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Inactivation of Rho Signaling Pathway Promotes CNS Axon Regeneration

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Regeneration in the CNS is blocked by many different growth inhibitory proteins. To foster regeneration, we have investigated a strategy to block the neuronal response to growth inhibitory signals. Here, we report that injured axons regrow directly on complex inhibitory substrates when Rho GTPase is inactivated. Treatment of PC12 cells with C3 enzyme to inactivate Rho and transfection with dominant negative Rho allowed neurite growth on inhibitory substrates. Primary retinal neurons treated with C3 extended neurites on myelin-associated glycoprotein and myelin substrates. To explore regeneration *in vivo*, we crushed optic nerves of adult rat. After C3 treatment, numerous cut axons traversed the lesion to regrow in the distal white matter of the optic nerve. These results indicate that targeting signaling mechanisms converging to Rho stimulates axon regeneration on inhibitory CNS substrates.

Key words: retinal ganglion cells; optic nerve; Rho GTPase; microcrush lesion; C3 toxin; myelin-associated growth inhibitory proteins; MAG

Axons in the CNS of mammals do not regenerate after injury, and one barrier to regeneration is growth inhibition by CNS myelin (Schwab et al., 1993). Myelin inhibits axon growth because it contains several different growth inhibitory proteins. Myelin-associated glycoprotein (MAG) inhibits axon growth both *in vitro* and *in vivo* (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Li et al., 1996; Schafer et al., 1996; Torigoe and Lundborg, 1998). Also, a different high molecular weight inhibitory activity is present in myelin (Caroni and Schwab, 1988). Neutralization of inhibitory activity with the IN-1 antibody allows some axons to regenerate in white matter (Schwab et al., 1993; Bregman et al., 1995). Inhibitory proteins expressed at the glial scar also block axon growth (McKeon et al., 1991). Therefore, multiple inhibitory proteins exist, and, for efficient axon regeneration in the adult CNS, it will be important to neutralize their inhibitory effects.

Although axons damaged in the CNS *in vivo* do not typically regrow, there have been some reports of long-distance axon extension in adult white matter. Such growth has been observed after transplantation of grafted neural tissue (Wictorin et al., 1990; Davies et al., 1994, 1997), but it is not completely understood. Suppression of the expression of inhibitory proteoglycans at the glial scar may be one determinant for successful neurite growth from transplanted neurons (McKeon et al., 1991; Davies et al., 1997). In other cases, priming with neurotrophic factors to increase neuronal cAMP levels would make cells less susceptible to growth inhibition (Cai et al., 1999). For the regeneration of injured adult neurons, current strategies to foster axonal regrowth in myelinated regions of the CNS are to bypass myelinated tracts (David and Aguayo, 1981; Cheng et al., 1996), remove myelin (Keirstead et al., 1992), or use IN-1 antibody to block myelin inhibitors (Schnell and Schwab, 1990; Bregman et al., 1995). However, none of these methods is directed toward neuronal signaling mechanisms that regulate axon growth. Neurotrophins have been tested *in vivo* for their ability to help axons regenerate, and they are known to delay retrograde cellular atrophy and apoptosis (Kobayashi et al., 1997; Bregman et al., 1998) and to promote local branching and sprouting (Schnell et al., 1994; Sawai et al., 1996). Likely, convergent signaling by multiple growth-promoting molecules is important in regeneration. Laminin, an extracellular matrix protein, is able to stimulate rapid neurite growth (Kuhn et al., 1995), and we have documented that, in the presence of laminin, neurites can extend directly on myelin substrates (David et al., 1995). Similarly, it has been documented that when the adhesion molecule L1 is expressed ectopically on astrocytes, it can partially overcome their nonpermissive substrate properties (Mohajeri et al., 1996).Therefore, neurons can, under appropriate conditions, grow axons on inhibitory substrates, demonstrating that the balance of positive-to-negative growth cues is a critical determinant for the success or failure of axon regrowth after injury and that multiple signals converge to regulate axon growth.

The Rho signaling pathway has been implicated in both positive and negative signaling events within neurons. Activation of the small regulatory GTPases may be an important link between signaling through integrins, signaling cascades of trophic factors, and regulation of cytoskeletal dynamics (Schlaepfer et al., 1996; Udagawa and McIntyre, 1996; Hall, 1998). Moreover, both MAG and the other myelin-derived growth inhibitory proteins block axon extension by causing growth cone collapse (Bandtlow et al.,

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1993; Li et al., 1996). Rho has been implicated in signaling to the growth cone cytoskeleton (Mackay et al., 1996; van Leeuwen et al., 1997) and in regulating growth cone collapse and the retraction of neurites (Jalink et al., 1994; Tigyi et al., 1996b; Katoh et al., 1998). These studies prompted us to first examine in PC12 cells and cultures of primary neurons the role of Rho in growth inhibition by MAG and by myelin. To investigate this possibility, we have made use of the C3 enzyme from *Clostridium botulinum* that selectively ADP-ribosylates Rho in its effector domain without affecting Rac and Cdc42, two other members of the Rho family (Rubin et al., 1988; Udagawa and McIntyre, 1996). Furthermore, we demonstrate axons regenerate *in vivo* after treatment of injured optic nerve with C3 to inactivate Rho.

MATERIALS AND METHODS

Preparation of growth substrates and recombinant proteins. Poly-L-lysine was obtained from Sigma (St. Louis, MO). Laminin was prepared from Engelbreth–Holm–Swarm tumors (Paulsson and Lindblom, 1994). Myelin was made from bovine brain corpus callosum, and native MAG was purified from myelin after extraction in 1% octylglucoside and separation by ion exchange chromatography (McKerracher et al., 1994). This preparation of native MAG has some additional proteins, including tenascin (Z. C. Xiao, P. Braun, S. David, and L. McKerracher, unpublished observations). Recombinant MAG (rMAG) was made in baculovirus-infected *Spodoptera frugiperda* (SF) cells as described previously (Shibata et al., 1998), except that the SF9 cells were transferred to serum-free conditions before collecting the culture supernatant. Test substrates were prepared as uniform substrates in 96-well plates or eight-chamber Lab-Tek slides (Nunc, Naperville, IL.). For all substrates, plates were precoated with poly-L-lysine (100 μg/ml) for 3 hr at 37°C and then washed and dried. MAG or myelin substrates were prepared in 96-well plates by drying down 4–8 μg of protein overnight. The plasmid pGEX2T-C3 coding for the glutathione *S*-transferase (GST)-C3 fusion protein was obtained from N. Lamarche (McGill University, Montréal, Québec, Canada), and recombinant C3 was purified as described previously (Ridley and Hall, 1992). Briefly, the GST fusion protein was cleaved by thrombin, and thrombin was removed by incubation with 100 μl of *p*-aminobenzamidine agarose beads (Sigma). The C3 solution was dialyzed against PBS and sterilized with a 0.22 μm filter. The C3 concentration was evaluated by protein assay (DC assay; Bio-Rad, Missassauga, Ontario, Canada), and C3 purity was controlled by SDS-PAGE analysis.

