Application of Rho Antagonist to Neuronal Cell Bodies Promotes Neurite Growth in Compartmented Cultures and Regeneration of Retinal Ganglion Cell Axons in the Optic Nerve of Adult Rats

Johanne Bertrand,1 Matthew J. Winton,1 Nieves Rodriguez-Hernandez,1 Robert B. Campenot,2 and Lisa McKerracher1,3
1Département de Pathologie et biologie cellulaire, Université de Montréal, Montréal, Québec, Canada, H3T 1J4, 2Department of Cell Biology, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7, and 3BioAxone Thérapeutique, Montréal, Québec, Canada, H2X 3P9

Inactivation of Rho promotes neurite growth on inhibitory substrates and axon regeneration in vivo. Here, we compared axon growth when neuronal cell bodies or injured axons were treated with a cell-permeable Rho antagonist (C3–07) in vitro and in vivo. In neurons plated in compartmented cultures, application of C3–07 to either cell bodies or distal axons promoted axonal growth on myelin-associated glycoprotein substrates. In vivo, an injection of C3–07 into the eye promoted regeneration of retinal ganglion cell (RGC) axons in the optic nerve after microcrush lesion. Delayed application of C3–07 promoted RGC growth across the lesion scar. Application of C3–07 completely prevented RGC cell death for 1 week after axotomy. To investigate the mechanism by which Rho inactivation promotes RGC growth, we studied slow axonal transport. Reduction in slow transport of cytoskeletal proteins was observed after axotomy, but inactivation of Rho did not increase slow axonal transport rates. Together, our results indicate that application of a Rho antagonist at the cell body is neuroprotective and overcomes growth inhibition but does not fully prime RGCs for active growth.

Key words: axon regeneration; optic nerve injury; sympathetic neuron; compartmented culture; RGC survival; slow axonal transport

Introduction

CNS neurons fail to spontaneously regenerate their axons after injury partly because of growth inhibitors present in myelin and at the glial scar (McKerracher and Winton, 2002; David and Lacroix, 2003). Growth inhibitory proteins activate Rho GTPase, which leads to growth cone collapse and growth inhibition (Jalink et al., 1994; Tigli et al., 1996; Jin and Strittmatter, 1997; Lehmann et al., 1999). In vitro, treatment with the Rho-specific antagonist C3-transferase inactivates Rho and stimulates neurite growth on inhibitory substrates (Dergham et al., 2002; Monnier et al., 2003). Cell-permeable versions of C3 have been used for more effective Rho inactivation (Winton et al., 2002; Shearer et al., 2003). From such studies, it is not known whether Rho antagonists act on the cell body or growth cone to promote neurite growth.

A simple paradigm to study CNS regeneration is the adult rat optic nerve lesion. Strategies to promote retinal ganglion cell (RGC) regeneration by blocking growth inhibition in vivo often use local delivery techniques after optic nerve injury (Weibel et al., 1994; Lehmann et al., 1999). In contrast, treatments aimed at boosting axonal growth capacity are successful when acting on the cell body of RGCs (Berry et al., 1996; Leon et al., 2000; Cui et al., 2003; Yin et al., 2003; Fischer et al., 2004b; Monsul et al., 2004). The relationship between regenerative effects on cell bodies versus injured axons is not well understood.

RGCs die after optic nerve injury, and this must be taken into account when studying regeneration. In rats, transection of the optic nerve close to the eye causes a delayed RGC death, with cells beginning to die ~4 d later. One week after injury, ~50% of RGCs have died, and by 2 weeks, <20% survive (Villegas-Perez et al., 1993; Mansour-Robaey et al., 1994). We previously demonstrated that Rho antagonists applied to injured optic nerve promote regeneration of RGC axons (Lehmann et al., 1999). Here, we determine the effect of Rho antagonist application to cell bodies versus axons. For in vitro studies, we used compartmented cultures, a well-characterized cell culture system in which cell bodies and proximal axons of superior cervical ganglion neurons are located in a separate fluid compartment from their distal axons (Campenot, 1977; Campenot and Martin, 2001). In vivo, injection into the eye after microlesion of the optic nerve provides an ideal model to examine regeneration after specific treatment of the cell body. We show that Rho antagonists promote axonal growth on inhibitory substrates when applied to either cell bodies or distal axons. As well, our results show that Rho antagonists promote RGC regeneration when ap-
plied to cell bodies, as previously observed when applied to injured axons (Lehmann et al., 1999), and that delayed treatments allow RGC axons to regenerate across the lesion scar. Intravitreal application of Rho antagonists also increased RGC survival. Despite its impact on RGC survival and regeneration, C3–07 does not blunt slow axonal transport rates that decrease after axotomy.

