investigate if Rho activation by growth inhibitory proteins leads to cell death, we studied the survival of cells plated on myelin substrate in the presence or absence of various apoptotic factors. To examine, *in vivo*, if myelin inhibitors promote cell death during CNS inflammation we investigated the extent of apoptosis after microinjection of myelin alone, lypopolysaccharide (LPS) alone, TNF-α alone or the co-microinjection of LPS/myelin, or TNF-α /myelin into the medial septum nucleus (MSN) of rats. LPS is a bacterial endotoxin that is widely used to initiate an acute inflammatory response. The inflammatory response induced by LPS is characterized by expression of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and TNF-α [16]. We provide both *in vitro* and *in vivo* evidence that myelin augments the apoptotic response of neurons to CNS inflammation in a Rho-dependent manner.

RESULTS

To date, no studies have reported the cell death of cultured neurons when plated on myelin substrates. Therefore to directly asses if myelin substrates can induce cell death we plated PC12 cells and cerebellar granule neurons (CGN) on myelin substrates and measured survival by 3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyl tetrasodium bromide (MTT) assay. Our results demonstrated that myelin did not adversely affect survival (Fig. 1a, b). Next, we challenged PC12 cell and CGN neurons with compounds known to induce apoptosis. No difference in cell survival was observed between neurons plated on myelin, or poly-L-lysine (PLL) substrates after treatment with the broad-spectrum protein kinase inhibitor staurosporine, NGF withdrawal, or KCl withdrawal (Fig. 1c). In contrast, treatment with TNF-α significantly decreased the survival of PC12 cell and CGN plated on myelin, as compared to PLL. We further found that purified medial septal neurons were sensitized to undergo apoptosis when exposed to both myelin and TNF-α (Fig. 1c). Cell death induced by TNF-α in the presence of myelin was apoptotic, as determined by TUNEL labeling (Fig.1d).

In neurons, growth inhibitory proteins and pro-inflammatory cytokines activate Rho [3,12]. To compare Rho activation levels after TNF, STS, and withdrawal treatments we used a pull down assay that only detects the active form Rho. Elevated levels of Rho activation were observed in PC-12 cells 0.5 hours following TNF-α treatment, which returned to control levels by 18 hours (Fig. 1e). By contrast, treatment with STS, or NGF withdrawal failed to increase levels of GTP-Rho at all time points tested (Fig. 1e). To better understand the role that Rho plays in the myelin-dependent increase in cell death of TNF-α treated cells, we examined PC-12 cells transfected with constitutively active Rho (V14RhoA) [10]. TNF-α treated V14RhoA PC-12 cells plated on myelin showed a

significant 18 % increase in cell death, as compared to TNF-α treated cells plated on PLL (Fig. 1f), indicating that Rho activation alone is not solely responsible for the increase in apoptosis and the presence of myelin-derived growth inhibitory proteins are needed to augment cell death.

Growth inhibition by myelin-derived growth inhibitory proteins can be blocked with antibodies [17], Nogo-66 receptor antagonists [18], or by the inactivation of the Rho signaling pathway [3]. To further understand the role that Rho activation and myelin/NgR signaling have on apoptosis we made use of C3-07 [Winton, 2002 #3;Bertrand, 2005 #128] a permeable Rho antagonist and NEP1-40 [18], a competitive peptide antagonist of NgR. The activity of both compounds verified was by neurite outgrowth assays (Supplementary Fig. 1). Next, we treated neurons with C3-07 or NEP 1-40, plated them on test substrates overnight, and exposed them to TNF-α for 24 hours. Inactivation of Rho by C3-07 increased the survival of TNF-α treated CGN plated on myelin by 21 % and NEP1-40 treatment increased the survival of CGN plated on myelin substrates by 22 % (Fig. 2a). There was no synergistic effect on survival when neurons were treated with both antagonists simultaneously (Fig. 2a), 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.

