

Rho Signaling Pathway Targeted to Promote Spinal Cord Repair

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The Rho signaling pathway regulates the cytoskeleton and motility and plays an important role in neuronal growth inhibition. Here we demonstrate that inactivation of Rho or its downstream target Rho-associated kinase (ROK) stimulated neurite growth in primary cells of cortical neurons plated on myelin or chondroitin sulfate proteoglycan substrates. Furthermore, treatment either with C3 transferase (C3) to inactivate Rho or with Y27632 to inhibit ROK was sufficient to stimulate axon regeneration and recovery of hindlimb function after spinal cord injury (SCI) in adult mice. Injured mice were treated with a single injection of Rho or Rho-associated kinase inhibitors delivered in

a protein adhesive at the lesion site. Treated animals showed long-distance regeneration of anterogradely labeled corticospinal axons and increased levels of GAP-43 mRNA in the motor cortex. Behaviorally, inactivation of Rho pathway induced rapid recovery of locomotion and progressive recuperation of forelimb–hindlimb coordination. These findings provide evidence that the Rho signaling pathway is a potential target for therapeutic interventions after spinal cord injury.

Key words: Rho GTPase; Rho-associated kinase; C3; Y27632; corticospinal tract; regeneration; BBB behavior scale; GAP-43; mouse

Mammalian neurons do not regenerate successfully after lesion. This is explained in part by myelin-derived inhibition (Caroni and Schwab, 1988; McKerracher et al., 1994; Mukhopadhyay et al., 1994; Chen et al., 2000) and the formation of a glial scar expressing inhibitory molecules (Snow et al., 1990; McKeon et al., 1991; Asher et al., 2000). However, numerous studies in animal models using methods designed to overcome the effect of growth inhibitory proteins have confirmed the regenerative potential of the injured spinal cord. These methods include the use of antibodies (Schnell and Schwab, 1990; Huang et al., 1999), peripheral nerve grafts (Cheng et al., 1996), transplantation of cells into the lesion site (Howland et al., 1995; Rapalino et al., 1998; Liu et al., 1999; McDonald et al., 1999; Ramon-Cueto et al., 2000) and limiting the formation of the glial scar (Davies et al., 1999; Moon et al., 2000).

The failure of regeneration in the adult CNS may also be caused by changes occurring in mature neurons (Li et al., 1995, 1996; Shen et al., 1999; Cai et al., 2001). Another approach to stimulate regeneration has been to target neurite growth signaling. For example, different neurotrophin treatments have increased the ability of neurons in adult CNS to regenerate and stimulate both axonal growth and sprouting after injury (Schnell et al., 1994; Sawai et al., 1996; Blesch and Tuszynski, 1997; Weidner et al., 1999; Coumans et al., 2001). Neurotrophins are known to delay apoptosis, prevent atrophy of axotomized neurons, and enhance the expression of growth-associated genes

(Fournier et al., 1997; Kobayashi et al., 1997; Bregman et al., 1998; Broude et al., 1999). Recent data suggest that neurotrophins might stimulate regeneration by increasing neuronal cAMP levels to overcome inhibitory signaling (Cai et al., 1999). Therefore, the decreased ability of the mature CNS to regenerate after injury may result from both the intrinsic properties of adult neurons and the extracellular inhibitory environment.

The Rho GTPase is a key intracellular regulator of cytoskeletal dynamics and cell motility (Hall, 1998). Rho is activated when growth cones collapse in response to chemorepulsive factors (Tigyi et al., 1996; Jin and Strittmatter, 1997; Kuhn et al., 1999; Wahl et al., 2000), and inhibiting Rho promotes neurite outgrowth in the presence of myelin (Jin and Strittmatter, 1997; Lehmann et al., 1999). Recently, Rho has been shown to regulate apoptosis (Liu et al., 2001; Trapp et al., 2001). An enzyme from *Clostridium botulinum*, C3 transferase (C3), blocks Rho function by ADP ribosylation of the effector domain (Dillon and Feig, 1995). Y27632 inhibits Rho-associated kinase (ROK), a serine–threonine protein kinase that is activated by Rho (Ishizaki et al., 1997; Uehata et al., 1997). Inactivation of ROK with Y27632 promotes neurite outgrowth (Katoh et al., 1998; Bito et al., 2000), but it is not known whether it is sufficient to block growth inhibition as was shown for inactivation of Rho (Lehmann et al., 1999). In the present study, we compare inactivation of Rho or ROK to promote axon growth on inhibitory substrates. We further study in fully adult mice whether inactivation of the Rho signaling pathway promotes axon regeneration and functional recovery after spinal cord injury (SCI). Animals treated to inactivate the Rho signaling pathway show significant improvement in locomotion by open field testing. Thus, inactivation of the Rho signaling pathway is an effective method to improve outcome after SCI.

