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Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis in the central nervous system

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Growth inhibitory proteins in the central nervous system (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 ~50% in both injured mouse and rat, showing a role for activated Rho in cell death after CNS injury. Next, we examined the role of the p75 neurotrophin receptor ($p75^{NTR}$) in Rho signaling. After SCI, an up-regulation 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 overactivation of Rho after SCI protects cells from $p75^{NTR}$-dependent apoptosis.

Introduction

Growth inhibitory proteins have long been known to inhibit axonal regeneration in the central nervous system (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 (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; Winton et al., 2002).

Early experiments demonstrated that lysophosphatidic 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 (Lehmann et al., 1999; Hara et al., 2000; Dergham et al., 2002). However, it is not known how CNS injury may affect Rho expression and activation.

The mechanism where growth inhibitory proteins may affect Rho signaling are beginning to be understood. It has been shown recently 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). Although $p75^{NTR}$ 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.,

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*Abbreviations used in this paper: CNS, central nervous system; MAG, myelin-associated glycoprotein; NgR, Nogo-66 receptor; $p75^{NTR}$, p75 neurotrophin receptor; RBD, Rho-binding domain; SCI, spinal cord injury.

Key words: spinal cord injury; RhoA; apoptosis; MAG; $p75^{NTR}$

1999). Therefore, we have focused on RhoA for our studies in neurons. In nonneuronal 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 (Jimenez et al., 1995; Aznar and Lacal, 2001; Coleman and Olson, 2002). 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 et al., 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 (Suwa et al., 1998; Fritz et al., 1999; Clark et al., 2000); 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 after 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 et al., 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 COOH terminal to help entry into cells (Winton et al., 2002). We show that C3–05 specifically inactivates Rho in vivo and prevents up-regulation of $p75^{NTR}$. Treatment of injured spinal cord with C3–05 not only effectively reversed Rho activation but also had cell protective effects.

Results

Rho is activated by inhibitory substrates

To examine the effect of growth-inhibitory proteins on Rho activation, we plated PC-12 cells on myelin, MAG, or poly-L-lysine 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 with poly-L-lysine controls (Fig. 1 A). The activation of Rho in cells plated on myelin or MAG was reversed by treatment with the Rho antagonist C3–05 (Fig. 1 B). The RBD beads incubated without lysate (buffer only) show no active Rho when overexposed GST-RBD is detectable (Fig. 1 C), 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).

Rho is activated after traumatic SCI

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 et al., 1999). In rats, we examined spinal cord regions after transection or contusion injury. Experiments are shown as paired control and injured CNS samples (Figs. 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 (Fig. 2, controls). By contrast, Rho activation is dramatically increased after injury (Fig. 2, A and B), increasing over 10-fold (Fig. 2 C). Expression levels of total RhoA, as detected by Western blots from tissue homogenates used for isolation of GTP-Rho, did not change (Fig. 2, A and B). These results show that Rho is massively activated in CNS tissue of rats and mice after SCI compared with 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 h, 24 h, 3 d, and 7 d after lesion. Interestingly, we found that Rho was active as early as 1.5 h after injury. The significant increase in activation observed by 24 h was sustained for at least 7 d (Fig. 2, B and C).

In neuronal-like cells (PC-12), Rho becomes activated when cells are plated on substrates of MAG or myelin (Fig. 1 A). It has also been shown that MAG is not only present in

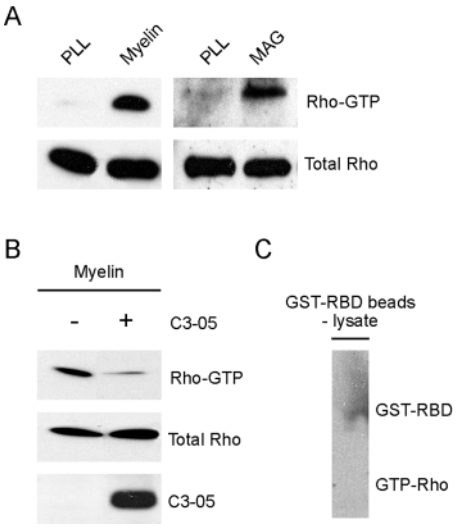

Figure 1. **RhoA is activated when cells are plated on growth inhibitory substrates.** (A) Rho activation levels were examined in vitro in PC-12 cells plated on either poly-L-lysine, myelin (8 μg), or MAG (8 μg). Active GTP-bound RhoA was isolated by pull-down assay 24 h after the cells were plated on substrates and detected by immunoblotting with anti-RhoA antibody. Total Rho levels were determined from whole cell lysates as shown in the 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-down assays and 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.