A known pathway for TNF-α induced neuronal death is by enhancing glutamate toxicity though alpha-amino-5-methyl isoxazole-4-propionate (AMPA) glutamate receptors [19-21]. We examined if methyl-D-aspartate (NMDA) or AMPA glutamate receptors are involved in the increased cell death of TNF-α treated neurons plated on myelin substrates. CGN were treated with MK-801, a NMDA glutamate receptor antagonist, or CNQX, an AMPA glutamate receptor antagonist, plated on myelin

substrates overnight and exposed to TNF-α for 24 hours. CNQX increased the survival of TNF-α treated CGN plated on myelin by 17 %, as compared to controls (Fig. 2b). Treatment with MK-801 showed no protective effect (Fig. 2b). Next we measured Rho activation levels in cell homogenates of CGN plated on myelin substrates and treated with CNQX or MK-801, or left untreated. As previous reported [3], neurons plated on myelin have increased levels of GTP-bound Rho (Fig. 2c). Treatment with CNQX decreased Rho activation levels while treatment with MK-801 had no affect (Fig. 2c). This finding that CNQX inactivates Rho agrees with a recent report that AMPA glutamate receptors activate Rho [22].

To investigate if myelin-derived growth inhibitory proteins contribute to apoptosis *in vivo*, we used a microinjection model of CNS inflammation to study neurons of the MSN. The MSN is easily identifiable and contains a sub-population of cholinergic neurons that can be identified by immunoreactivity to choline acetyltransferase (ChAT). First we determined that cholinergic neurons express NgR, and that their neurite growth is blocked by myelin (Supplementary Fig. 2a-d). Next, we verified the accuracy of our stereotactic injections by microinjection of DiI to label the injection tract (Fig. 3a). Then, microinjected of PBS, myelin, LPS, TNF-α, or combination of myelin\LPS or myelin\TNF-α into the MSN of adult rats. Following microinjection, apoptotic cells were identified by TUNEL labeling and counted. The average number of TUNEL positive cells per section did not significantly differ after microinjection of PBS, LPS, myelin or TNF-α alone. By contrast, combined microinjection of LPS\myelin or TNF-α\myelin significantly increased the number of TUNEL positive cells, as compared to single injection controls (Fig. 3b). To confirm that apoptotic cells were neuronal, we performed

stereological cell counts of ChAT-positive neurons located in the MSN. Microinjection of LPS\myelin or TNF-α\myelin significantly decreased the number of ChAT positive cells, as compared to PBS, LPS, myelin or TNF-α alone (Fig 4a, b). Combined injections also caused a significant reduction of the average cell body diameter of the ChAT positive neurons (Fig 4c), indicating that neurons appear to atrophy faster with myelin and inflammation.

DISCUSSION

In neurodegenerative diseases or after traumatic CNS injury, it is well known that the inflammatory response contributes to disease progression [23,24]. However, it has not previously been recognized that myelin growth inhibitory proteins may play a significant role in inducing neuronal death in neuroinflammatory disease. We show here with both *in vitro* and *in vivo* experiments that TNF-α and myelin together induce neuronal apoptosis. Further, we show that cholinergic neurons, one of the neuronal types that are susceptible to cell death in Alzheimer disease, die by apoptosis after the combined exposure to myelin and TNF-α. In the intact CNS, TNF-α expression is low, but it is dramatically increased in response to disease and traumatic insult, including multiple sclerosis, Alzheimer's disease and Parkinson's disease [11,25]. In MS, TNF-α is detected at the site of demylinating lesions and is suggested to be responsible for myelin and oligodendrocyte damage [26,27]. 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 [25].

Inflammation has been implicated in the loss of neural tissue during brain and spinal cord trauma, stroke, Amyotrophic Lateral Sclerosis, Parkinson's disease and Alzheimer's diseases (AD) [28,29]. Blocking inflammation through the administration of glucocorticoids or non-steroidal anti-inflammatory drugs (e.g.: prostaglandin synthesis inhibitors) can prevent extensive tissue loss following CNS trauma and to slow down the progression of neurodegenerative diseases.

In the present study, we investigated whether myelin inhibitors contribute to neuronal cell death in the presence of CNS inflammation. The bacterial endotoxin LPS was

microinjected in close proximity of the medial septum nucleus to trigger an acute inflammatory response in the basal forebrain of our animals. The intraparenchymal LPS injection model has been used by several other groups to study the local expression and release of classic proinflammatory cytokines, including TNF-α, and the recruitment of inflammatory cells toward CNS tissues [30-32]. Although treatment with TNF-α induces apoptotic cell death in culture [13], it appears that a single injection of this cytokine in the CNS parenchyma tends not to cause neuronal cell death [31]. Nadeau and Rivest have also reported that LPS alone is unable to provoke neuronal cell loss when microinjected acutely in the CNS, as determined by TUNEL, Fluoro-Jade B and cleaved Caspase-3 staining [31], a finding supported by our result that LPS alone does not induce cell death at 24 hours. We found that the number of TUNEL positive cells only increased with combined injections of TNF-α/myelin and LPS/myelin, and there was a concomitant decrease in cholinergic neurons. These results show that myelin debris together with TNF-α leads to neuronal apoptosis. Further, it was recently reported that TROY, a member of the TNF receptor family, is a co-receptor in the NgR signaling complex responsible for mediating growth inhibition [33,34]. In the presence of myelin-derived growth inhibitory proteins, cells co-expressing of TROY, NgR and Lingo-1, another NgR co-receptor, have high Rho activation levels. Rho is activated by growth inhibitory proteins [9] and by TNF [12].