Cell culture. We used PC12 cells obtained from three different sources: the American Type Culture Collection, Dr. Phil Barker (Montréal Neurological Institute, McGill University), and Gabor Tigyi (University of Tennessee, Memphis, TN). We found that all lines were inhibited by both myelin and MAG in contrast to a different PC12 line tested under different experimental conditions (Rubin et al., 1995). PC12 cells were grown in DMEM with 10% horse serum and 5% fetal bovine serum. Human wild-type RhoA was obtained from Dr. A. Hall (University College, London, UK), and a dominant negative mutation was generated by replacing Thr19 for Asn (N19TRhoA). This mutated RhoA was cloned into the *Bgl*II site of the pEXV mammalian expression plasmid, and N19TRhoA or mock (empty vector)-transfected PC12 cells were selected, cloned, and characterized (Sebok et al., 1999). To identify Rho proteins expressed by PC12 cells, cell lysates were prepared and ribosylated with C3 and [^{32}P]NAD+ as described previously (Dillon and Feig, 1995), and the different Rho proteins were detected by two-dimensional gel electrophoresis and identified as described previously (Santos et al., 1997). For C3 experiments, PC12 cells were washed once with scraping buffer (in mM: 114 KCl, 15 NaCl, 5.5 $MgCl_2$, and 10 Tris-HCl) and then scraped with a rubber policeman into 0.5 ml of scraping buffer in the presence or absence of 40 μg/ml C3 transferase. The cells were pelleted and resuspended in 2 ml of DMEM, 1% FBS, and 50 ng/ml nerve growth factor before plating. Quantitative analysis of neurite outgrowth was with the aid of Northern Eclipse software (Empix Imaging, Mississauga, Ontario, Canada). Data analysis and statistics were with Microsoft (Seattle, WA) Excel. At least four experiments, each done in duplicate, were analyzed for each treatment. Experiments on MAG substrates were analyzed by phase-contrast microscopy. Because myelin is phase dense, experiments with myelin substrates were by fluorescent microscopy with DiI-labeled cells (McKerracher et al., 1994). For each well, four images were collected with a 20× objective using a Zeiss (Oberkochen, Germany) Axiovert microscope. For each image, the number of cells with and without neurites was counted, and the length of the longest neurite per cell was determined.

To culture retinal neurons, retinas were removed from postnatal day 1 (P1)–P5 rat pups, and the cells were dissociated with 12.5 U/ml papain in HBSS, 0.2 mg/ml DL-cysteine, and 20 μg/ml bovine serum albumin. The dissociated cells were washed and then triturated with C3 or buffer in culture media. Cells were plated on test substrates in the presence of 50 μg/ml BDNF in DMEM with 10% FBS, vitamins, and penicillin–streptomycin in the presence or absence of 25 or 50 μg/ml C3 transferase. Quantitative analysis was done with cells treated with 25 μg/ml C3. Neurons were visualized by fluorescent microscopy with anti-βIII tubulin antibody, which detects growing retinal ganglion cells (RGCs) (Fournier and McKerracher, 1997).

To examine the efficiency of C3 scrape loading, PC12 cells or retinal neurons were treated with C3 or scrape-loading buffer as described above. After 2 d in culture, cells were washed with PBS and lysed in 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin. Lysates were cleared by centrifugation, and protein concentrations evaluated by DC assay (Bio-Rad). Ten micrograms of protein was separated on 11% acrylamide gels and transferred to nitrocellulose, and membranes were blocked with TBS containing 0.1% Tween 20 and 5% nonfat milk powder, incubated in blocking buffer with anti-RhoA antibody (Upstate Biotechnology, Lake Placid, NY), and revealed with and HRP-based chemiluminescent kit (Boehringer Mannheim, Laval, Quebec, Canada). Membranes were reprobed with polyclonal antibody against Cdc42 (Upstate Biotechnology) and secondary alkaline phosphatase-linked anti-rabbit antibody and revealed with nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indyl-phosphate (NBT–BCIP) (Canadian Life Technology, Burlington, Ontario, Canada).

C3 treatment of crushed optic nerve in adult rats. Rats were anesthetized with 0.6 ml/kg hypnorm, 2.5 mg/kg diazepan, and 35 mg/kg ketamin. To make microcrush lesions, the left optic nerve was exposed by a supraorbital approach, the optic nerve sheath was slit longitudinally, and the optic nerve was lifted out from the sheath and crushed 1 mm from the globe by constriction with a 10.0 suture held for 60 sec (see Fig. 5*a*). To verify the lesion was complete, Fluorogold (Flurochrome Inc., Englewood, NJ) was applied bilaterally to the superior colliculus ($n = 3$ animals), and the left (microcrush-lesioned) and right retinas were visualized as whole mounts (Selles-Navarro et al., 1996). Lesions were also examined by anterograde tracing 24 hr after lesion (see below; $n = 4$ animals). For C3 treatment and buffer controls, Gelfoam soaked in PBS or 2 mg/ml C3 transferase was placed on the nerve at the lesion site. Two 3-mm-long tubes of Elvax (Sefton et al., 1984) loaded with buffer or 20 μg of C3 were inserted in the Gelfoam near the nerve for continued slow release of C3 (see Fig. 5*a*). To anterogradely label RGC axons, 5 μl of 1% cholera toxin β subunit (CT) (List Biologic, Campbell, CA) was injected into the vitreous, for either 2 d [dichlorotriazinyl amino fluorescein (DTAF)-labeled] or 3 hr [3,3′-diaminobenzidine tetrahydrochloride (DAB)-labeled]. Two weeks after optic nerve crush, the animals were fixed by perfusion with 4% paraformaldehyde, and the eye with attached optic nerve was removed and post-fixed in 4% paraformaldehyde. Optic nerve were treated in one of two ways. (1) Longitudinal 14 μm cryostat sections were processed for immunoreactivity to CT with goat anti-CT at 1:12,000 (List Biologic), followed by rabbit anti-goat biotinylated antibody (1:200; Vector Laboratories, Burlingame, CA) and DTAF–streptavidin (1:500; Jackson ImmunoResearch, West Grove, Pa) and viewed with epifluorescence. Some of these sections were further examined for confirmation of the location of the crush and for myelin staining by a Luxol fast blue–cresyl violet procedure. After photomicrographs of the fluorescent images were taken, the coverslips were removed, and the slides were soaked in PBS, passed through 95% ethanol, and stained in 1% Luxol fast blue overnight. The slides were rinsed in water, differentiated in 0.005% lithium carbonate, and then left in 70% ethanol until the unmyelinated fibers in the retina cleared. The slides were counterstained with 0.05% cresyl violet, dehydrated, and mounted with Permount. (2) Optic nerves were embedded in 20% gelatin, further fixed in 4% paraformaldehyde for 6–8 hr, and cryoprotected in 30% sucrose, and longitudinal 30 μm cryostat sections were cut and processed as free-floating sections. The nerves were treated with goat anti-CT as above, incubated with avidin-biotin HRP complex (ABC; Vector Laboratories), and rinsed in 0.1 M potassium phosphate buffer, pH 7.2. The color reaction was by incubating sections in 0.05% DAB, 0.01% cobalt chloride, and 0.01% nickel sulfate for 5 min before adding 0.006% H_2O_2 for 3–5 min.