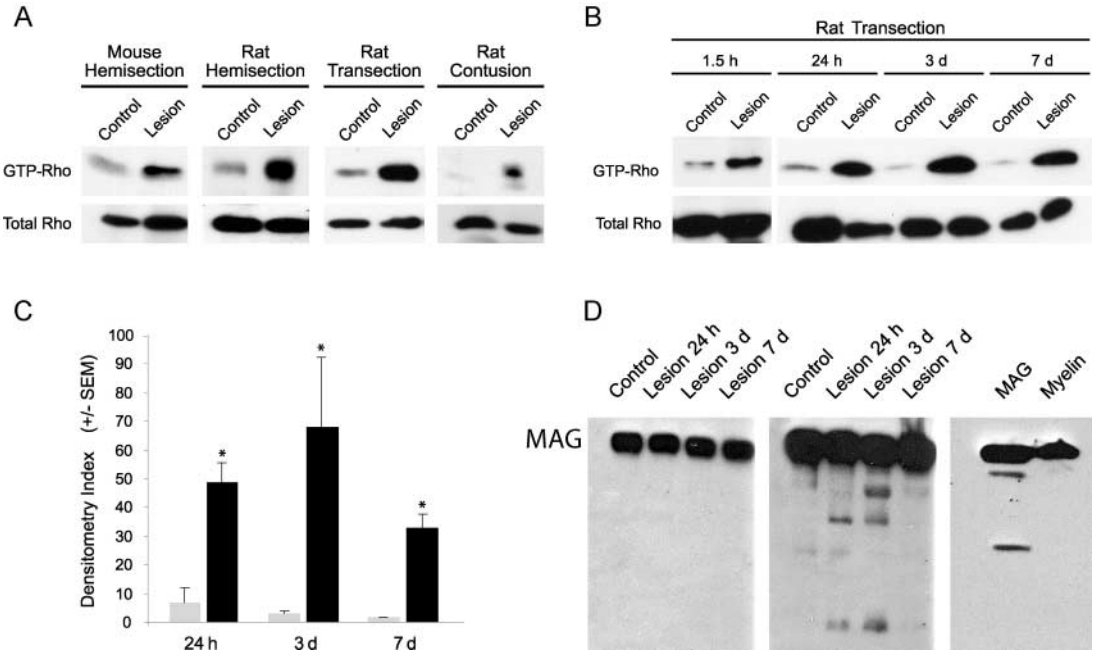

Figure 2. **RhoA activation in normal and injured spinal cord tissue.** (A) RhoA was examined in normal rat and mouse spinal cord homogenates (control) and homogenates prepared 24 h after transection or contusion injury, as indicated (lesion). Active GTP-RhoA was isolated by pull-down assay and detected 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 h postinjury, and activation was sustained for at least 7 d after injury. (C) Quantitative analysis by densitometry of GTP-RhoA after transection of rat spinal cord shown as mean ± SEM for all animals examined; 24 h ($n = 5$), 3 d ($n = 3$), and 7 d ($n = 3$). n represents the number of animals. *$P < 0.05$ 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 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.

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 Fig. 2 B were probed with a monoclonal antibody raised against MAG. MAG levels remain unchanged; minimal protein degradation was visible in overexposed blots (Fig. 2 D). The sustained activation of Rho after SCI may result, in part, from contact of damaged cells with myelin-derived inhibitory proteins.

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 h later. Treatment with C3–05 inactivated Rho, bringing the RhoA activation levels back to the normal basal state (Fig. 3, A and B). To determine if the reversal of Rho activation was sustained after a single injection of the compound, we examined rats 7 d after transection injury and treatment. Even 7 d after C3–05 treatment, Rho activation still remained at basal levels (Fig. 3, A and B). 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 (Fig. 3 A). 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 (Fig. 3 C), showing that endogenous cells from the spinal cord take up C3–05 in vivo.

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 et al., 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 gray (Fig. 4, panels 1 and 2) and white matter (Fig. 4, panel 3) of injured spinal cord. We also found that Rho was activated both rostral (Fig. 4, panel 1) and caudal (Fig. 4, panel 2) to the lesion site. At further distances from the lesion site, staining for active Rho was very faint or absent (Fig. 4, panel 4). Rho-GTP was not detected in uninjured spinal cord (Fig. 5 A, left) or after C3–05 treatment was used to reverse the increase in Rho activation after SCI