MATERIALS AND METHODS

Cell culture. Primary cortical neurons were isolated from embryonic day (E) 15–E18 rat fetuses. The cortex was cut into small pieces into Ca²⁺-

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and Mg^{2+} -free HBSS, 0.3 M HEPES buffer, pH 7.3, and penicillin/streptomycin, and then dissociated with 0.25% trypsin, 0.1% DNase at 37°C for 15 min. The dissociated cells were washed and diluted to 2×10^5 cells per milliliter in minimal essential media (Invitrogen, Burlington, ON), N2 supplement (Invitrogen), 5% fetal bovine serum, 1% ovalbumin, and 1 mM pyruvate. Cells were then tritirated with 25 μ g/ml C3 or buffer, or with 31 or 3.1 μ M Y27632. Cells were plated in eight-well chamber slides coated with 25 μ g/ml poly-L-lysine or with test substrates. Myelin substrates were made by coating with 8 μ g purified bovine brain myelin dried overnight at room temperature. Chondroitin sulfate proteoglycan (CSPG) substrates were made by incubating 0.5 μ g/ml mixed CSPG solution (Chemicon, Temecula, CA) overnight in poly-L-lysine-coated chamber slides. Mixed substrate was made by coating and drying 8 μ g of myelin in 150 μ l of CSPG solution. The plates were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde after 12, 24, or 48 hr, and neurons were identified by immunocytochemistry using a β III-tubulin antibody (Sigma, Oakville, ON). The longest neurite per neuron was measured on an average of 400 cells per experiment with a minimum of three experiments per condition. Doses for C3 and Y27632 were chosen on the basis of previous experiments (Lehmann et al., 1999; Bito et al., 2000; M. Winton and L. McKerracher, unpublished observations).

Preparation of recombinant C3 and Y27632. Recombinant C3 exoenzyme was prepared as a glutathione *S*-transferase–C3 fusion protein and stored at -80°C (Lehmann et al., 1999). Y27632 was synthesized from α -methylbenzylamine and exhibited identical ^1H and ^{13}C nuclear magnetic resonance spectra as reported in United States Patents 4,997,834 and 5,478,838. Before *in vivo* use, the activity of C3 and Y27632 was tested in tissue culture with retinal neurons plated on myelin substrates (Lehmann et al., 1999).

Spinal cord injury and delivery of Rho and Rho-kinase inhibitors. BALB-c female mice ($n = 70$) of ~ 20 gm were anesthetized with 0.4 ml/kg hypnorm and 5 mg/kg diazepam. A segment of the thoracic spinal cord was exposed using fine rongeurs to remove the bone, and a dorsal over-hemisection was made at T7. Fine scissors were used to cut the dorsal part of the spinal cord, which was cut a second time with a fine knife to ensure that the lesion extended past the central canal. A fibrin adhesive delivery system was prepared using a Tisseel VH kit (ImmunoAG, Vienna, Austria). According to manufacturer's instructions for slow polymerization, lyophilized fibrinogen was reconstituted in an aprotinin solution, thrombin was reconstituted in a calcium chloride solution, and both solutions were warmed to 37°C. Fifty microliters of 1 mg/ml C3 or Y27632 were added to 25 μ l of the thrombin solution. This was mixed with 25 μ l of the fibrinogen solution just before application to the spinal cord to allow infiltration of the mixture into the lesion site before polymerization. In some C3-treated animals and in all Y27632-treated animals, 10 μ l of the 1 mg/ml solution was applied to the lesion site immediately after the cord was cut. As controls, a second group of animals received fibrin adhesive with buffer, and a third group was left untreated. C3-containing collagen gels were formed as follows. C3 was lyophilized (40 μ g per mouse) and then reconstituted in 10 μ l of 7.5% NaHCO_3 , and then 25 μ l of 0.7 mg/ml rat tail collagen was added. As with fibrin, 10 μ l of C3 was added to the lesion cavity before the C3-containing collagen gel was applied. For retranssections 3 weeks after SCI, the spinal cords were cut at T6 as described above, and the animals were tested using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale on days 1, 2, and 6 after the second surgery.