Alzheimer's disease is a progressive neurodegenerative disorder with significant effects on cholinergic neurons [35] A characteristic pathological feature of AD is the accumulation of extracellular deposits of amyloid. Amyoid plaques result from the aggregation of amyloid-β peptides (Aβ), which are formed by processing of amyloid

precursor protein (APP) by β- and γ-secretases. Studies with non-steroidal anti-inflammatory drugs (NSAIDs) which are known to reduce inflammation show a link between inflammation and Rho signaling [36]. Inhibitors of Rho signaling reduce aggregation *in vitro*, and amyloid plaques *in vivo*. These data are consistent with the role of both myelin and inflammation in the activation of Rho, and suggest a mechanism whereby NSAIDS reduces pathological Aβ and lower the risk of developing AD [37]. A potential role of Rho in AD is further strengthened by studies on Rho kinase. Another hallmark of AD, is the hyperphosphorylated Tau, wihich forms filamentous inclusions [38]. Two kinases that phosphorylate Tau are activated by active Rho, glycogen synthase kinase (GSK-3β) [39] and Rho kinase [40]. One last line of evidence on cholesterol homeostasis suggests the potential link between growth inhibitory signaling, Rho, and AD. Use of statins to treat hypercholesterolemia results in a significant reduction in the prevalence of AD [41,42] and in the formation of Aβ plaques [43]. In addition, several studies have documented the effect of statins on Rho activation levels [44,45]. Together studies on amyloid plaques, tangles of tau, and cholersterol suggest that inflammation and growth inhibitory proteins may together contribute to plaques and tangles in AD though Rho signaling mechanisms.

In CNS disease or neurotrauma, the disruption of myelin, leads to the release of growth inhibitory proteins [46] and TNF- α is secreted by activated microglia . Recently, Nogo-A was shown to be involved in autoimmune-mediated demyelination in an experimental autoimmune encephalomyelitis (EAE) model of MS, [47]. We speculate that activation of Nogo receptor by myelin inhibitors in the presence of TNF-α contributes to neuronal cell death in many different CNS diseases. Treatments that neutralize Nogo,

Nogo receptors or signaling to Rho may be potential neuroprotective therapies for inflammatory disorders. The beneficial effects of Rho, or NgR antagonists after SCI are well documented [6,18]. These studies demonstrate that myelin-derived growth inhibitory proteins augment neuronal death in the presence of CNS inflammation. Further, this increase in apoptotic cell death is blocked by treatments that reduce Rho activation levels. Therefore, in addition to being of therapeutic value for stimulating regeneration and cell survival after SCI, Rho antagonists may also slow the pathological progression of neurodegenerative diseases.

METHODS

Cell culture and measurement of neuronal cell death *in vitro*

PC-12 cells were cultured as previously described [3,10]. Primary cultures of rat cerebellar granule neurons were prepared from postnatal day 6 to 8 Sprague Dawley rats, as previously described [48]. Primary cultures of rat septal neurons were prepared from embryonic day 16 to 17 Sprague Dawley rats, as previously described [48]. All cells were plated on PLL or myelin substrates [10] and incubated overnight at 37°C. To induce apoptosis, PC-12 cells, were treated for 24 hours with 300 nm STS (Sigma, Oakville, Ontario), 100 ng/ml TNF-α (Chemicon,Temecula, CA) or NGF withdrawal. NGF was withdrawn by several washes with NGF-free medium and cells were then maintained in NGF-free media for 24 hours. Granule neurons were treated for 24 hours with either 300 nm STS 100 ng/ml TNF-α or KCl withdrawal [14]. KCl was withdrawn by several washes with KCl-free medium and cells were then maintained in KCL reduced media (i.e. 25 mM to 5 mM) for 24 hours cells. Cell survival was quantified by MTT assay (Sigma). The data were normalized to respective controls. For each experiment, the number of surviving neurons was expressed as percent survival and unpaired student-t tests ($p \leq 0.05$) were completed. For all cell culture experiments, at least four separate experiments were performed in duplicate and data represents the average of all of these experiments.