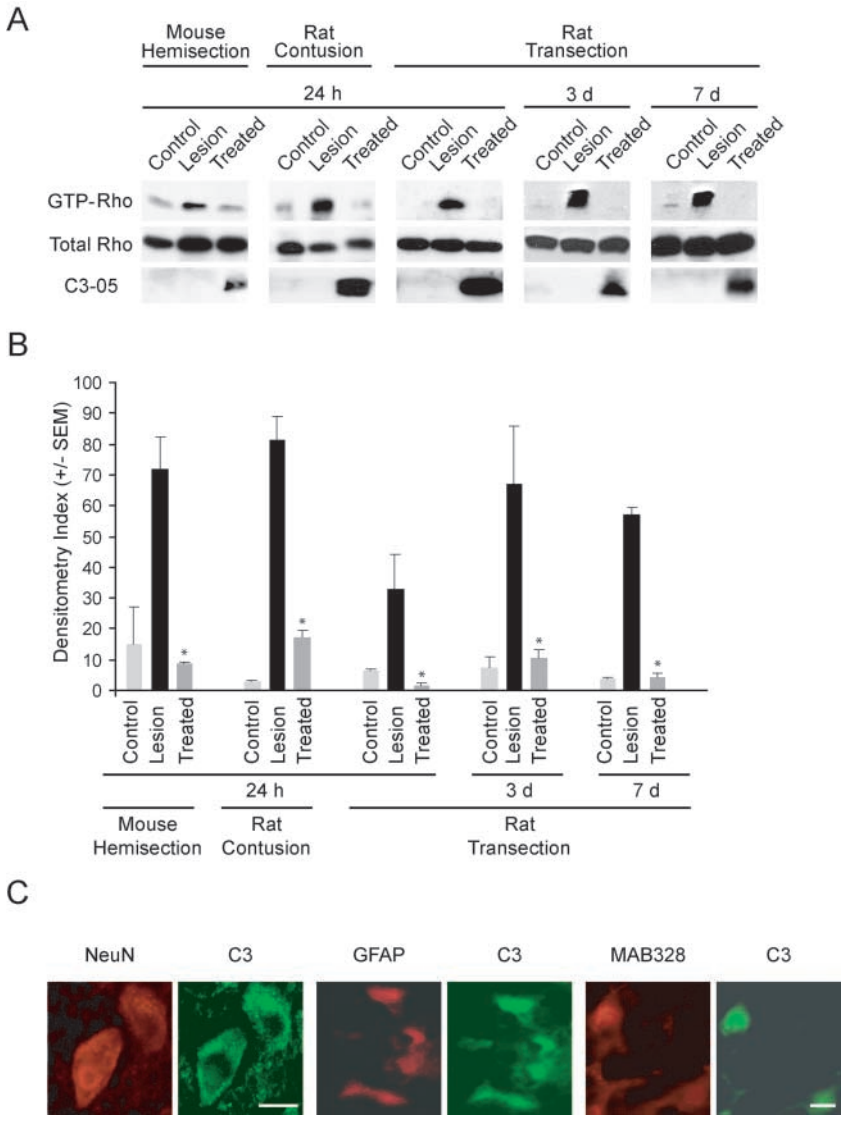

Figure 3. **Treatment with the Rho antagonist C3–05 after contusion or transection of the spinal cord reverses RhoA activation after injury.** (A) Injection of C3–05 into the injury site reversed RhoA activation to basal levels after SCI. Active GTP-RhoA was isolated by pull-down assay and detected with 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 d (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 h ($n = 3$); 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 with 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. Bars, 50 μm.

(Fig. 5 A, middle). To assess the specificity of the technique, we incubated sections with GST without RBD, and no positive cellular active Rho staining is visible (Fig. 5 A, 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 h, 3 d, and 7 d after injury (Fig. 5 B). Therefore, the activation of Rho observed after injury (Figs. 2 and 3) is an endogenous cellular response (Fig. 5 B). The ability to detect GTP-Rho without prior transfection (Li et al., 2002) confirms the high level of endogenous active Rho in neurons and glia after SCI.

Treatment with C3–05 protects cells from apoptosis after injury

Both neurons and glia undergo apoptosis after SCI in rat, which leads to the formation of a large lesion cavity (Liu et al., 1997; Shuman et al., 1997; Grossman et al., 2001). 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 TUNEL (Fig. 6 A, top), similar to that observed after rat SCI (Fig. 6 A, bottom). Importantly, in both mice and rats treated with C3–05, the number of TUNEL-labeled cells was significantly reduced by ~50% after SCI (Fig. 6 B). Not only was C3–05 present in neurons, astrocytes, and oligodendrocytes (Fig. 3 C), but most cells containing C3–05 were not TUNEL positive (Fig. 6, C and D). The small number of cells double labeled with C3 and TUNEL (16%) suggests 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 SCI lead to improved functional recovery (Liu et al., 1997).

Inhibitory substrates regulate Rho activation after injury by a p75-dependent mechanism

It has been shown recently 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 (Fig. 2 D). To determine the mechanism by which Rho is activated after