Materials and Methods

Rho antagonists C3–05 and C3–07. C3–05 is a modified version of C3-ADP-ribosyltrasferase from Clostridium botulinum with an added transport sequence that facilitates entry into cells. C3–05 was prepared as described previously (Winton et al., 2002) by affinity purification and was ~95% pure. During the course of our experiments, a newer version of C3–05 was made to increase purity and yield. C3–07 has the same enzymatic activity and transport sequence as C3–05, and its ability to inactivate Rho was verified by Rho pull-down assay, as described for C3–05 by Dubreuil et al. (2003) (data not shown). C3–07 differs from C3–05, because the DNA sequence for the GST tag used for affinity purification of C3–05 was removed before subcloning into a pET vector. C3–07 was purified by fast-protein liquid chromatography (FPLC), as described previously (Han et al., 2001), and activity was verified by neurite outgrowth assay, as for C3–05 (Winton et al., 2002). The FPLC-purified protein was ~99% pure.

Axonal growth after axotomy in Campenot chambers. Superior cervical ganglion (SCG) from postnatal day 0 (P0) to P1 were dissociated and plated into compartmented cultures (Tyler Research, Edmonton, Alberta) as described previously (Campenot and Martin, 2001). Briefly, compartmented cultures were constructed in collagen-coated 35 mm culture dishes using a Teflon divider that separates the neurons into individual compartments (i.e., cell bodies and distal axons) (see Fig. 1A). The dish floor was patterned into 20 parallel collagen tracks bordered by scratches made with a specially constructed pin rake. To form the compartments, a Teflon divider was sealed to the dish floor with silicone grease. This sealing method was shown to prevent the exchange of media between the individual compartments (Campenot, 1977, 1979; Macinnis et al., 2003). Neurons were then plated in the center compartment and extended axons under the grease barrier into the two side compartments. The cells were maintained in L15CO2 medium supplemented with 2.5% rat serum, 20 ng/ml NGF (Cedarlanes Laboratories, Hornby, Ontario), vitamin C, and 10 μM cytosine arabinoside (Sigma, Oakville, Ontario). Distal axons were axotomized 5–7 d after plating by spraying cold, sterile water through a 0.22 gauge needle into the two side chambers. This process was repeated ~3–4 times, until all distal axons had been axotomized (Kimpinski et al., 1997; Macinnis et al., 2003). After axotomy, 2.5 μg/ml C3–07 was added to the various compartments (i.e., cell body compartment only, distal axon compartments only, or all three compartments), and axonal growth into side chambers was measured 3 d after axotomy. The media was changed every 48 h, and C3–07 was re-added to the fresh media in treated compartments. Axonal growth was measured from the grease barrier to the tip of the longest axon in a track. Measurements were made with an inverted microscope fitted with a digitizer that tracks stage movements to an accuracy of ±5 μm (Campenot and Martin, 2001). Experiments were completed in triplicate with a minimum of 10 tracks per culture chamber.