Anterograde labeling. Three weeks to 3 months after injury, corticospinal tract (CST) fibers were labeled by injection of the anterograde tracer wheat germ agglutinin–horse radish peroxidase (WGA-HRP) into the motor cortex as described (Huang et al., 1999). Two days later, the animals were perfused transcardially with saline and then 4% paraformaldehyde, and the spinal cords and brains were removed. Serial longitudinal cryostat sections of the spinal cord were cut at 30 μ m, reacted for HRP (Huang et al., 1999), and counterstained with neutral red. Measurement of axon regeneration was assessed independently by two reviewers. Lesion depth was assessed by measuring the depth of damaged tissue in the spinal cord as a percentage of total spinal cord width.

In situ hybridization. GAP-43 mRNA was detected by *in situ* hybridization on coronal cryostat sections through the motor cortex of mice treated with PBS ($n = 2$) or C3 ($n = 3$). *In situ* hybridization was performed as described previously (Fournier et al., 1997) with an ^{35}S -labeled GAP-43 cRNA probe derived from a plasmid provided by Dr. Pate Skene (Duke University Medical Center) (Basi et al., 1987). After the *in situ* hybridization procedure, sections were Nissl stained and bright-field and dark-field digital micrographs were taken. On the basis of

Nissl staining and retrograde labeling of motor cortex by Fluorogold in previous animals, a region was circled on bright-field micrographs to include axotomized layer V neurons. Corresponding dark-field micrographs were black and white inverted and thresholded in Northern Eclipse (Empix imaging, Mississauga, ON), and autoradiographic grain clusters >30 pixels in size were counted. Background-corrected grain cluster densities were calculated on 4–10 sections per animal using the following formula: [(number of counted grain clusters in circled region/area of circled region)/(number of counted grain clusters in background/area of background sampled)].

Behavioral testing. Behavioral recovery was assessed for 1 month after SCI in an open field environment by the BBB method (Basso et al., 1995). We modified the 21 point BBB scale to a 17 point score because mice do not exhibit differences in toe drag that can be monitored visually. Thus, scale points 16, 17, and 18 were removed from the scale. Mice raise their tails early in their recovery, and score 19 for tail up position was removed, leaving a 17 point total score. The mouse modified BBB score was as follows: (0) no observable hindlimb (HL) movement; (1) slight movement of one or two joints; (2) extensive movement of one joint and/or slight movement of one other joint; (3) extensive movement of two joints; (4) slight movement of all three joints of the HL; (5) slight movement of two joints and extensive movement of the third; (6) extensive movement of two joints and slight movement of the third; (7) extensive movement of all three joints of the HL, walking with little/no weight support; (8) extensive movement of all three joints, walking with weight support; (9) frequent to consistent dorsal stepping with weight support; (10) frequent plantar stepping with weight support; (11) consistent plantar stepping with weight support, no coordination; (12) consistent plantar stepping with consistent weight support, occasional forelimb (FL)–HL coordination; (13) consistent plantar stepping with consistent weight support, frequent FL–HL coordination; (14) consistent plantar stepping with consistent weight support, consistent FL–HL coordination; predominant paw position during locomotion is rotated internally or externally, or consistent FL–HL coordination with occasional dorsal stepping; (15) consistent plantar stepping with consistent weight support, consistent FL–HL coordination; predominant paw position is parallel to the body; frequent to consistent curled toes, trunk instability; (16) consistent plantar stepping with consistent weight support, consistent FL–HL coordination; predominant paw position is parallel to the body, flat toes, some trunk instability; (17) consistent plantar stepping with consistent weight support, consistent FL–HL coordination; predominant paw position is parallel to the body, flat toes and consistent stability in the locomotion. For scoring, each animal was videotaped for 3 min, and two reviewers participated. In the late phase of recovery, the BBB score was determined from sequences of four steps or more from digitized videos projected on a computer screen at one-fourth speed. Detailed patterns of front paw and foot placements were assessed, as shown in Figure 5, *F* and *G*, for untreated ($n = 6$) and C3-treated ($n = 6$) animals.