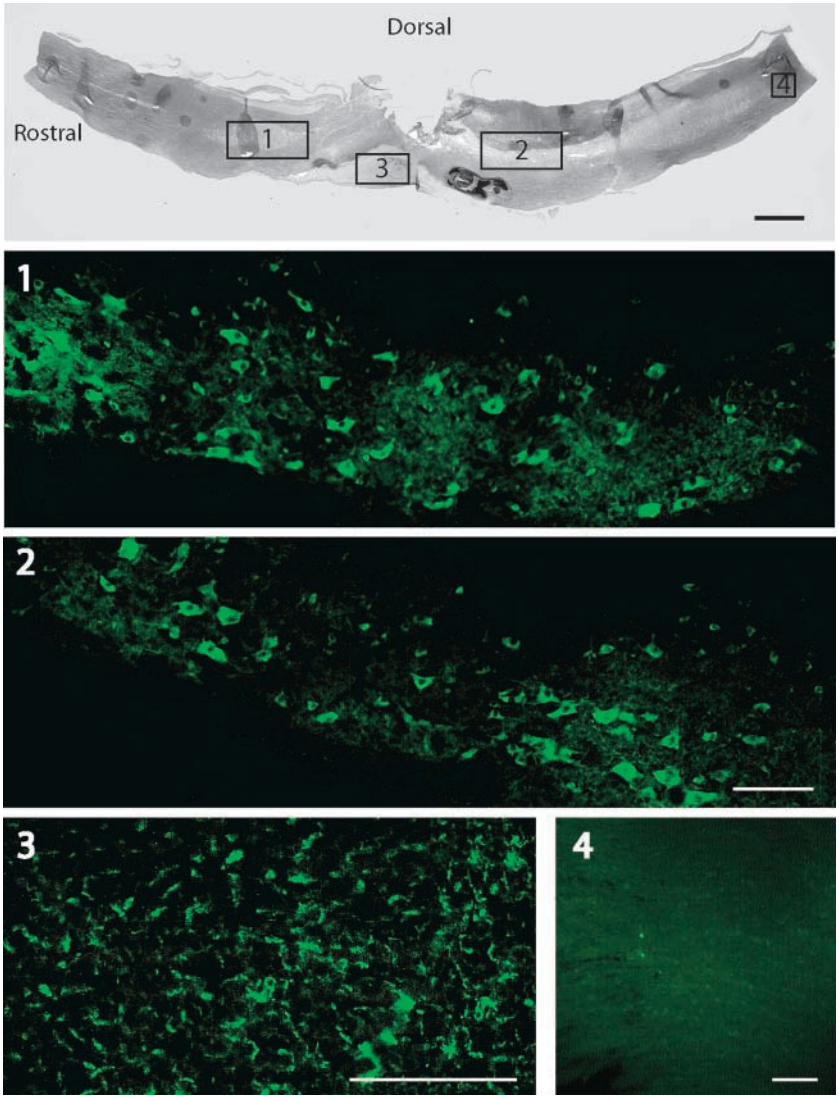

Figure 4. **Rho is activated in a large population of cells rostral and caudal to the lesion site.** (Top) Nissel-stained longitudinal section of rat spinal cord 24 h after dorsal over-hemisection. Bar, 1 mm. Higher magnification of areas spanning the section are shown boxed and numbered. Magnified panels 1–4 show active Rho (GST-RBD detection) in a large number of cells spanning the lesion site. Panel 1 shows active Rho in gray matter rostral to the lesion, panel 2 shows active Rho in gray 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. Bars, 100 μm.

injury, we examined if Rho activation was $p75^{NTR}$ dependent. We first examined if $p75^{NTR}$ was present in cells containing active Rho. We found that $p75^{NTR}$ colocalizes with active Rho in both gray (Fig. 7 A, top) and white matter (Fig. 7 A, bottom) 24 h 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 h after SCI (Fig. 7 B). Rho activation was, however, detected in these animals 3 d after injury (Fig. 7 C). These results indicate that Rho is activated through a $p75^{NTR}$-dependent 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; Frade and Barde, 1999; Dechant and Barde, 2002). In addition to counting TUNEL cells after SCI (Fig. 6 B), we counted $p75^{NTR}$-labeled cells and double labeled cells. Many cells express $p75^{NTR}$ after SCI (Fig. 8 B), 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 (Fig. 8, A 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 SCI, we probed the homogenates of transected spinal cord with $p75^{NTR}$-specific antibodies using the same homogenates as shown in the bottom panel of Fig. 8 C. There was very low $p75^{NTR}$ detected in Western blots in the adult spinal cord (Fig. 7 B and Fig. 8 C, controls). Levels of $p75^{NTR}$ increased as early as 24 h after SCI, and high levels were detected 3 and 7 d postinjury, a finding consistent with previous reports of $p75^{NTR}$ up-regulation after SCI (Casha et al., 2001; Widenfalk et al., 2001; Beattie et al., 2002). Treatment with C3–05 not only blocked the increase in $p75^{NTR}$ protein levels after SCI, as detected by Western blot (Fig. 8 C), but also reduced the number of $p75^{NTR}$-TUNEL–labeled cells (Fig. 8 B). 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 $p75^{NTR}$ up-regulation after SCI.

Discussion

It is now well established that neurons in the CNS respond to negative growth inhibitory cues and positive growth-promoting signals. Much work has underlined the impor-