After axonal growth was measured and with the Teflon dividers still in place, the cultures were washed twice with cold Tris-buffered saline and lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl2, 1 μg/ml leupeptin (Roche, Laval, Quebec), 1 μg/ml aprotonin (Roche), 1 mM phenylmethyl-sulfonyl fluoride (PMSF) (Sigma)). Separate lysates from cell bodies/proximal axons and distal axon compartments were pooled across three cultures, clarified by centrifugation at 14,000 × g for 10 min at 4°C, and stored at −80°C. Proteins in the lysates were separated on 12% acrylamide gels and transferred to nitrocellulose. The membranes were blocked in 5% powdered milk, incubated in blocking buffer with a polyclonal C3 antibody (1:4000) (Winton et al., 2002), and the immunoreactive bands revealed by HRP-based chemiluminescent reaction (Pierce, Rockford, IL). Protein levels were determined by protein assay and verified by Ponceau Red staining.

Axonal growth on myelin-associated glycoprotein in Campenot chambers. Myelin-associated glycoprotein (MAG)-coated dishes were prepared 1 d before use. MAG was purified as described previously (Mckerracher et al., 1994) and applied to collagen-coated 35 mm dishes at 4 μg of total protein per dish and allowed to dry overnight in a laminar flow hood. MAG was applied only in areas that would correspond to the side chambers of the compartmented culture. After the MAG substrate dried, the dishes were washed with water, the compartmented cultures were assembled, and dissociated SCG neurons were plated in the center compartments. After 1 d in culture, C3–07 (2.5 μg/ml) was added to either the cell body/proximal axon compartment only, the distal axon compartments only, or all three compartments, and axonal growth into the side compartments was measured for three consecutive days. The media was changed after 48 h, at which time the C3–07 was replenished in the fresh media in treated compartments. Experiments were completed in triplicate with a minimum of 10 tracks measured per culture chamber.

Intraorbital microlesion surgery and C3–05/07 treatment. Intraorbital microlesions effectively axotomize all RGC axons and provide a well-defined lesion site (Selles-Navarro et al., 2001). Microlesions were performed on female Sprague Dawley rats (180–200 g; Charles River, St-Constant, Quebec, Canada) under gas anesthesia with 2.5–3% isoflurane in oxygen. The left optic nerve was exposed, the dural sheath was slit longitudinally, and the nerve was crushed 1 mm from the globe by holding a tight knot made with a 10–0 suture for 60 s. The suture was then completely removed. Treated animals were injected intravitreally with 10 μg of C3–05 or 1 μg of C3–07 in 5 μl of PBS immediately after the microlesion or 4 d later. Control animals were injected with 5 μl of PBS. The injections were made with a fine glass tube attached to a 10 μl Hamilton syringe (Hamilton, Reno, NV). Care was taken not to touch the lens during the injection, and animals that showed signs of lens injury were excluded from the study. The needle was slowly removed, and the injection site was sealed with surgical glue (Indermuc; Tyco Health Care Canada, Montréal, Québec, Canada). The skin was sutured with 4–0 silk. The vascular integrity of the retina was verified by fundus examination, and animals whose retina showed ischemic damage were excluded from the study. Rats were examined for regeneration after 2 or 4 weeks. To quantify regeneration 2 weeks after optic nerve injury and immediate treatment, 11 animals were treated with the Rho antagonists C3–05 or C3–07, and 10 animals served as buffer and lesion controls. We used five C3–07 treated and 10 control rats in similar experiments in which regeneration was allowed to continue for 4 weeks. We used eight C3–07 treated and five control rats to evaluate regeneration 2 weeks after lesion when application of C3–07 was delayed until 4 d after lesion. All animals were used at a mean weight of 0.015 mg. Animals were killed with an overdose of chloral hydrate (100 mg/kg ip). Animals were kept in a controlled environment where they were exposed to a 12 h light/dark cycle and had ad libitum access to food and water. All animal procedures followed guidelines from Canadian Council of Animal Care.

Anterograde labeling, immunohistochemistry, and quantification of axon growth. Regenerating axons in the optic nerve were identified by anterograde labeling. Animals received an intravitreal injection of 5 μl of 1% cholera toxin β subunit (CT/β) (Sigma) 24 h before they were killed. Animals were deeply anesthetized with a 7% chloral hydrate overdose, followed immediately by intracardial perfusion with saline and then 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.2. The eyes were dissected in cold PBS, and the optic nerve was separated from the eye behind the globe. The nerves were cryoprotected overnight in 30% sucrose in PBS, embedded in Tissue-Tek (VWR Scientific Products, Montréal, Québec, Canada), and frozen in 2-methylbutane in a liquid nitrogen bath. Longitudinal cryostat sections of optic nerves (14 μm) were mounted on SuperFrost Plus slides (Fisher Scientific, Montréal, Québec, Canada) and stored at −80°C.