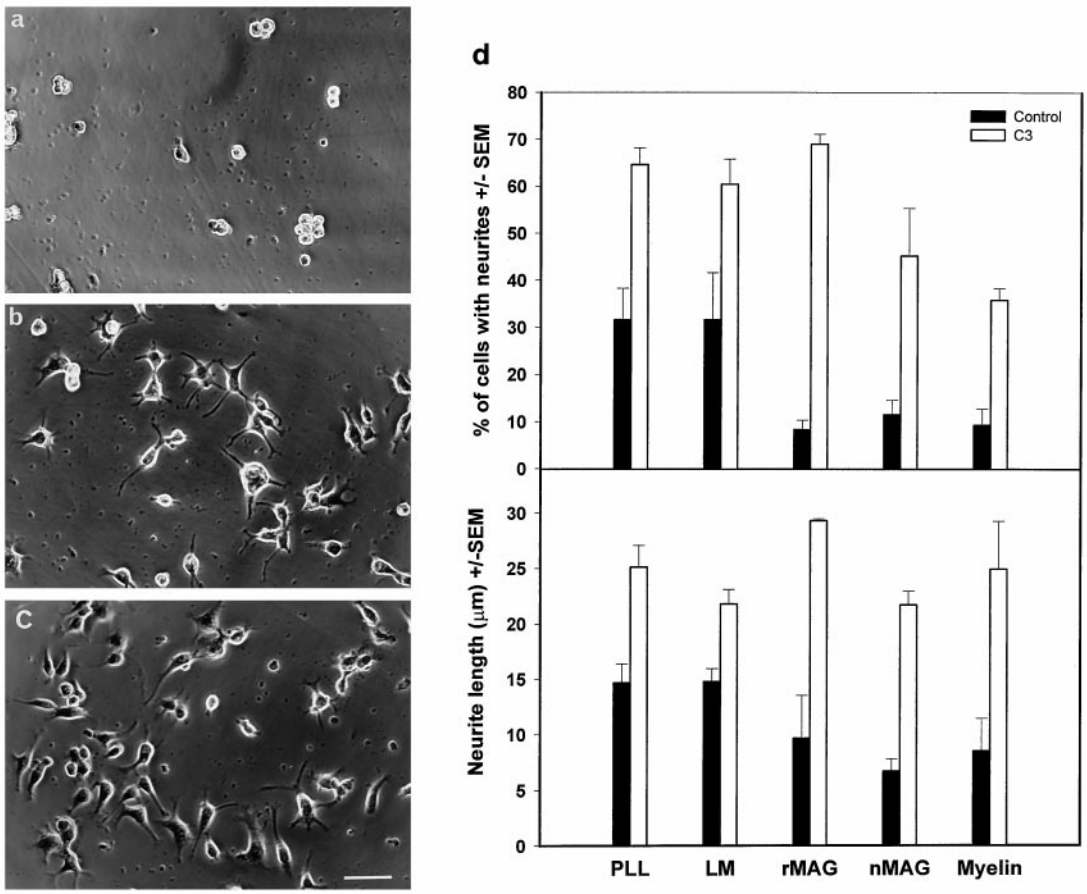

Figure 1. C3 treatment of PC12 cells plated on inhibitory MAG and myelin substrates. *a–c*, PC12 cells plated on MAG remained rounded and did not extend neurites (*a*), but cells plated on MAG in the presence of C3 (*b*) grew neurites. *c*, Poly-L-lysine (*PLL*) controls. Scale bar, 50 μm. *d*, Quantitative analysis of neurite growth with C3 treatment (*open bars*) or in scrape-loaded buffer controls (*filled bars*) when PC12 cells were plated on poly-L-lysine, laminin, rMAG, native MAG (*nMAG*), or myelin. The number of cells that extended neurites after 18–24 hr of treatment was counted (*top*), and the length of the longest neurite per cell was measured (*bottom*).

For a quantitative analysis, the numbers of axons per section were counted at distances of 100, 250, and 500 μm, and at least four sections per animal were analyzed (see Fig. 9).

RESULTS

Effect of C3 transferase on PC12 cells

PC12 cells typically extend neurites in response to NGF, but, when plated on myelin substrates, the cells remain round and do not extend neurites (Rubin et al., 1995). We plated three different lines of PC12 cells on both native and recombinant MAG substrates (Fig. 1). All of the lines of PC12 cells showed reduced cell spreading and remained round without neurites in the presence of NGF. Next, we inactivated Rho in PC12 cells by scrape loading cells with purified recombinant C3 at 40 μg/ml before plating the cells on the test substrates (Fig. 2). On MAG substrates, in which neurite formation is inhibited, C3 had a dramatic effect on the ability of cells to extend neurites (Fig. 1*a–c*). On control substrates of poly-L-lysine and laminin, treatment with C3 increased both the number of cells with neurites and the length of neurites (Fig. 1*d*). Moreover, on both MAG and myelin substrates, significantly more cells extended neurites, and neurite length was significantly longer after C3 treatment (Fig. 1*d*). These results demonstrate that C3 treatment elicits neurite growth from PC12 cells plated on growth-inhibitory myelin or MAG substrates.

To ensure that the effect of C3 treatment resulted from uptake

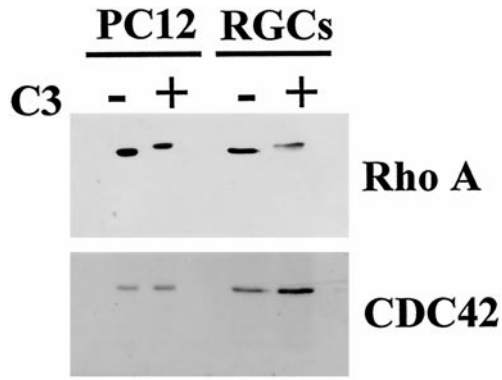

Figure 2. ADP-ribosylation of Rho by C3 detected in cultured cells. PC12 cells or retinal neurons were cultured in the presence (+) or absence (−) of C3 for 2 d. The cells were lysed, and 10 μg of protein from each sample was separated on a 11% acrylamide gel. The proteins were transferred to nitrocellulose, probed with mouse anti-RhoA antibody and anti-mouse-HRP antibody, and revealed by a chemiluminescent reaction (*top*). The membranes were then reprobed with rabbit anti-Cdc42 and anti-rabbit alkaline phosphatase and revealed with NTB/BCIP color reaction. Treatment of cells with C3 results in an ADP-ribosylation-induced decrease in the mobility of RhoA. The mobility of Cdc42 does not change with C3 treatment.