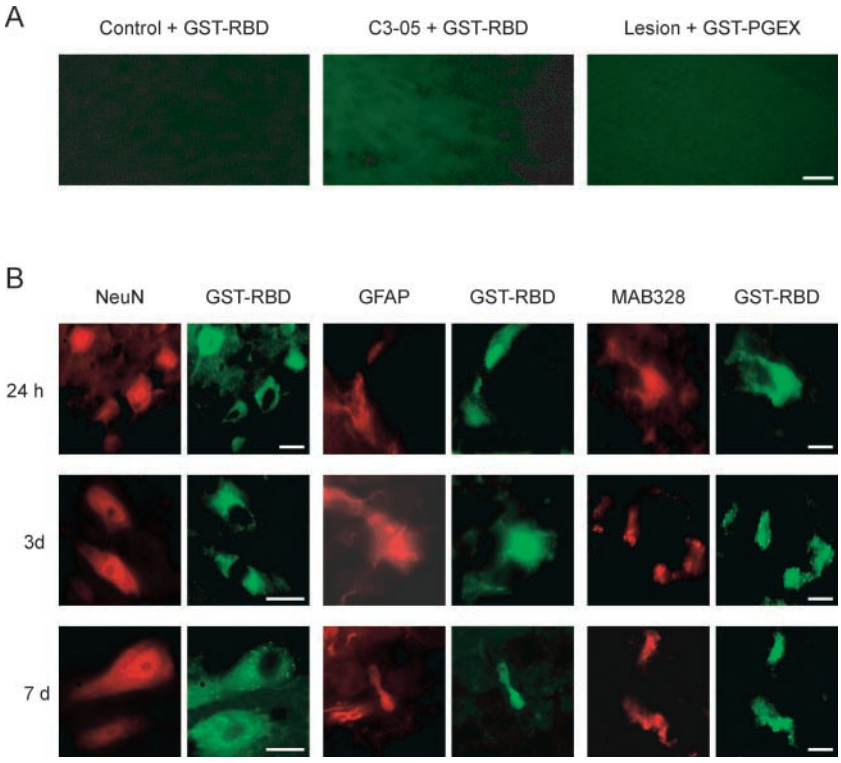

Figure 5. **Rho is active in neurons and glial cells after SCI, detected by in situ pull-down assay.** (A) Control uninjured animals (control + GST-RBD) and C3–05 treatment of animals after SCI (C3–05 + GST-RBD) probed with GST-RBD show no active Rho. Sections of animals after SCI incubated with lysate 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. Bar, 50 μm. (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 h, 3 d and 7d), GTP-Rho was detected in neurons (NeuN), astrocytes (GFAP), and oligodendrocytes (MAB328) after SCI. Bars, 50 μm.

tance of myelin-derived growth inhibitory proteins expressed by oligodendrocytes and present in white matter (Schwab et al., 1993; Schwab, 2002). 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 (Jin and Strittmatter, 1997; Lehmann et al., 1999; Dergham et al., 2002; Niederost et al., 2002; Fournier et al., 2003) and to stimulate axon regeneration after SCI (Hara et al., 2000; Dergham et al., 2002; Fournier et al., 2003). 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.

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 PC-12 cells are plated on inhibitory substrates (Fig. 1). Soluble Nogo fusion proteins can also activate neuronal Rho (Niederost et al., 2002; Fournier et al., 2003). 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 et al., 2000). Preliminary evidence from our lab indicates that astrocytes plated on inhibitory substrates show Rho activation (unpublished data). 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 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 (Schwartz and Fehlings, 2002; Popovich and Jones, 2003). 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 d 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 known to be present after SCI (Donovan et al., 1997; Wahl et al., 2000; De Winter et al., 2002; Shirvan et al., 2002; Swiercz et al., 2002). 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 (Donovan et al., 1997; Citron et al., 2000; Lee et al.,