To detect CT/β in RGC axons, slides were postfixed in 4% PFA in 0.1 M PB and blocked for 1 h with 5% normal rabbit serum, 3% bovine serum albumin, 0.2% Triton X-100, and 1 mM sodium azide. Slides were then incubated overnight with a goat choleragenoid antibody (1:4000) from List Biological Laboratories (Cedarlanes Laboratories, Hornby, Ontario, Canada).
and an enlargement of the neurons on a single track among the 16–20 tracks in an individual culture. Compartmented cultures
both.

Four experiments on a Western blot are shown (dashed line), with or more experiments, each using triplicate cultures.

Statistical significances were established using Student’s t test (***, p < 0.001; **, p < 0.01; *, p < 0.05). Statistical tests were performed using In Stat (Graph Pad, San Diego, CA).

Western blots of retina and optic nerve homogenates. Microlesion of the left optic nerve was performed as described above, and 5 μg of C3–07 was injected intravitreally. Animals were killed 3 d later by a 7% chloral hydrate overdose, followed by an intracardial perfusion with saline. The retina and first 2 mm segment of the optic nerve were removed and stored at –80°C. Tissues were homogenized in 160 μl (retinas) or 20 μl (optic nerve segments) of NP-40 lysis buffer [20 msi Tris, pH 8.0, 137.5 msi NaCl, 10% glycerol, 1% NP40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin (Roche), 1 mm PMSE]. Lysates were clarified by high-speed centrifugation for 5 min at 4°C. Supernatants were removed and centrifuged at high speed for 15 min. Samples (20 μl) were separated on a 12% acrylamide gel and transferred to nitrocellulose. Membranes were blocked in 5% powdered milk and incubated overnight at 4°C with a polyclonal C3 antibody.

The signals were revealed by HRP-based chemiluminescent reaction (Pierce).

Retrograde labeling and quantitation of RGC survival. RGCs were retrogradely labeled from the superior colliculus with Fluorogold (Fluorochrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was exposed, the pia matter overlying the superior colliculus was removed, and small pieces of gel-chrome, Denver, CO). The right midbrain was Experiments. Two groups were A, Axons on MAG substrates treated with C3–07 grow >2 mm. D, Axons plated on collagen substrates grow >2 mm. E, Measurement of the average axonal length. Data represent the average of four or more experiments performed in triplicate.
jejion immediately after the lesion \((n = 4)\), another received a PBS injection \((n = 3)\), and the last group had no injection \((n = 4)\).

In another experiment, animals did not have an optic nerve crush but received a C3–07 injection 6 d after labeling and were killed 8 d later \((n = 3)\).

Evaluation of slow axonal transport. Six or 14 d after radiolabeling, animals used for slow axonal transport experiments were killed by a 7% chloral hydrate overdose, followed immediately by intracardial perfusion with saline. The left optic nerve was removed, cut into 2 mm segments, and stored at \(-80^\circ\text{C}\). Each 2 mm segment of optic nerve was homogenized in 60 \(\mu\)l of SDS-PAGE sample buffer using a motorized Teflon-in-glass homogenizer. The samples were clarified by high-speed microcentrifugation for 15 min. Samples were then loaded on 7.5% acrylamide gels for electrophoretic separation. The gels were stained with Coomassie Blue, destained in 10% acetic acid, impregnated with Amplify (Amersham Biosciences, Montréal, Québec, Canada), dried, and exposed to preflashed Hyperfilm MP (Amersham Biosciences) for periods varying from 1 to 6 d. Slow axonal transport was assessed by following the advance of the 150 kDa middle neurofilament subunit (NF-M) labeling front across the five optic nerve segments. The position of the front was determined as the segment preceding the one in which the protein density dropped to 10% of its highest value for that animal. The expression level of NF-M is 85% of controls 2 weeks after an intracranial crush of the optic nerve (Mckerracher et al., 1993). NF-M lanes of an autoradiograph were scanned for densitometry using an Epson perfection 1200U scanner, transferred to Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA), and the images were analyzed with the densitometry IQ MAC 1.2 software (Molecular Dynamics, Sunnyvale, CA).