Measurement of neuronal survival *in vitro*

Prior to plating on test substrates, CGN were treated with Rho antagonist C3-07 (2.5 μg/ml), NEP 1-40 (1 μm) (ADI, San Antonio, Tx), 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.

C3-07 is a cell permeable form of C3-transferase related to C3-05 [Winton, 2002 #3;Bertrand, 2005 #128]. TNF-α was added to culture media for 24 hours and cell survival was quantified by MTT assay. The data were normalized to controls and expressed as a percentage. Statistical unpaired student-t tests ($p \leq 0.05$) were completed.

Pull down assays

Rho-GTP pull down assay was performed as previously described [9]. GTP-bound and total Rho levels were detected by western blot with a monoclonal Rho antibody (1:1000) (Santa Cruz, Santa Cruz, CA) and revealed by HRP-based chemiluminescent reaction.

TUNEL assay

Purified CGN were plated on PLL or myelin substrates in 8-well chamber slides (Nunc, Naperville, IL), incubated overnight, followed by 24 hour treatment with TNF-α (100 ng/ml). TUNEL labeling was performed using the Fluorescein-FragEL DNA Fragmentation Kit (Oncogene, Boston, Massachusetts) as previously described [9].

Medial septum microinjection

A total of 58 adult male Sprague-Dawley rats weighing 200-225g were used for the *in vivo* study. All surgical procedures were approved by the Laval University Animal Care Committees and followed the Canadian Council on Animal Care guidelines. Adult rats were anesthetized with ketamine-xylazine and placed in a stereotaxic frame (David Kopf, Tujunga, CA). An incision was made to expose the skull and a small hole was drilled at co-ordinates A/P 0.83 mm, M/L 0.5mm relative to the bregma. A stainless steel

internal cannula (28 gauge, Plastics One, Roanoke, VA) connected to a Hamilton syringe via polyethylene tubing (PE-50) was then descended into the MSN at V/D 7mm. The syringe was driven by an injection pump at a rate of 0.5μl/min. One μl of PBS, myelin (6μg/μl), TNF-α (100ng/μl), LPS (2.5μg/μl), myelin/TNF-α, or myelin/LPS was then injected over a two minutes period. The cannula was left in place for an additional 2 min following the end of the injection to permit diffusion of the drugs. Animals were sacrificed at survival times of 12 hrs and 1 day, following MS injection.

Immunohistochemistry

Immunohistochemistry with an anti-NgR antibody (ADI) or an anti-ChAT antibody (Chemicon) were preformed on coronal sections of 30-μm thickness. For ChAT staining free-floating sections were used. Sections were incubated with primary antibodies for 24 hours at 4°C and revealed by 3, 3'-diaminobenzidine. For TUNEL staining, sections of 14-μm were used, and the reaction was performed as described above.

Quantitative analysis of TUNEL positive and ChAT-positive neurons

To determine the number of TUNEL and ChAT positive neurons, every third 14-μm and 30-μm section, respectively, through the entire MSN was examined. The number of ChAT-immunoreactive neurons on the ipsilateral and contralateral sides of the medial septum was expressed as an average per section. Cell counts were corrected according. to the formula: $N = n \times T / (T+D)$, where N = the total number, n = counted profile, T = section thickness and D = average cell diameter [49]. The average cell diameter was

determined from measurements of 35 randomly chosen ChAT-positive neurons per animal. ANOVA ($p \leq 0.05$) were completed with Graph Pad In Stat.

FIGURE LEGENDS

Figure 1. The effect of myelin substrates on apoptosis. (a) Survival of PC-12 cells plated on poly-L-lysine (PLL) or myelin (Mye) substrates. Bars represent mean +/- S.E.M., asterisk $p \leq 0.05$ in this an all subsequent experiments. (b) Survival of CGN plated on test substrates. (c) Survival of PC12 cells (PC), CGN and SN plated on PLL (black bars) or myelin (grey bars). Apoptosis was induced by NGF or KCl withdrawal (WD), staurosporine (STS) or TNF-α. (c) TUNEL staining of control and TNF-α treated neurons on PLL and myelin substrates compared to Dapi staining. White arrows show TUNEL-positive cells. (d) Pull down assay for GTP-bound Rho after plating cells on myelin and inducing apoptosis. Western blots show GTP-Rho (top) and total Rho (bottom) (e) Survival of TNF-α treated V14 RhoA PC-12 cells (constitutively active) plated on PLL (black bars) or myelin (grey bars).