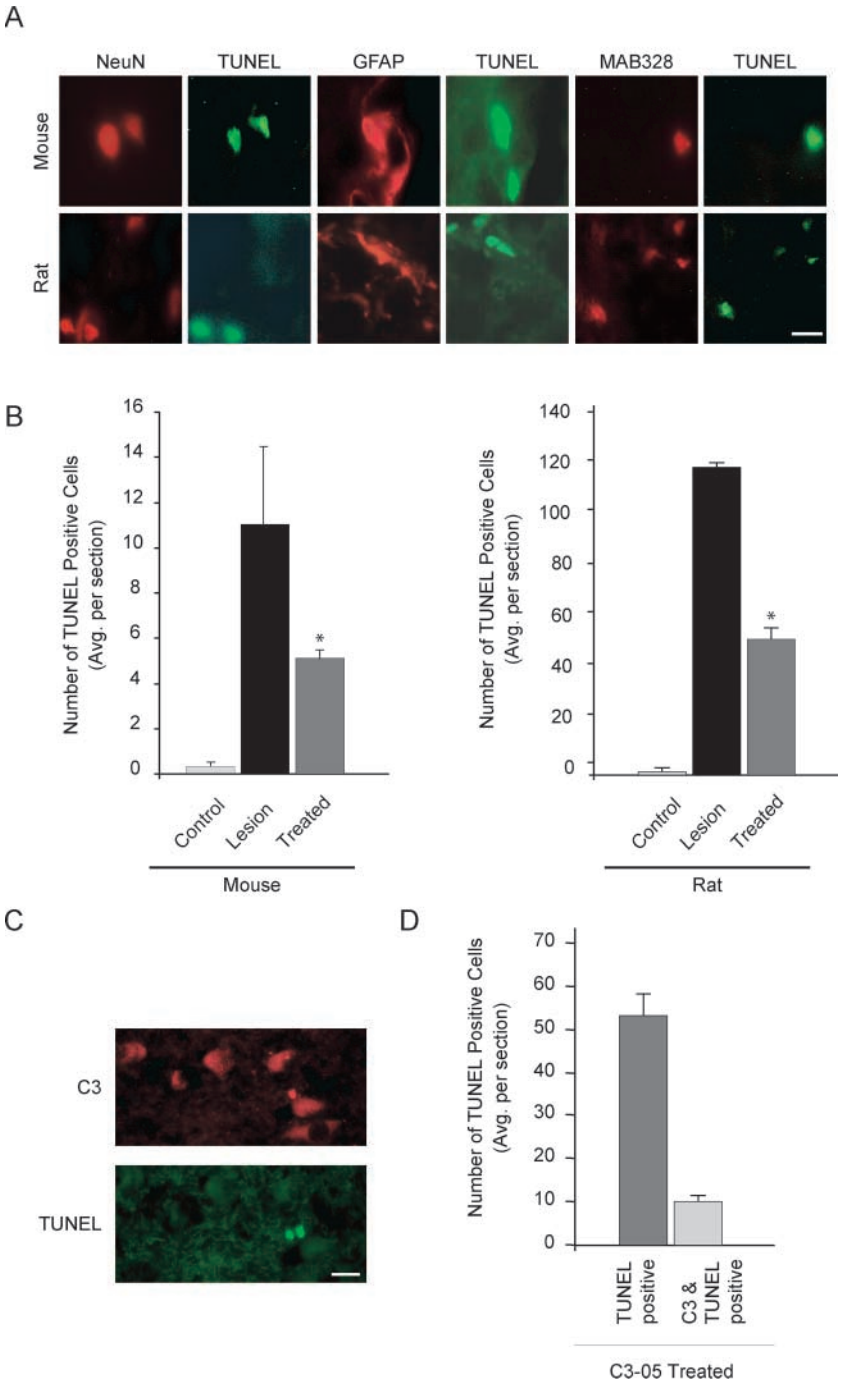

Figure 6. **Inhibition of Rho activation with C3–05 protects cells from apoptosis.** (A) Sections of injured spinal cords from mouse (top) and rat (bottom) were double labeled with specific cell markers NeuN, GFAP, or MAB328 (red) and by TUNEL (green) to detect apoptotic cells. Bar, 50 μm. (B) Treatment 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 three animals examined per group. *$P < 0.05$ compared with 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. Bar, 50 μm. (D) C3-labeled cells are less likely to be TUNEL positive. C3 and TUNEL cells were counted and compared with the number of TUNEL-labeled cells in C3–05–treated animals.

2000). Our experiments with the $p75^{NTR}$ knockout 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 after 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 d. Therefore, multiple local signals may activate Rho in CNS cells. We speculate that continued presence of growth inhibitory molecules at the site of a CNS lesion contributes to sustained activation of Rho in neurons and glia after SCI.

Rho activation leads to apoptosis after SCI

Rho-GTPases are known regulators of apoptosis in various cell types. In nonneuronal cells, such as epithelial cells (Fiorentini et al., 1998a,b), endothelial cells (Hippenstiel et al., 2002), T cells (Moorman et al., 1996; Gomez et al., 1997), and some fibroblasts (Bobak et al., 1997), inactivation of Rho causes apoptosis through a Bcl-2–dependent mechanism. In other cells, such as PC-12 cells (Mills et al.,

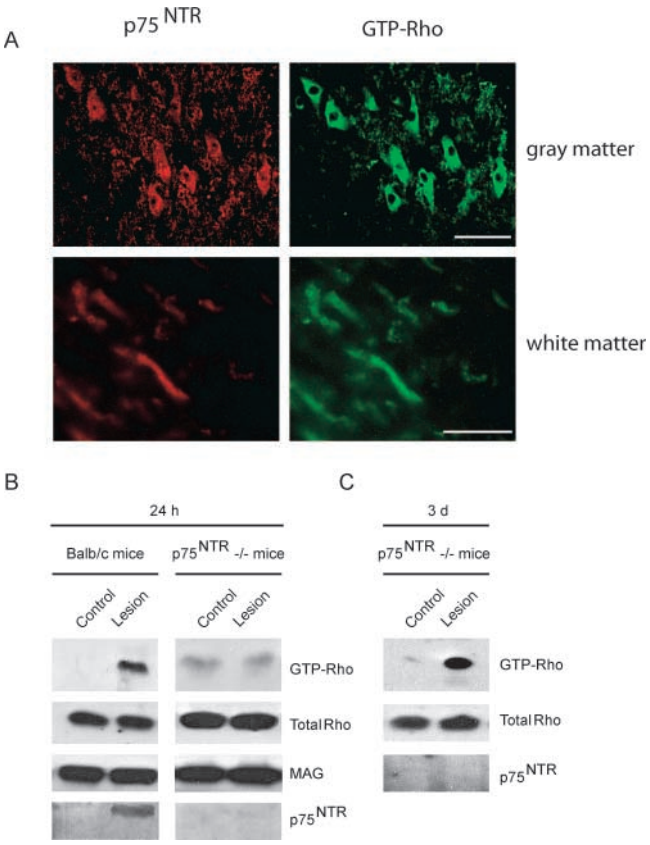

Figure 7. **Rho is activated after SCI by a p75^{NTR}-dependent mechanism.** (A) p75^{NTR}-labeled cells colocalize with active GTP-Rho in injured spinal cord. Cells labeled with p75^{NTR} (red) and GST-RBD (green) in gray matter (top) and in white matter (bottom). Bars, 100 μM. (B) Rho activation after 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). In p75^{NTR}−/− mice, 24 h after injury only basal levels of active Rho are detected compared with 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 kD). The p75^{NTR} levels (apparent MW 75 kD) are shown in bottom panel. In control uninjured animals low levels of p75^{NTR} are detected, with p75^{NTR} only being up-regulated after injury. (C) Active Rho is detected in p75^{NTR}−/− mice 3 d after SCI.