Results
Localized application of C3–07 after axotomy
To determine any effects of C3–07 on normal axon growth, we applied cell-permeable Rho antagonist C3–07 to cell bodies or distal axons in sympathetic neurons plated in compartmented cultures (Fig. 1A). The cultures were maintained for 5–7 d, at which time axons in the distal chambers were axotomized. Immediately after axotomy, media with C3–07 (2.5 \(\mu\)g/ml) was added to selected compartments, and axonal regrowth was measured 3 d later. C3–07 did not significantly increase the rate that axons extended along the tracks after axotomy, but there was \(\sim 15\%\) enhancement of growth rate, independently of the site of application (Fig. 1B). Treatment of both cell bodies and axons did not further increase the response to the treatment.

After axonal regrowth was measured in the above experiment, lysates from cell body compartments and distal axon compartments were prepared, and the proteins were separated by electrophoresis. Western blot analysis revealed that the preponderance of C3–07 localized to the cellular regions within the compartments in which it was directly applied, although a relatively small amount of C3–07 applied to cell bodies traveled into distal axons and vice versa (Fig. 1C).

Localized application of C3–07 promotes axonal growth on MAG substrate
Inactivation of Rho stimulates neurite outgrowth on inhibitory substrates when neurons in culture are exposed to Rho antagonists (Lehmann et al., 1999; Dergham et al., 2002; Winton et al., 2002; Monnier et al., 2003). We determined whether C3–07 application to either the cell body compartment or the distal axon compartments was sufficient to overcome growth inhibition by MAG. The MAG substrate was added to the side compartments of collagen-coated dishes, in areas that would correspond to the side chambers of the compartmented cultures. Sympathetic neurons were plated in the center compartment, and after 1 d in culture, when axons were just entering the side compartments, C3–07 (2.5 \(\mu\)g/ml) was added to the culture media of the cell body compartment, both distal axon compartments, or all three
Intravitreal injection of C3–05 or C3–07 stimulates regeneration in the optic nerve

Previous results demonstrated that in the adult rat, an application of C3 at the site of an optic nerve lesion promotes axon regeneration (Lehmann et al., 1999). To test whether treatment of the RGC cell bodies promoted regeneration in vivo, we injected Rho antagonists into the vitreous immediately after microlesion of the optic nerve 1 mm behind the optic disc. In the first experiments, we used C3–05 (n = 4), an affinity-purified cell-permeable variant of C3, until a more highly purified version, C3–07 (n = 7), became available. Both have the same enzymatic activity and transport sequence. Control animals received a PBS injection (n = 5) or microlesion alone (n = 5). Axon regeneration in the optic nerve was evaluated 14 d later, after an injection of the anterograde tracer CTB.

Two weeks after microlesion, virtually no CTβ-positive axons were detectable in control animals (Fig. 3A), whereas a large number of axons were visible past the lesion site in C3–05 (Fig. 3B)– and C3–07-treated rats (Fig. 3C). The microcrush injury model provides a clearly defined lesion site with little or no cavitation (Selles-Navarro et al., 2001), and the lesion site was identified by dark-field microscopy and/or CTβ staining. The number of axons present at different distances past the lesion site was then counted. Animals treated with C3–05 (Fig. 3D) or C3–07 (Fig. 3E) had a significantly higher number of regenerating axons per section than controls, at distances of 50, 100, and 250 μm from the lesion site. Regeneration in animals injected with C3–07 was similar to that of C3–05-treated animals, but we observed a greater number of longer axons in some animals treated with the more highly purified C3–07 (Fig. 3C).