Figure 2. AMPA, NgR and Rho antagonists prevent increase in cell death by myelin. (a) Survival of CGN treated with NEP 1-40, C3-07, NEP 1-40 + C3-07, or Ctrl and exposed to TNF-α on test substrates. Black line shows the survival of TNF-α treated CGN on myelin (65%). (b) Survival of CGN treated with MK-801, CNQX, or left untreated (Ctrl) and exposed to TNF-α. (c) GTP-bound Rho in CGN plated on myelin and treated with CNQX, MK-801 or with no treatment (Ctrl).

Figure 3. Microinjection of myelin and TNF together induce neuronal cell death *in vivo*. (a) Schematic of coronal section through the medial septum and micrograph of

microinjection with DiI to show the injection site. (b) Apoptotic cell death in the medial septum determined by counting TUNEL-labeled cells after injections of PBS, LPS, myelin or TNF alone or a combination of myelin and LPS or myelin and TNF. Error bars represent the S.E.M.; asterisk, statistically significant $p \leq 0.05$.

Figure 4. Myelin and TNF together induce cell death of cholinergic neurons (a) ChAT expression detected in the medial septum nucleus of adult rats by immunohistochemistry, Scale; 200 μM. (b) Cell counts of the ChAT positive neurons in the medial septal nucleus. (c) The average cell diameter of ChAT positive neurons.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Rho and NgR antagonists promote neurite outgrowth on inhibitory substrates. (a) The percentage of cells that extend neurites longer than one cell body diameter (neurite outgrowth). (b) The length of the longest neurite per cell (neurite length). (c) Micrographs showing CGN treated with NEP 1-40 (1 μM) or C3-07 (2.5 μg/ml). PLL and myelin controls were left untreated.

Supplementary Figure 2. Medial septal neurons express NgR. (a) Expression of NgR in lysates of cultured medial septal neurons using an anti-NgR antibody. The specificity of the anti-NgR antibody was verified by antigen blocking. (b) Comparison of NgR expression in septal and cortical neurons. (c) Micrographs showing septal neurons plated on PLL and myelin and labeled with anti-βIII tubulin, or anti-ChAT antibodies. (d) Percentage of neurite outgrowth on PLL or myelin substrates. (e) NgR expression

detected in the medial septum of adult rats by immunohistochemistry (right) compared to secondary antibody only control (left).