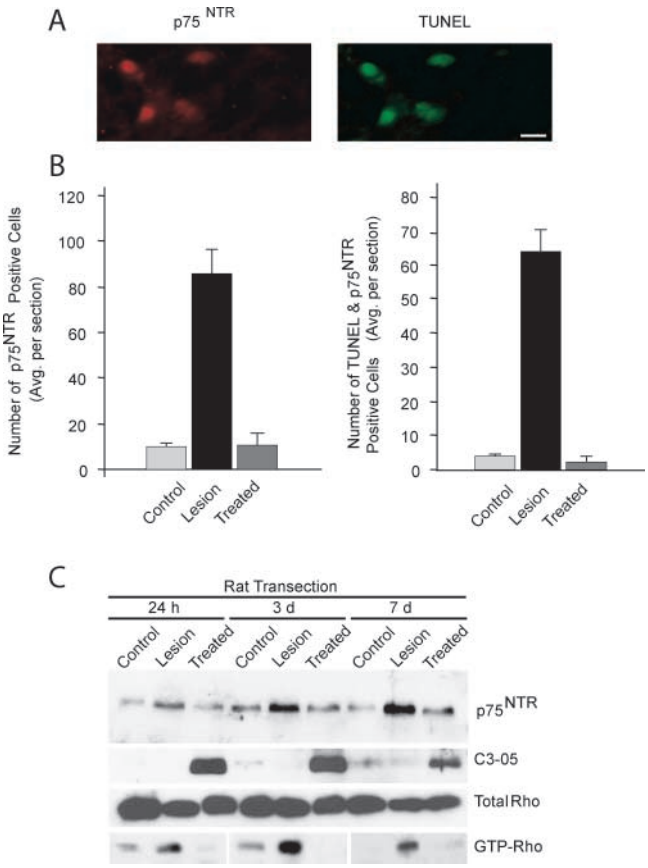

Figure 8. **Rho regulates p75^{NTR} expression after injury.** (A) Transverse sections throughout rat lesion sites show cells double labeled with p75^{NTR}-specific antibody (red) and with TUNEL (green). Bar, 50 μm. (B) The number of p75^{NTR}-labeled cells (left) and p75^{NTR} cells positive for TUNEL (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 active 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.

1998) and endogenous cells of the spinal cord, as we show here, inactivation of Rho protects cells from apoptosis. In NIH 3T3 fibroblasts, overexpression of active Rho induces cell death upon serum withdrawal (Jimenez et al., 1995). Thus, the cell background is critical in the effect of Rho signaling and cell death. In PC-12 cells, a neural cell line, Rho proteins have been shown to induce Rho-dependent 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-dependent apoptosis. Treatment with C3 reversed the thrombin-induced apoptosis of astrocytes and neurons by ∼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 SCI. 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.

Mechanism of Rho activation

Our results show that blocking the activation of Rho after SCI prevents an increased synthesis of p75^{NTR} protein (Fig. 8 C), 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 dependent (Fig. 9). It is known that Rho is involved in the activation of transcription factors in the nucleus that control synthesis of proapoptotic

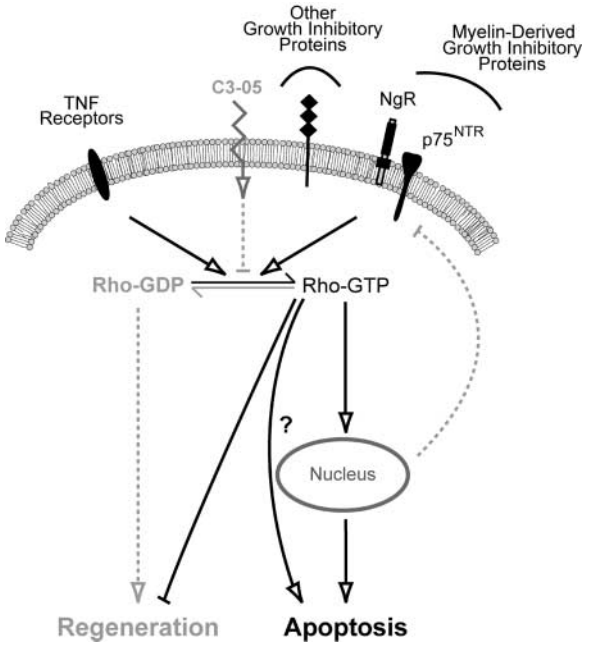

Figure 9. **Schematic diagram showing possible apoptotic cascade mediated by Rho after SCI.** Both myelin-derived growth inhibitory proteins (Fig. 1) and TNF (Neumann et al., 2002) directly activate Rho. $P75^{NTR}$ activates Rho in the absence of neurotrophin binding (Yamashita et al., 1999). The inactivation of Rho by C3–05 after SCI blocks the increase of $p75^{NTR}$ protein levels (Fig. 8 C) and inhibits apoptosis (Fig. 6, B and C). Inactivation of Rho with C3–05 both prevents apoptosis, as shown in this paper, and stimulates regeneration (Lehmann et al., 1999; Dergham et al., 2002). Gray lines indicate C3–05 treatment and inactivation of Rho; black lines indicate the effects of active GTP-bound Rho.

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 proapoptotic proteins such as $p75^{NTR}$.