C3–05 was also used to evaluate regeneration 4 weeks after microlesion. At that time point, we still observed significantly more axons growing past the lesion site in the C3–05-treated group (n = 5) than in the control groups (PBS, n = 5; microlesion alone, n = 5) at distances of 50, 100, and 250 μm (Fig. 4).

These results indicate that application of Rho antagonists C3–05/07 to RGC cell bodies can promote optic nerve regeneration after microlesion. To examine whether treatment had a sustained effect on axon growth, we compared the average length of the longest axon in treated optic nerves 2 and 4 weeks after axotomy. No significant differences in axon length were detected at 4 weeks compared with 2 weeks (data not shown), suggesting that a single treatment does not result in sustained long-term growth.

To examine the localization of C3–07 after injection in the eye, we injected 5 μg of C3–07 in the eye after microlesion of the optic nerve. We prepared retina and optic nerve homogenates for Western blots 3 d later and probed them with an anti-C3 antibody. The specific C3–07 band was compared with recombinant C3–07 protein run in a separate lane (data not shown). We observed full-length C3–07 in optic nerve lysates when C3–07 was injected in the eye (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), in agreement with in vitro studies with compartmented cultures.

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

By 24 h after microlesion, a chondroitin sulfate proteoglycan (CSPG)-positive scar is well formed (Selles-Navarro et al., 2001). To determine whether a delayed treatment with C3–07 stimulated regeneration of RGCs through the lesion scar, we injected C3–07 (n = 8) in the vitreous 4 d after microlesion of the optic nerve and examined regeneration 10 d later. Control animals were injected with PBS (n = 5). A large number of CTβ-positive axons could be seen past the lesion site in treated animals, whereas very few were observed in PBS controls (Fig. 5A). Animals treated with C3–07 had a significantly higher number of regenerating axons per section than controls, at distances of 50, 100, 250, and 500 μm from the lesion site (Fig. 5B). A comparison of the number of axons per section showed similar numbers of regenerating axons in animals treated with an immediate (Fig. 3D) or delayed (Fig. 5B) injection of C3–07. The average longest axon was significantly longer in animals receiving either an immediate or a delayed C3–07 treatment than in PBS controls (Fig. 5C). These results demonstrate the existence of a therapeutic window for a Rho antagonist treatment after optic nerve injury and indicate that inactivation of Rho allows RGC axons to grow across the lesion scar.

Intravitreal injection of C3–07 increases RGC survival

We reported previously that inactivation of Rho prevented apoptotic cell death after spinal cord injury (Dubreuil et al., 2003). After injury of the optic nerve, approximately one-half of the RGCs die by apoptosis by 1 week. To determine whether a single intravitreal injection of C3–07 protected RGC from cell death, we
examined RGC survival in retinal whole mounts. RGCs were retrogradely labeled with Fluorogold 1 week before optic nerve axotomy, and surviving RGCs were counted 7 or 14 d later in animals treated with C3–07 (n = 7 at 7 d; n = 5 at 14 d) or vehicle control (n = 3 at 7 d; n = 4 at 14 d). Treatment with C3–07 completely rescued RGCs 1 week after axotomy, compared with 40% survival in vehicle-injected animals (Fig. 6). RGC survival after a single injection was not sustained, and RGC numbers decreased after 1 week. However, at 14 d, cell survival was still significantly better with C3–07 treatment, with more than twice the number of RGCs in treated animals compared with controls.