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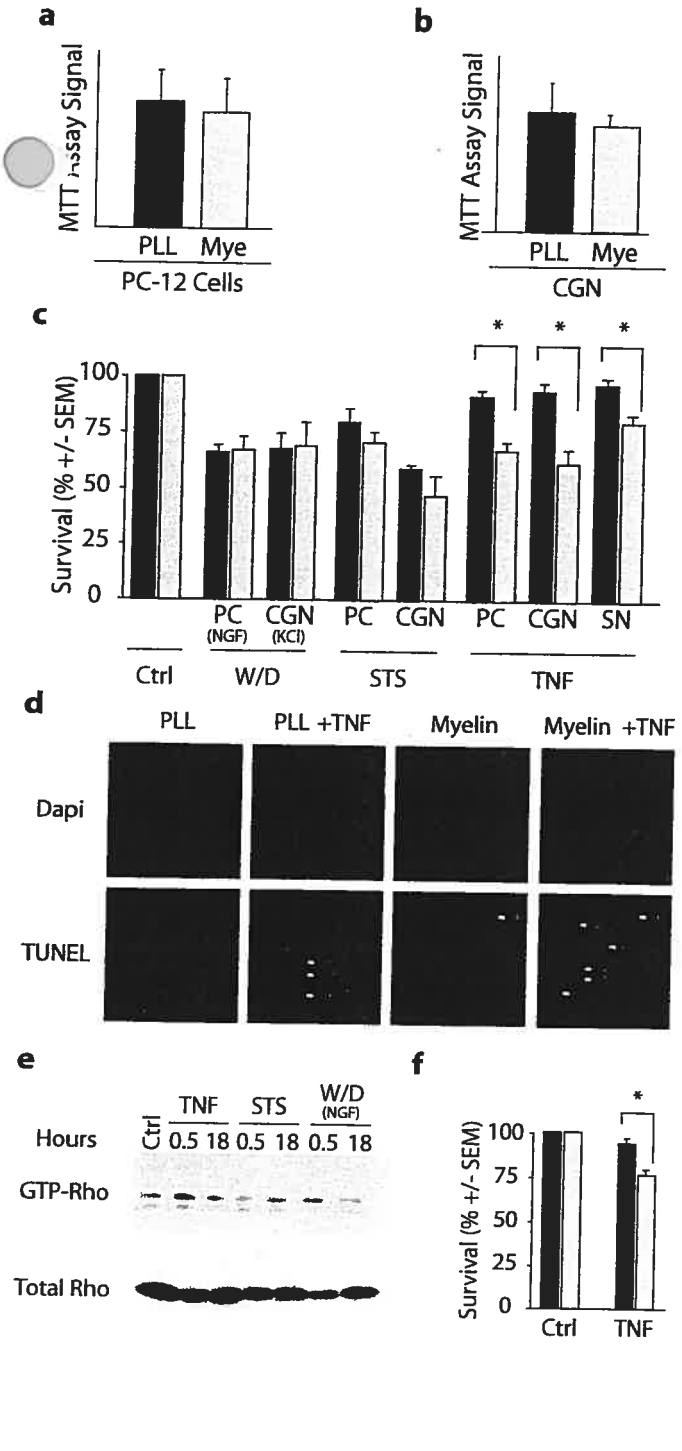
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PLL
Mye
PC-12 Cells
b
MTT Assay Signal
PLL
Mye
CGN
c
Survival (% +/- SEM)
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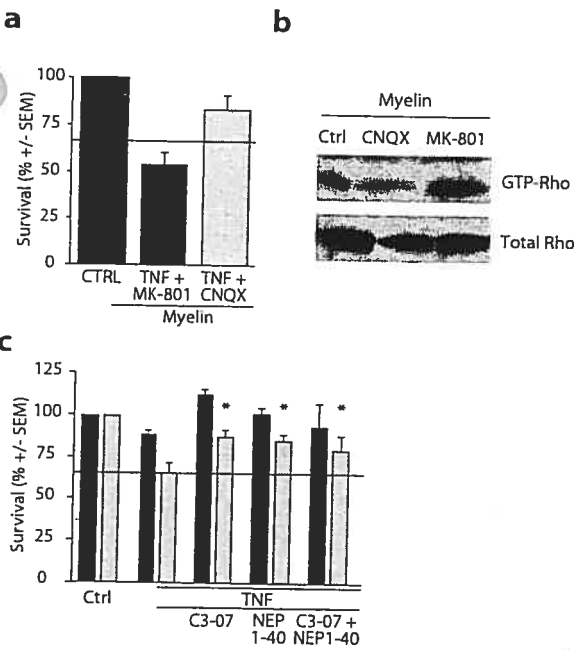
75
50
25
0
PC (NGF)
CGN (KCl)
PC
CGN
PC
CGN
SN
Ctrl
W/D
STS
TNF
*
*
*
d
PLL
PLL +TNF
Myelin
Myelin +TNF
Dapi
TUNEL
e
Hours
Ctrl
TNF
0.5
18
STS
0.5
18
W/D (NGF)
0.5
18
GTP-Rho
Total Rho
f
Survival (% +/- SEM)
100
75
50
25
0
Ctrl
TNF
*