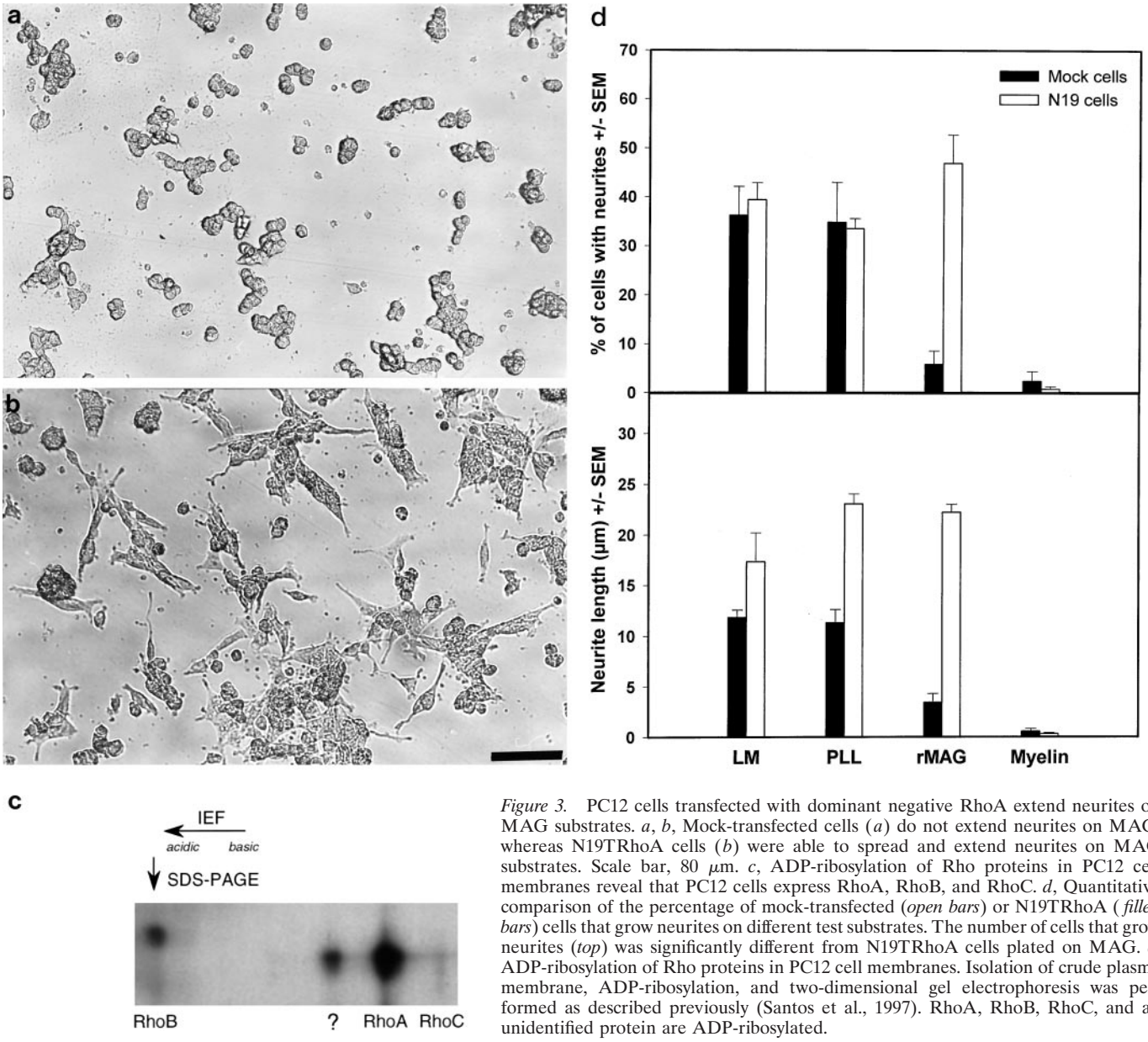

Figure 3. PC12 cells transfected with dominant negative RhoA extend neurites on MAG substrates. *a*, *b*, Mock-transfected cells (*a*) do not extend neurites on MAG, whereas N19TRhoA cells (*b*) were able to spread and extend neurites on MAG substrates. Scale bar, 80 μm. *c*, ADP-ribosylation of Rho proteins in PC12 cell membranes reveal that PC12 cells express RhoA, RhoB, and RhoC. *d*, Quantitative comparison of the percentage of mock-transfected (*open bars*) or N19TRhoA (*filled bars*) cells that grow neurites on different test substrates. The number of cells that grow neurites (*top*) was significantly different from N19TRhoA cells plated on MAG. *c*, ADP-ribosylation of Rho proteins in PC12 cell membranes. Isolation of crude plasma membrane, ADP-ribosylation, and two-dimensional gel electrophoresis was performed as described previously (Santos et al., 1997). RhoA, RhoB, RhoC, and an unidentified protein are ADP-ribosylated.

of C3 into the cells, we examined by Western blot the electrophoretic mobility of Rho in PC12 cells treated with C3 or with scrape-loading buffer as a control (Fig. 2). It has previously been shown that ADP-ribosylation of Rho results in decreased mobility of Rho on SDS-acrylamide gels (Narito and Narumiya, 1995). Western blots of cell lysates with anti-RhoA antibody revealed an increase in the apparent molecular weight of RhoA in cells treated with C3. As a control for the specificity of the effect, we probed the same blots for another small GTPase of the Rho family, Cdc42. Cdc42 did not show any change in mobility after treatment with C3 (Fig. 2), demonstrating the specificity of C3 treatment under our experimental conditions.

Growth of dominant negative Rho-transfected cells on MAG substrates

PC12 cells transfected with dominant negative RhoA (N19TRhoA) show enhanced neurite extension after exposure to NGF (Sebok et al., 1999). The N19TRhoA cells and the mock-transfected cells were compared for their ability to extend neurites on different inhibitory substrates (Fig. 3*a*,*b*). N19TRhoA cells plated on rMAG substrates were able to extend neurites, and the neurites were significantly longer than those of the mock-transfected cells plated on rMAG (Fig. 3*d*). On myelin substrates, the N19TRhoA cells were unable to extend neurites (Fig. 3*d*). To examine whether other members of the Rho family are also present in PC12 cells, we examined by ADP-ribosylation of membrane proteins the Rho proteins expressed in PC12 cells (Fig. 3*c*). These experiments revealed that PC12 cells express RhoA, RhoB, RhoC, as reported for brain (Dillon and Feig, 1995). The inability of the N19TRhoA cells to extend neurites on myelin is consistent with the report of incomplete inhibition of Rho activity by dominant negative mutations (Qiu et al., 1995). Inactivation of all of the Rho proteins or of a threshold amount of RhoA may be necessary for neurites to extend on myelin substrates.

Effect of C3 on primary cells

To test the involvement of Rho in the response of primary neurons to MAG and to myelin substrates, we purified retinal