Slow axonal transport after C3–07 treatment
Axonal transport in normal and injured optic nerve of adult rat has been well described (McQuarrie et al., 1986, 1989; McKerracher et al., 1990) and is detected by examining the movement of 35S-methionine-labeled proteins along 2 mm optic nerve segments. In uninjured optic nerve, the rate of slow axonal transport of neurofilament, as detected by the 150 kDa middle neurofilament subunit (NF-M), is ~0.5 mm/d (McQuarrie et al., 1986), and the transport rate decreases significantly when the optic nerve is crushed intracranially near the optic chiasma (McKerracher et al., 1990). To determine whether C3–07 had an impact on slow axonal transport, the optic nerve was crushed 6 d after labeling, a time when tubulin and neurofilament proteins enter the optic nerve (Fig. 7A). Control animals received no injection or were injected with PBS, and treated animals received an intraocular injection of C3–07 immediately after the crush. Optic nerves were removed 8 d after crush, a total of 14 d after labeling, and were cut into five segments of 2 mm each (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

The NF-M front in uninjured control animals reached the second segment 6 d after labeling (Fig. 7A) and progressed to the fifth segment by 14 d after labeling (Fig. 7B). In untreated animals whose nerve was crushed 6 d after labeling, the NF-M front reached the third segment 14 d after labeling (Fig. 7C,D). This distance is similar to that observed in animals who were killed 6 d after labeling (Fig. 7A), showing a dramatic decrease in slow axonal transport after optic nerve crush. When C3–07 was injected in the vitreous immediately after the optic nerve crush (Fig. 7E), the NF-M front traveled no further than it did in control axotomized animals with no injection (Fig. 7C) or PBS injection (Fig. 7D). Therefore, C3–07 did not increase slow axonal transport after injury. We also tested whether C3–07 affected slow axonal transport in uninjured nerve. The distance traveled by the NF-M front in uninjured animals after C3–07 injection at day 6 after labeling was not changed at 14 d (data not shown).

Discussion
These findings show that C3-based Rho antagonists have a growth-promoting effect on distal axons even when applied to neuronal cell bodies. Our in vitro experiments with compartmented cultures indicate that C3–07 promotes axonal growth on inhibitory substrates when applied to either cell bodies or distal axons. Previous treatments tested in compartmented cultures, such as NGF (Campenot, 1994) or glial cell-conditioned medium (Hayashi et al., 2004), promoted axonal growth when applied in distal axon compartments but not when applied in the cell body chamber. In vivo, treatment of cell bodies with an intraocular application of C3–05 or C3–07 promoted axon regeneration similar to that obtained with an application of C3 to injured optic nerve (Lehmann et al., 1999). Therefore, both in vitro and in vivo...
experiments suggest that the site of application of Rho antagonists is not determinant for promoting axonal growth and regeneration in the presence of growth inhibitors.

We are the first to directly compare the effect of the application of a Rho antagonist to cell bodies versus injured axons. Other studies show growth-promoting factors are effective in stimulating axonal regeneration when applied to the eye, such as factors secreted in the retina after lens injury or intravitreal implantation of peripheral nerve graft (So and Aguayo, 1985; Leon et al., 2000). Intravitreal grafting of acellular peripheral nerve has a small regenerative effect on RGCs (Berry et al., 1996), whereas acellular grafts connected to the cut end of the optic nerve do not (Berry et al., 1988; Hall and Berry, 1989). In contrast, inhibitors of Nogo have only been tested after application to the injured optic nerve (Weibel et al., 1994; Cui et al., 2004).

The similarity of results on axonal growth and regeneration when C3–07 is applied at the cell bodies or injured axons can be explained, at least in part, by diffusion or active transport of C3–07 between cell body and axon. C3–07 initially applied to the cell body was later detected in untreated axons in our in vitro and in vivo experiments. Therefore, the effect of Rho antagonists on regeneration could result from known actions on the actin cytoskeleton at the growth cones. C3–07 might prevent growth cone collapse through its known action on blocking inhibitory signaling. The increased survival observed 2 weeks after lesion in C3–07-treated animals might also contribute to the higher level of regeneration, although survival strategies alone are not sufficient to promote regeneration (Mansour-Robaey et al., 1994; Inoue et al., 2002).