The $p75^{NTR}$ contributes to initial apoptotic cascades that follow injury in the CNS. In oligodendrocytes, an increase in the expression of $p75^{NTR}$ after SCI leads to apoptotic cell death (Casha et al., 2001; Beattie et al., 2002). 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 after 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 (Fig. 5 B). Moreover, reversal of Rho with C3–05 reduced apoptosis (Fig. 6 B) and prevented $p75^{NTR}$ up-regulation (Fig. 8, B and C). Together these results indicate that Rho activation induces apoptosis in a $p75^{NTR}$-dependent manner early on after SCI and that inactivation of Rho is cell protective.

Materials and methods

Surgical procedures

Rats were anaesthetized under 2–3% isoflurane. For SCI, 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 h to 7 d after SCI, animals received an overdose of chloral hydrate anaesthetic, were perfused with saline, and the spinal cords were 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) were anaesthetized with hypnorm (20 ml/Kg) and diazepam (1 mg/Kg). Dorsal over-hemisections were performed at T8: $n = 5$ at 24 h and $n = 5$ at 3 d. 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 two to three times per day. Rats were given 5 ml of 0.9% saline subcutaneously twice a day for 1 wk 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) 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 PBS without fibrin was injected into rat contusion injury sites. All animal procedures followed guidelines from the Canadian Council of Animal Care.

Cell culture

PC-12 cells were grown in DME with 10% horse serum, 5% FBS, 1% penicillin-streptomycin. PC-12 cells were grown on poly-L-lysine (0.1 μg/ml) (Sigma-Aldrich) or myelin (8 μg per well) or MAG- (8 μg) coated 6-well culture dishes. After the cells settled (3–6 h 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 h later, washed with ice cold 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 PMSF). Cell lysates were clarified by centrifugation at 13,000 *g* for 10 min at 4°C and kept at −80°C.

Pull-down assays and immunoblotting

Purification of GST-RBD was performed as described previously (Ren and Schwartz, 2000). Bacteria expressing GST-RBD in a pGEX vector (a gift from John Collard, Division of Cell Biology, Netherlands Cancer Institute, Amsterdam, Netherlands) were grown in L-broth with 100 μl/ml ampicillin. Overnight cultures were diluted 1:10 into 3,600 ml L-broth and incubated in a shaking bacterial incubator at 37°C for 2 h. Isopropyl-β-D-thiogalactopyranoside (0.5 mM) was then added to the incubating cultures for 2 h. Bacteria were collected by centrifugation at 5,000 *g* for 15 min. The pellets were resuspended in 40 ml lysis buffer (50 mM Tris, pH 7.5, 1% Triton X, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF). After sonication, the lysates were spun at 14,000 rpm for 30 min at 4°C. The clarified bacterial lysate was then incubated with glutathione agarose beads (0.6 ml wet volume; preswelled with water) (Sigma-Aldrich) for 60 min at 4°C. The coupled beads were then washed six 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 ml of the wash buffer containing 10% glycerol and stored overnight at −80°C. Frozen tissue was homogenized 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 PMSF). The homogenates and cell lysates were clarified by two 10-min centrifugations at 13,000 *g* at 4°C. They were then incubated for 50 min at 4°C with GST-RBD (a gift from John Collard, Division of Cell Biology, Netherlands Cancer Institute, Amsterdam, Netherlands) coupled beads (20–30 μg/sample). The beads were then washed four 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 Biotechnology, Inc.). Bands were visualized with peroxidase-linked secondary antibodies (Promega) and an HRP-based chemiluminescence reaction (Pierce Chemical Co.). 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). For all blots, 20 μg 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 analyzed with the densitometry IQ MAC 1.2 software (Molecular Dynamics). 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).

For in situ pull-down assays, rat spinal cord cryosections (16-μm thickness, fresh) were postfixed 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 three times in TBS, blocked in 3% BSA for 1 h at room temperature, and incubated with anti-GST antibody (New England Biolabs, Inc.) and with cell type–specific antibodies (NeuN, GFAP, and MAB328; Chemicon) or with antibody raised against $p75^{NTR}$ (Promega) overnight at 4°C. Sections were washed in TBS and incubated for 2 h at room temperature with FITC, Texas red, or rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories).

TUNEL labeling and immunohistochemistry

Spinal cord samples of 3 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 pieces were postfixed in 4% PFA, washed, and embedded in paraffin. Transverse sections of 6-μm thickness were cut, deparaffinized in xylene, and rehydrated by ethanol washes. TUNEL labeling was performed using the Fluorescein-FragEL DNA Fragmentation kit (Oncogene Research Products). The sections were costained with Hoechst 33342 (Sigma-Aldrich), 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 three 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 three animals examined per group. Immunohistochemistry with cell type–specific antibodies (NeuN, GFAP, and MAB328; Chemicon) or with a polyclonal C3 antibody (Winton et al., 2002) was performed on paraffin sections. After deparaffinization, transverse sections were treated with 2× saline sodium citrate at 80°C for 20 min. Sections were blocked in TBS containing 3% BSA and 2% goat serum and incubated overnight with primary antibody at 4°C followed by a 2-h incubation with FITC or Texas red–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). All pictures were taken with northern eclipse software and transferred to Adobe Illustrator® 9.0.

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