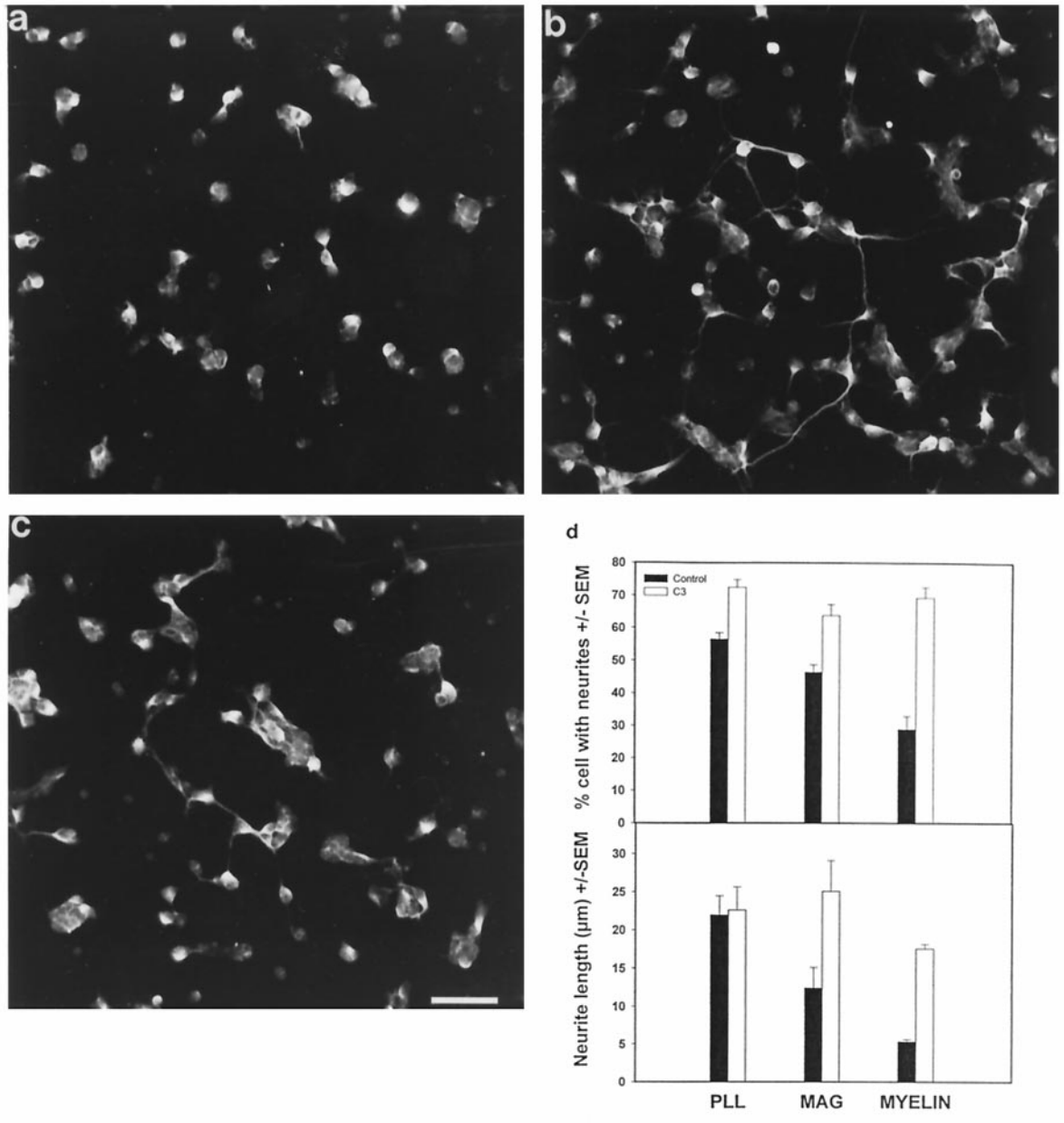

Figure 4. Treatment of retinal neurons with C3 stimulates neurite growth on MAG substrates. On native MAG substrates, neurite growth is inhibited (*a*), but after C3 treatment, retinal neurons plated on native MAG substrates extend neurites (*b*). Growth of neurites from retinal neurons plated on poly-L-lysine (*c*). Scale bar, 50 μm. *d*, Quantitative analysis of neurite growth of retinal neurons on poly-L-lysine, MAG, and myelin substrates, as described in the legend of Figure 1. Significantly more cells extended longer neurites on MAG and myelin substrates with C3 treatment than with buffer-treated controls. Scale bar, 50 μm.

neurons and treated them with C3. Neurite outgrowth from these cells was inhibited by MAG (Fig. 4*a*) and myelin (Fig. 4*d*). Treatment of retinal neurons with C3 allowed neurite extension on the growth-inhibitory MAG substrates to an extent similar to that observed on control substrates (Fig. 4*b*,*c*). A quantitative analysis revealed that C3 treatment of retinal neurons plated on MAG or myelin substrates had significantly longer neurites, and significantly more cells extended neurites (Fig. 4*d*). Also, we documented that, in retinal neurons treated with C3, a shift in the mobility of Rho, but not Cdc42, was detected (Fig. 2). These experiments demonstrate that inactivation of Rho by ADP-ribosylation allows retinal neurons to extend neurites on growth-inhibitory substrates.

Effects of C3 on retinal ganglion cell axon growth *in vivo*

It is known from studies with retrograde tracers that damaged axons can take up externally applied substances. Therefore, we explored the possibility that transected axons treated with C3 would foster regeneration *in vivo*. The RGC response to injury has been well documented (Vidal-Sanz et al., 1987; Villegas-Perez et al., 1988; Ajemain, David, 1994; Berkelaar et al., 1994; Berry et

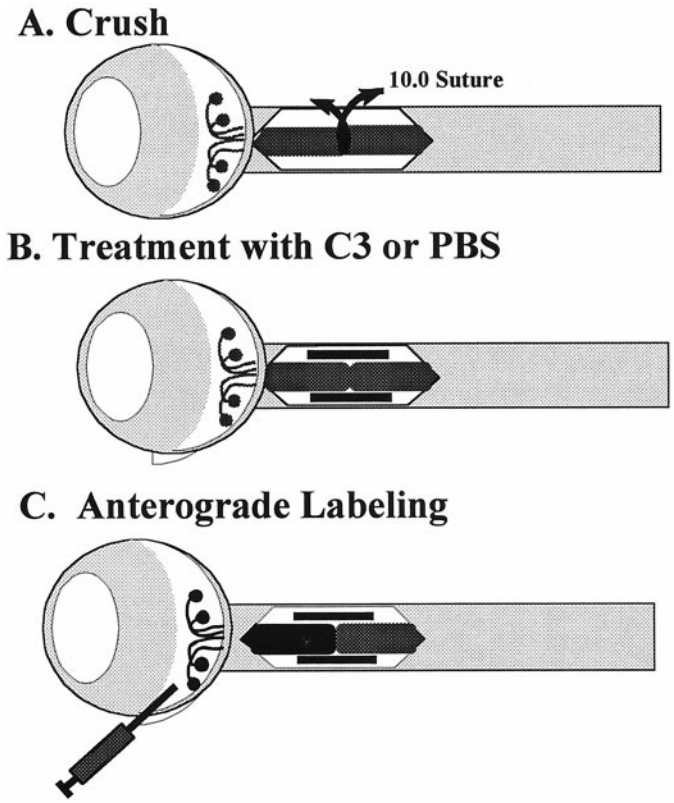

Figure 5. Illustration of methods used to study the effect of C3 on injured RGC axons. *a*, The optic nerve was removed from the sheath before crushing with 10.0 sutures. *b*, C3 was applied in Gelfoam and Elvax tubes immediately after crushing the optic nerve. *c*, Retinal ganglion cell axons were detected by anterograde labeling with CT.

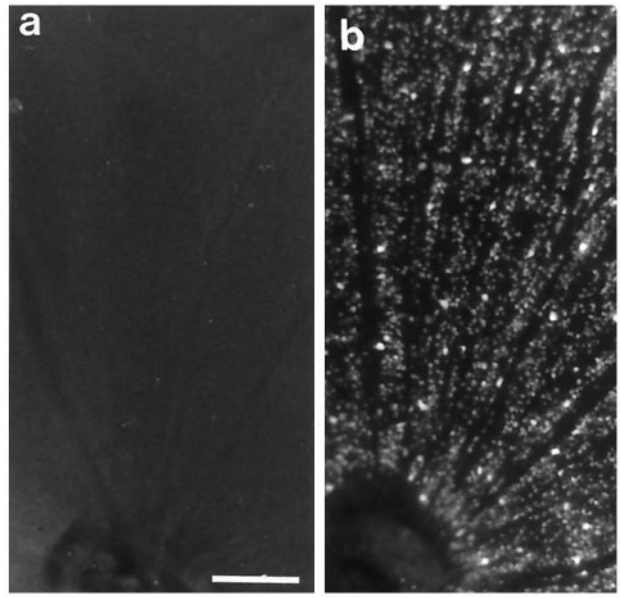

Figure 6. Retinal whole mounts visualized after the application of Fluorogold to the tectum demonstrate the microcrush lesion is a complete lesion. *a*, Retinas are not labeled by Fluorogold applied to the tectum after a microcrush lesion. *b*, A control retina to show the normal pattern of retrograde labeling with Fluorogold.

al., 1996), and we examined regeneration of RGC axons in the optic nerve 2 weeks after injury. Recently, it has been shown that microlesions in the CNS reduce the extent of the glial scar to allow axonal growth from transplanted adult neurons into CNS white matter (Davies et al., 1997). To reduce possible effects of the glial scar, we made microcrush lesions of optic nerve to axotomize RGC axons (Fig. 5). To verify that this method completely axotomized RGC axons, we applied the retrograde tracer Fluorogold to the superior colliculus at the time of microcrush lesion and examined retinal whole mounts for the presence of labeled cells. After microcrush lesion ($n = 3$), the RGCs failed to become labeled, indicating that the lesion was complete (Fig. 6*a*). With unlesioned optic nerves ($n = 3$), the RGC population was normally labeled (Fig. 6*b*). In addition, anterograde labeling (Fig. 5*d*) of microcrush-lesioned RGC axons 24 hr after injury verified that RGC axons were effectively axotomized ($n = 4$ animals; data not shown).