Here, we report that treatment with C3–07 prevented death of RGCs for at least 1 week, a finding consistent with a recent report on RGCs transfected in vivo with a C3–construct in which increased RGC survival was reported (Fischer et al., 2004b). However, one difference in the two studies is that Fischer et al. (2004b) reported only a partial increase in RGC survival, perhaps explained by transfection efficiency. Possibly, the application of cell-permeable C3–07 in our study affected other cells, such as Muller cells, that might also influence cell survival. It is important to note that the survival rates that we report here are comparable with the best results obtained when the neurotrophic factor BDNF is injected in the vitreous after axotomy (Mansour-Robaey et al., 1994; Peinado-Ramon et al., 1996). BDNF is also able to rescue all RGCs from axotomy at 1 week after intracocular injection, and RGCs begin to die between 7 and 14 d. Similarly, the effect of C3–07 is not sustained after a single application, and RGCs begin to die between 1 and 2 weeks after axotomy.

Our results clearly show that inactivating Rho with C3–07 applied to the cell bodies promotes regeneration of RGC axons. However, regenerating axons did not grow for long distances after crossing the lesion site, a result consistent with findings from most studies on regeneration in the rat optic nerve (Leh-
mann et al., 1999; Leon et al., 2000; Ellezam et al., 2003; Sapieha et al., 2003; Fischer et al., 2004a). Treatments such as vaccination with a spinal cord homogenate or application of C3 to the lesion site can induce a much longer regeneration in the spinal cord (Huang et al., 1999; Dergham et al., 2002) than in the optic nerve (Lehmann et al., 1999; Ellezam et al., 2003), a finding not well understood. The number of regenerating axons is another important determinant when examining regeneration. Although numbers of regenerating RGCs are significant after Rho inactivation, the number of RGCs that regenerate are small, even though 0.5% of RGC axons extend past the lesion site. This small number could be explained in part by the finding that <1% of postnatal RGCs retain rapid axonal elongation capabilities (Goldberg et al., 2002a). In contrast, many more RGCs can grow in the permissive environment of a peripheral nerve (PN) graft (Villegas-Perez et al., 1988; Cui et al., 2003). It was shown recently that the combination of Rho inactivation with lens injury could increase dramatically the success of regeneration, compared with C3 alone, even though not all RGCs were transfected with the C3 construct (Fischer et al., 2004b). It has been suggested that combination therapies offer the best hope for robust and substantial regeneration, a suggestion supported by other recent investigations of RGC regeneration. Combining CNTF treatment with Nogo-neutralizing antibody IN-1 (Cui et al., 2004) was more effective than either strategy alone. Dominant-negative Nogo receptor expressed in RGCs in vivo does not promote regeneration, but when combined with lens injury, there was a robust regeneration better than lens injury alone (Fischer et al., 2004a). RGCs lose their trophic responsiveness after axotomy (Shen et al., 1999), and RGCs require “priming,” such as by treatment with cAMP, to help increase their responsiveness to neurotrophic factors (Goldberg et al., 2002b). Together, current studies suggest that combining Rho inactivation with treatments that prime mature RGCs into a growth mode enhances the number of RGCs regenerating over long distances into an inhibitory environment.

Our experiments confirmed a previous report that slow axonal transport is almost completely stopped after an intracranial crush of the optic nerve (McKerracher et al., 1999). Typically, RGCs do not regenerate with intracranial injury, even when a PN graft is provided (Richardson et al., 1982; You et al., 2000). However, combining intraocular growth factors with a PN graft promotes RGC regeneration after distal axotomy (Cui et al., 1999). Slow axonal transport is critical to regeneration because it supplies cytoskeletal proteins that are required to support axon elongation. To test the effect of Rho inactivation on slow axonal transport, we axotomized optic nerves near the chiasma, which is almost 1 cm farther than the site of axotomy used for our regeneration experiments. This change was necessary to provide a sufficient length of optic nerve for the transport experiments, but it should be noted that there are differences in RGC survival and regenerative ability with intraorbital and intracranial axotomy (Villegas-Perez et al., 1993; You et al., 2000). C3–07 did not prevent the decrease in slow axonal transport after intracranial axotomy, suggesting that, under these conditions, C3–07 does not have a priming effect, even though it can promote RGC survival.

References
Lehmann M, Fournier A, Selles-Navarro I, Dergham P, Sebok A, Leclerc N,