Figure 1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b
Survival (% +/- SEM)
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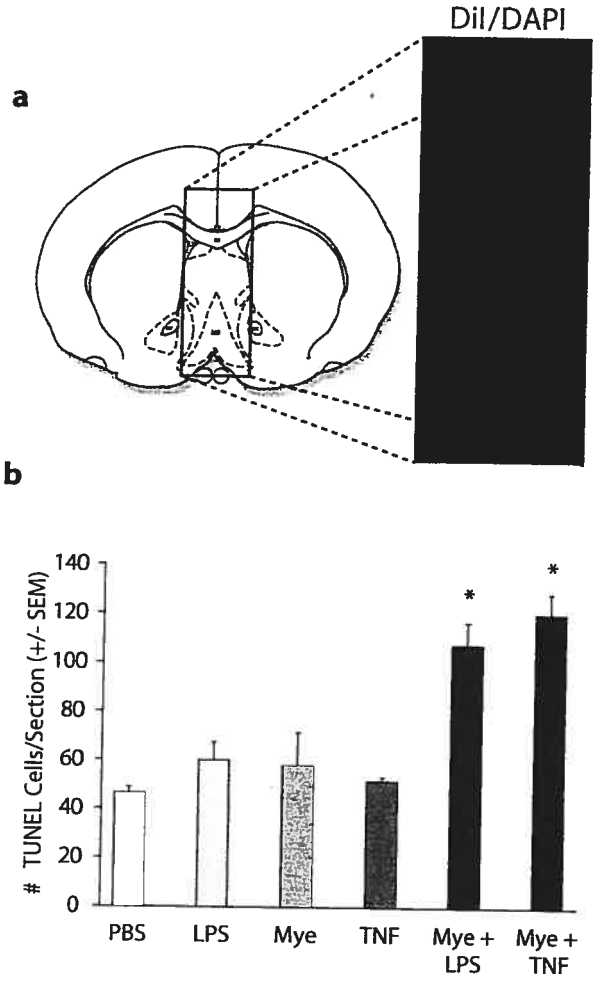
75
50
25
0
CTRL
TNF + MK-801
TNF + CNQX
Myelin
Myelin
Ctrl
CNQX
MK-801
GTP-Rho
Total Rho
c
Survival (% +/- SEM)
125
100
75
50
25
0
*
*
*
Ctrl
TNF
C3-07
NEP 1-40
C3-07 + NEP1-40

Figure 2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DiI/DAPI
b
TUNEL Cells/Section (+/- SEM)
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120
100
80
60
40
20
0
*
*
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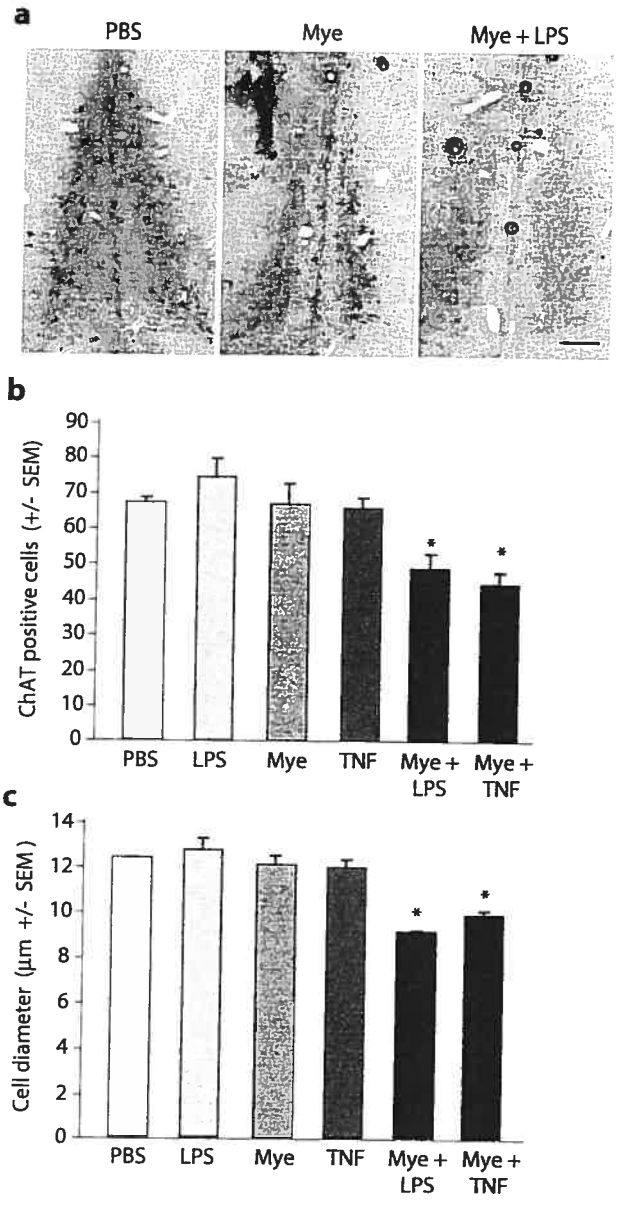
LPS
Mye
TNF
Mye + LPS
Mye + TNF

Figure 3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Mye
Mye + LPS
b
ChAT positive cells (+/- SEM)
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80
70
60
50
40
30
20
10
0
PBS
LPS
Mye
TNF
Mye + LPS
Mye + TNF
*
*
c
Cell diameter (µm +/- SEM)
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12
10
8
6
4
2
0
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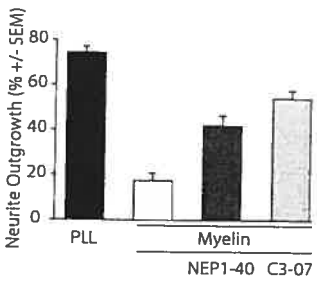
LPS
Mye
TNF
Mye + LPS
Mye + TNF
*
*