To apply C3 to microcrush-lesioned optic nerves, C3 in Gelfoam was wrapped around the site of crush, and two Elvax tubes, each loaded with C3, were positioned for sustained slow release (Fig. 5). For these experiments, 16 animals were treated with C3, 10 animals were treated with Gelfoam and Elvax tubes with buffer as controls, and four animals received microcrush lesion only. All animals were examined 2 weeks after surgery. Regenerating axons were visualized by anterograde labeling with CT injected into the eye, and longitudinal cryostat sections of the optic nerves were examined for cholera toxin immunoreactivity. In all 10 of the buffer-treated animals, most anterogradely labeled axons stopped abruptly at the crush site (Figs. 7*a*, 8*c*), although a few axons did extend past the crush (Fig. 8*c*, *arrows*). In these controls, axon extension past the crush site was typically restricted to the edge of the optic nerve. After treatment with C3, large numbers of axons extended through the site of the crush, both along the edge (Fig. 8*c*) and in the middle of the optic nerve (Figs. 7*b*,*c*, 8*d*). This observation of regenerating axons throughout the thickness of the optic nerve was confirmed by examining serial sections (Fig. 7). After C3 treatment, many of the axons that extended past the lesion site showed a twisted path of growth, supporting their identification as regenerating axons (Fig. 8*e*). Counterstaining of the fluorescently labeled sections with Luxol fast blue–cresyl violet confirmed that the fluorescently labeled axons extended past the crush and into regions of the nerve that remained myelinated (Fig. 8*b*). To examine quantitatively the differences between C3 and buffer-treated animals, we counted the number of axons in each section at distances of 100, 250, and 500 μm past the lesion site in all of the animals examined with the two immunolabeling methods (Fig. 9*a*). 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 and 250 μm (Fig. 9*b*). Therefore, C3 applied to injured RGC axons can enter axotomized axons and promote robust but short-lived axon regeneration in the environment of the optic nerve.

DISCUSSION

Here, we report that the small GTP binding protein Rho is a key intermediate in the neuronal response to neurite growth-inhibitory signals. Although it is known that treatment of neurons with C3 to inactivate Rho can stimulate axon outgrowth of cells plated on poly-L-lysine or laminin (Nishiki et al., 1990; Jin and Strittmatter, 1997; Kozma et al., 1997), we demonstrate here that treatment with C3 can also overcome growth inhibition by inhibitory substrates. Treatment of cultured PC12 cells and retinal neurons with C3 enzyme to inactivate Rho allowed neurites to

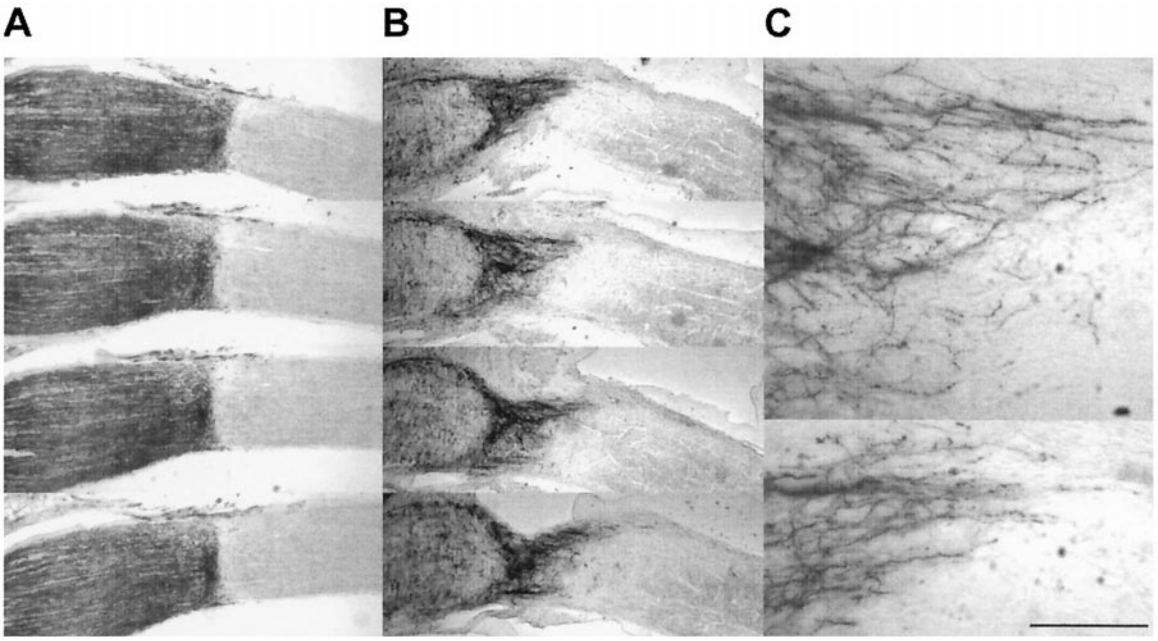

Figure 7. Anterogradely labeled RGC axons detected past the crush in longitudinal sections of C3-treated optic nerve. *a*, A series of four sections through one optic nerve to show that most axons do not extend past a microcrush lesion without C3 treatment. *b*, A series of four sections though a C3-treated optic nerve to show that many axons extend past the lesion throughout the thickness of the optic nerve. *c*, Higher magnification view of the third and first section shown in *b*. Scale bar: *a*, *b*, 500 μm; *c*, 100 μm.

extend directly on inhibitory substrates of MAG or myelin. Also, PC12 cells transfected with dominant negative RhoA extended neurites on MAG substrates. Therefore, the Rho signaling pathway is likely to play a key role in the integration of both permissive and inhibitory substrate cues in axon growth and regeneration.

Regulation of neurite growth by Rho family members

There is now good evidence that members of the Rho family regulate axon outgrowth in development. Both activating and null mutations in Rac block the extension of axons in *Drosophila* (Luo et al., 1994). Activating mutations of Rho disrupt axonal pathfinding in *Caenorhabditis elegans*, implicating Rho in coupling guidance cues to process outgrowth (Zipkin et al., 1997). Recently, it has been shown that the guidance molecule collapsin acts through a Rac-dependent mechanism (Jin and Strittmatter, 1997). In transgenic mice that express constitutively active Rac in Purkinje cells, there are alterations in the development of axon terminals and dendritic arborizations (Luo et al., 1996). The introduction of mutated Rac, Rho, or Cdc42 into cortical neurons affects dendritic morphology (Threadgill et al., 1997). Immunocytochemical observations of DRG neurons indicate that Rho protein is concentrated in growth cones (Renaudin et al., 1998). Therefore, members of the Rho family regulate axon and dendrite growth in development.

In PC12 cells, dominant negative Rac disrupts neurite outgrowth in response to NGF (Hutchens et al., 1997; Daniels et al., 1998), whereas treatment of PC12 cells with lysophosphatidic acid, a mitogenic phospholipid that activates Rho, or treatment with constitutively active Rho causes neurite retraction (Tigyi et al., 1996b; Kozma et al., 1997). Rapid neurite growth consistently follows treatment with C3 enzyme to inactivate all Rho family members in PC12 cells and primary neurons (Nishiki, 1990, Jalink et al., 1994; Tigyi et al., 1996b; Jin and Strittmatter, 1997; Kozma et al., 1997). We report here that C3 inactivation of Rho can promote neurite growth of PC12 cells and retinal neurons on MAG and myelin. A recent study reports that both active RhoA and active Rac protect chick motor neurons from growth cone collapse by myelin (Kuhn et al., 1999), but dominant negative Rho and C3 were not tested to permit a direct comparison with our results. The difference between our findings could relate to differences in neuronal cell type. Also, it is possible that different Rho isoforms (i.e., RhoA, B, and C) contribute differently to regulating growth, as found for Rac. Activated rRac1B expressed in retinal neurons stimulates neurite growth, whereas activation of Rac1A did not (Albertinazzi et al., 1998). We found that dominant negative RhoA expressed in PC12 cells promoted neurite growth on MAG but not on myelin, perhaps because Rho inhibition by dominant negative constructs can be low (Qiu et al., 1995). We and others (Jin and Strittmatter, 1997) have observed robust neurite growth on myelin substrates when neurons are treated with C3. We suggest that inactivation of the multiple forms of Rho by treatment with C3 is the most effective way to overcome growth inhibition by myelin.