Figure 4

a



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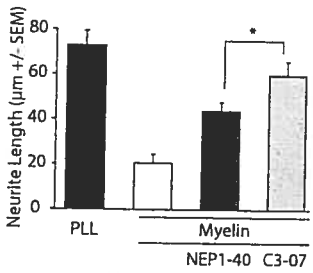

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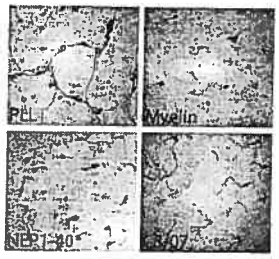
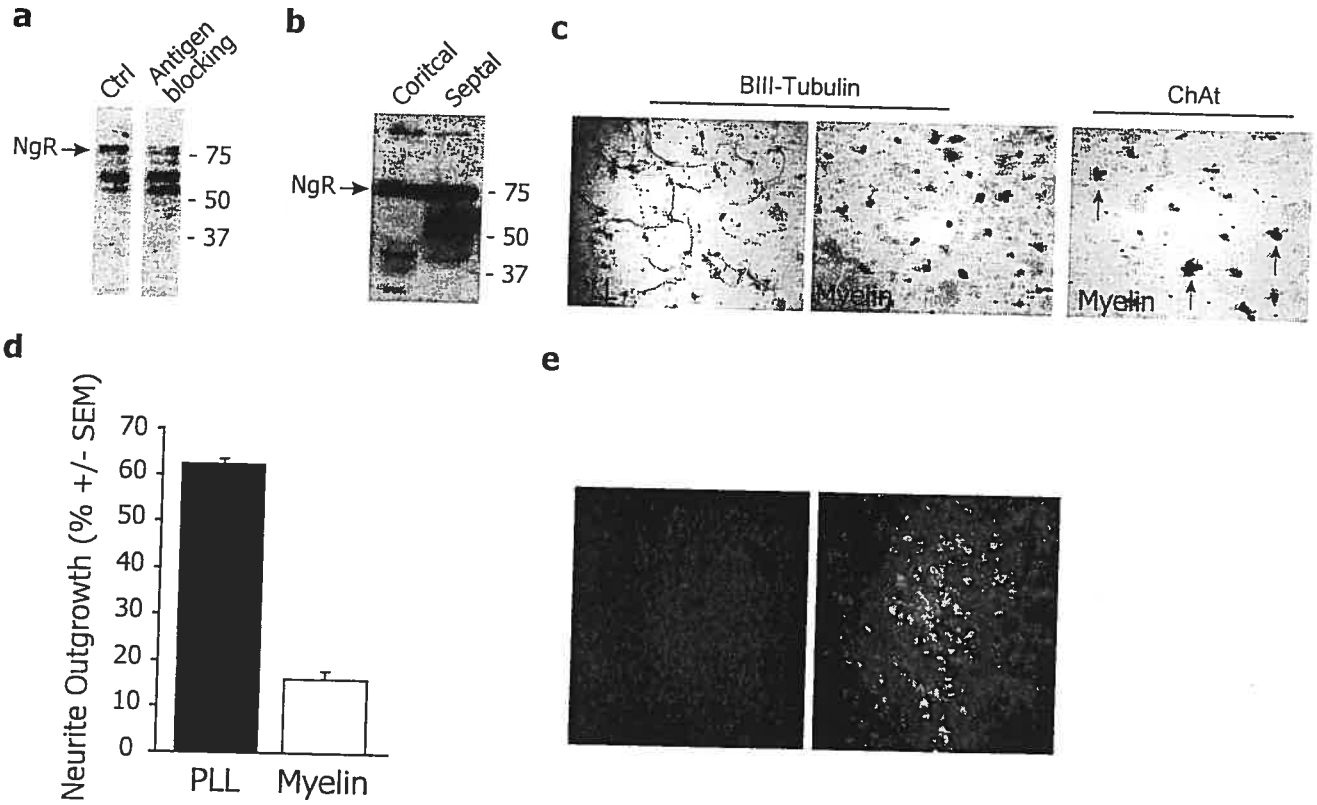


SUPPLEMENTARY FIGURE 1



SUPPLEMENTARY FIGURE 2