In non-neuronal cells, a complementary hierarchy of signaling between Rho, Rac, and Cdc42 has been proposed (Nobes and Hall, 1995). In contrast, Rac and Rho may have opposite effects on neurite growth (Kozma et al., 1997; van Leeuwen et al., 1997): inactivation of Rho stimulates rapid neurite outgrowth (Nishiki et al., 1990; Jalink et al., 1994; van Leeuwen et al., 1997; Katoh et al., 1998), whereas activation of Rac stimulates neurite extension (Kozma et al., 1997; van Leeuwen et al., 1997; Daniels et al., 1998; Albertinazzi et al., 1998). Rho and Rac may have additive effects on growth cone morphology, with activated Rho and inactive Rac cooperating to give a spread growth cone morphology, with lower rates of growth (Jin and Strittmatter, 1997). Kuhn et al. (1999) found that activation of Rho prevented growth cone collapse by

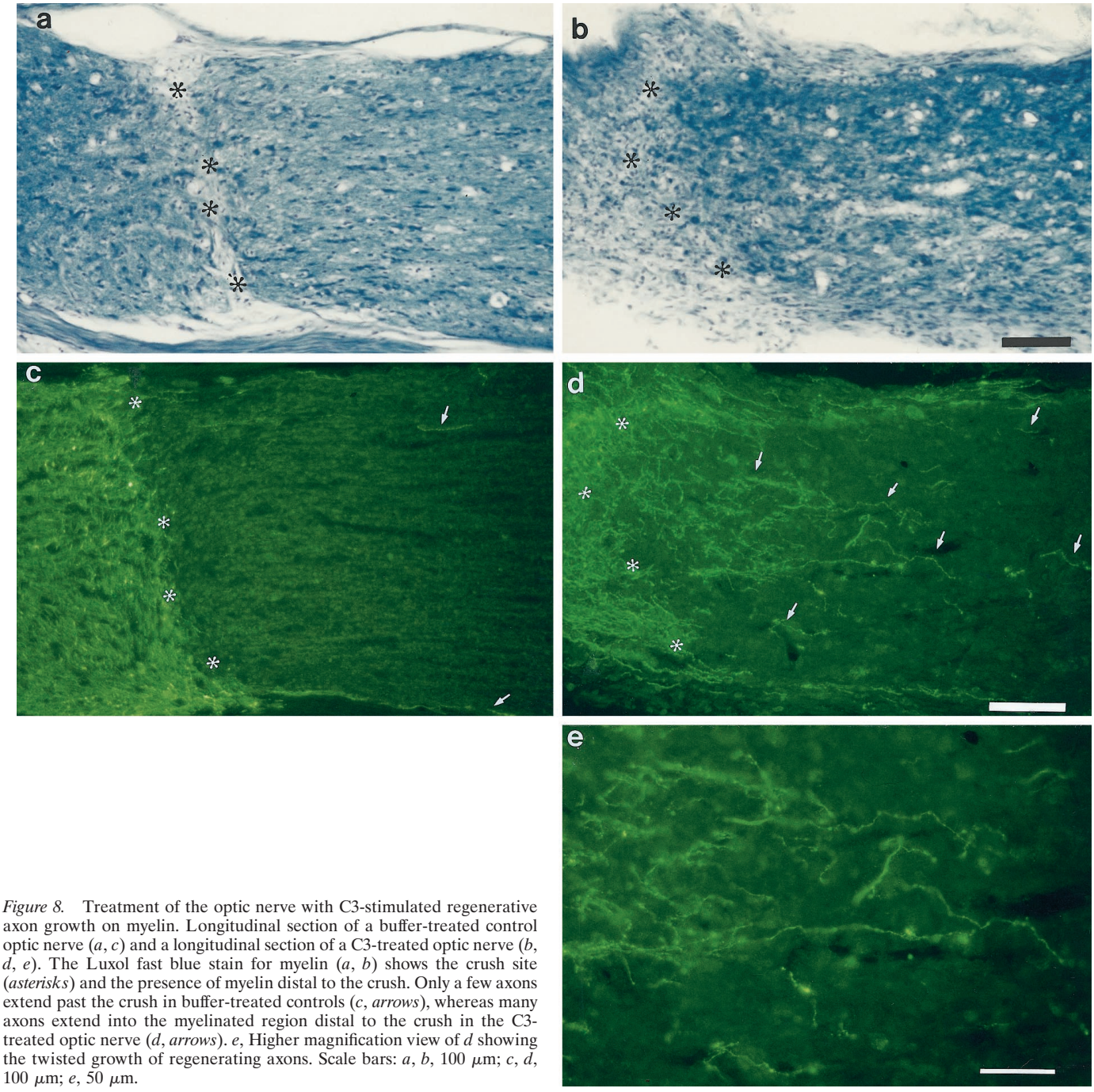

Figure 8. Treatment of the optic nerve with C3-stimulated regenerative axon growth on myelin. Longitudinal section of a buffer-treated control optic nerve (*a*, *c*) and a longitudinal section of a C3-treated optic nerve (*b*, *d*, *e*). The Luxol fast blue stain for myelin (*a*, *b*) shows the crush site (*asterisks*) and the presence of myelin distal to the crush. Only a few axons extend past the crush in buffer-treated controls (*c*, *arrows*), whereas many axons extend into the myelinated region distal to the crush in the C3-treated optic nerve (*d*, *arrows*). *e*, Higher magnification view of *d* showing the twisted growth of regenerating axons. Scale bars: *a*, *b*, 100 μm; *c*, *d*, 100 μm; *e*, 50 μm.

myelin, but growth cone morphology is not always predictive of the growth state. Rapid neurite elongation in the presence of C3 occurs with a collapsed growth cone morphology (Jin and Strittmatter, 1997), and *in vivo*, rapidly extending axons are bullet-shaped (Mason and Wang, 1997). Possibly, the prevention of myelin-derived growth cone collapse by activated Rho (Kuhn et al., 1999) reflects the cooperative affects of Rac and Rho on growth cone morphology.

Recently, it was found that priming cells with neurotrophins increases cAMP levels to block the inhibitory response to MAG (Cai et al., 1999). We note that, under our experimental conditions with retinal ganglion cells, the neurons were not primed before treatment with C3 to inactivate Rho. However, our data that suggest the Rho signaling pathway is a key target for regulating growth cone motility is relevant to the finding that cyclic nucleotides regulate growth cone responses to inhibitory proteins. Growth cone repulsion by MAG can be converted into attraction by elevation of intracellular cAMP levels to activate protein kinase A (PKA) (Song et al., 1998). Experiments with non-neuronal cells have implicated cAMP in the regulation of Rho because elevation of cAMP and activation of PKA inhibit Rho activation (Lang et al., 1996; Laudanna et al., 1996; Dong et al., 1998). Moreover, PKA directly phosphorylates Rho, and this phosphorylation decreases the ability of Rho kinase to interact

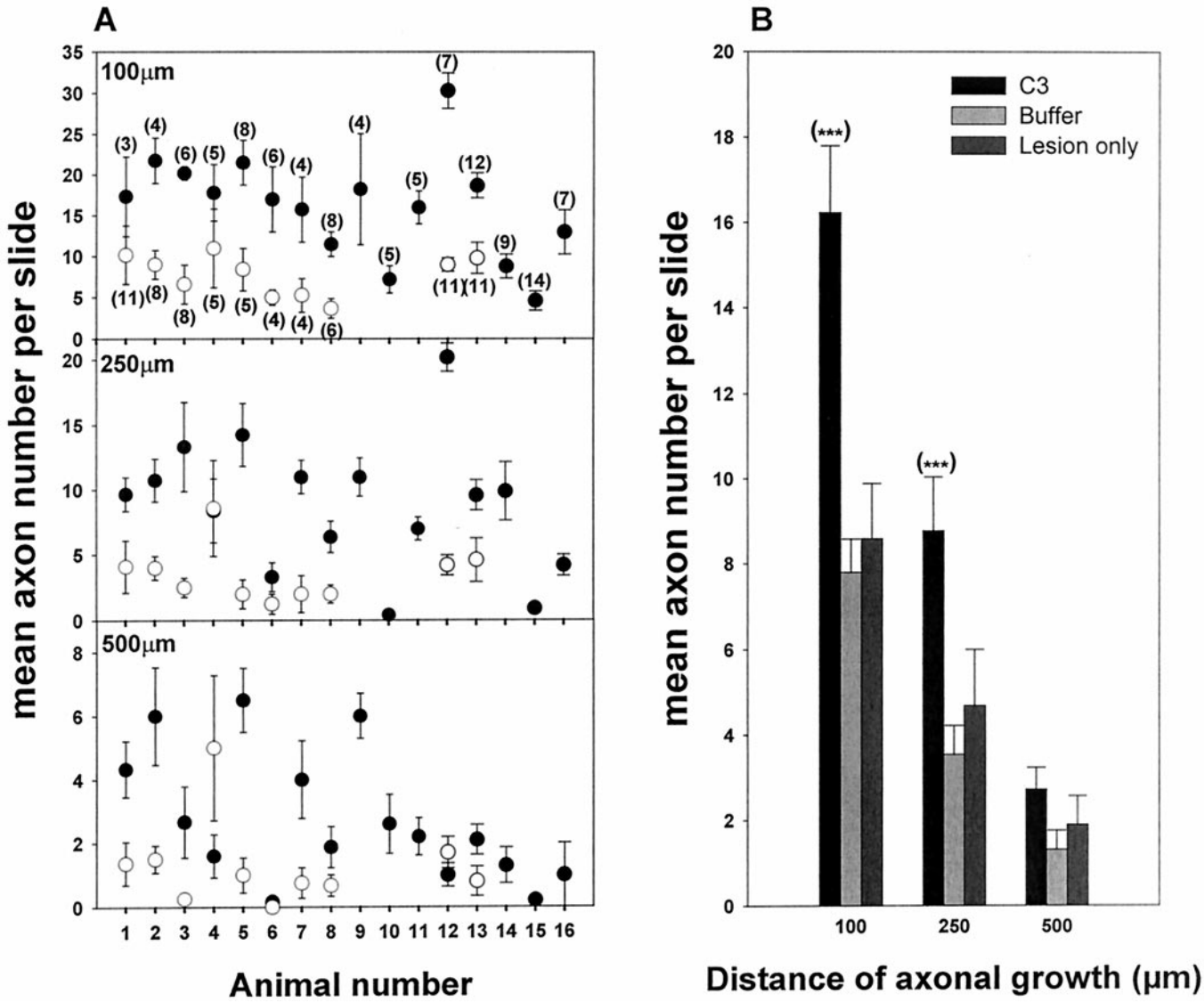

Figure 9. Average axon growth in sections of optic nerves from individual animals. *A*, Quantitative analysis of the average number of RGC axons per section measured at 100, 250, and 500 μm for C3-treated (●) and buffer-treated (○) animals. Each *point* represents data from one animal, with the *number* of sections analyzed for each animal shown in parentheses. Animals 1–11 were examined by fluorescent microscopy (DTAF) and animals 12–19 by an HRP–DAB reaction. *B*, Pooled results for the three groups of animals: C3-treated, buffer-treated controls, and microcrush lesion alone. Average axon growth after C3 treatment was significantly greater than buffer control or lesion alone at 100 and 250 μm.

with activated Rho (Lang et al., 1996; Dong et al., 1998). In PKA-deficient PC12 cells, elevation of cAMP fails to protect from the activation of Rho by lysophosphotydic acid (Tigyi et al., 1996a). It is likely, therefore, that PKA-dependent regulation of Rho occurs in growth cones as well.

Not all of the myelin-derived inhibitory molecules are known to date, and less is known about the neuronal receptors for growth inhibitory molecules. Several different MAG binding partners have been identified (Yang et al., 1996; Collins et al., 1997), and specific neuronal receptors to myelin inhibitors are likely to exist. Targeting intracellular signaling mechanisms converging to Rho rather than individual receptors may be the most practical way to overcome growth inhibition *in vivo*. The advantage of inactivating Rho to stimulate regeneration is that axons can regenerate directly on the native terrain of the CNS and thus may be more likely to find their natural targets.

The response of adult rat retinal ganglion cells to axonal transection

Remarkably, we observed that RGC axons crossed the lesion site to enter the distal optic nerve after treatment of injured optic nerve with C3. The striking feature of our results was the large number of axons that crossed the lesion into the distal white matter compared with buffer-treated controls or after microcrush lesion alone. Studies of RGC regeneration after treatment with IN-1 antibody to block myelin inhibitors have demonstrated that RGC axons do not regenerate long distances compared with axons in the spinal cord (Bartsch et al., 1995). One further barrier to axonal regeneration is the cell death by apoptosis that follows axonal injury. This has been thoroughly characterized for RGCs in which the type of injury (cut or crush) and distance of the lesion from the retina influence the extent of cell death (Villegas-Perez et al., 1988, 1993; Berkelaar et al., 1994). Treatment of the

optic nerve with C3 is unlikely to prevent the apoptosis that follows injury. The number of axons that we observed to regenerate likely represents <1% of the normal RGC population, but only 5–18% of retinal ganglion cells are expected to be alive 2 weeks after intraorbital lesion (Villegas-Perez et al., 1988; Berkelaar et al., 1994). When RGC do regenerate their axons after grafting of a peripheral nerve, which also provides some trophic support (Villegas-Perez et al., 1988), an average of only 3% of RGC axons regrow (Vidal-Sanz et al., 1987).

Our observations of microcrush-lesioned optic nerves after treatment with C3 provide the first evidence that treatment of injured white matter tracts with C3 can help foster regeneration after injury. Whereas the *in vitro* experiments showed that C3 can affect directly the growth of neurites from retinal cells, it is likely that the effects we observed after application of C3 to the optic nerve *in vivo* are more complex. In some C3-treated animals, the crush zone was constricted compared with controls (Fig. 8*b*), suggesting that C3 may affect non-neuronal cells such as fibroblasts and astrocytes. Also, C3 is known to affect cell migration (Hall, 1998) and could influence macrophage invasion in the injured nerve. The effects of C3 on astrocytes and macrophages need be further examined both *in vivo* and *in vitro* to better understand the implications of C3 treatment for stimulating axon growth *in vivo*.

C3 is a 24 kDa protein, and, although it may efficiently enter transected axons, growing or mature axons may not take up C3 very efficiently. The inability of intact growing axons to take up C3 may explain why the robust regeneration that we observed was not sustained for longer distances. It is known that injured axons take up exogenously applied retrograde tracers such as Fluorogold, but intact axons do not. Our interpretation of our results is that C3 has a dramatic but short-lived effect on RGC axons because it is taken up immediately after axon transection but is not taken up by axons once they begin to regenerate. Antagonists of Rho activity that can cross the plasma membrane of growing axons may improve the extent of regeneration. Also, it will be interesting to test C3 in spinal cord models of axon injury in which axon growth can be almost an order of magnitude greater that that observed in injured optic nerve after treatment with IN-1 antibody (Bartch et al., 1995). Nonetheless, our data of C3 treatment of injured optic nerve provide compelling evidence that C3 can promote neurite growth on inhibitory substrates *in vitro* and helps to overcome growth inhibition *in vivo*.

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