RESULTS

Inactivation of Rho or ROK promotes growth of primary neurons plated on complex inhibitory substrates

We tested first whether treatment of primary cortical neurons with C3 or with Y27632 was sufficient to stimulate growth on complex inhibitory substrates typical of the glial scar and white matter. Neurons plated on different test substrates were examined at 12, 24, and 48 hr, and similar results were observed at all time points. Neurons plated on CSPG, purified myelin, or a mixture of both did not extend long neurites and had a rounded shape (Fig. 1*A,B*). After treatment with C3 (Fig. 1*C*) or Y27632 (Fig. 1*D*), neurons were able to extend neurites. Measurements at 24 hr showed that treatment with either C3 or Y27632 significantly increased the length of neurites compared with untreated cells plated on myelin, CSPG, or mixed myelin/CSPG substrates (Fig. 1*A*). Quantitation at 12 hr showed similar results (data not shown), and at 48 hr growth of treated neurons was too extensive to measure neurite length. C3 was significantly better than Y27632 in promoting neurite growth (*t* test; $p < 0.05$). These results demonstrate that inactivation of Rho or inhibition of ROK

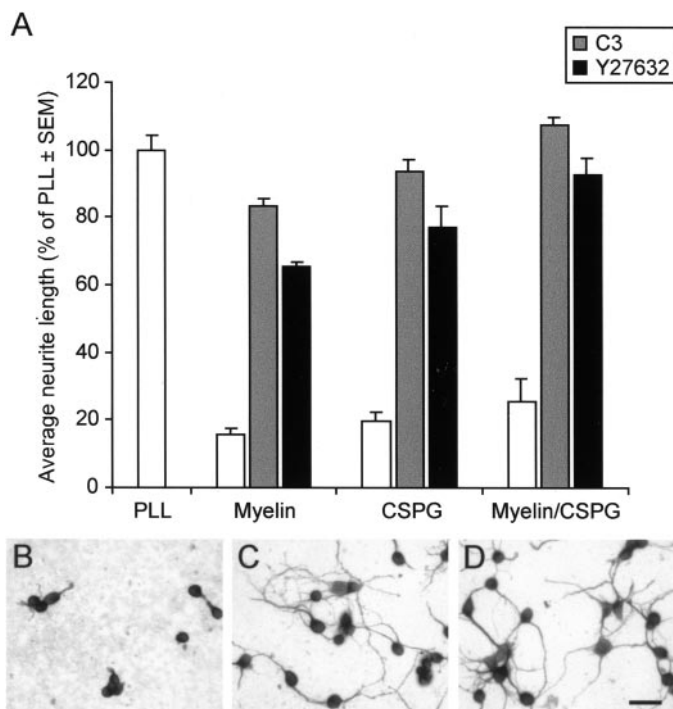


Figure 1. Effect of Rho antagonist C3 or Rho-associated kinase inhibitor Y27632 on neurite outgrowth of primary cortical neurons plated on inhibitory substrates. *A*, Neurite outgrowth was analyzed quantitatively by measuring the longest neurite per cell 24 hr after plating on poly-L-lysine, myelin, CSPG, or mixed substrates and treatment with buffer (white), 25 μ g/ml C3 (gray), or 31 μ M Y27632 (black). Differences between treated and untreated cells were significant on all test substrates (*t* test; *p* < 0.05). *B–D*, Representative micrographs of cortical neurons plated on mixed inhibitory substrates either untreated (*B*) or treated with C3 (*C*) or Y27632 (*D*). Scale bar, 25 μ m.

stimulates cortical neurons to extend neurites on complex growth inhibitory substrates.

Treatment of injured spinal cord promotes long-distance regeneration

To assess the potential of Rho inactivation to treat SCI, we cut the spinal cord of adult mice at T7 by a dorsal over-hemisection (Huang et al., 1999). We tested C3 by local delivery either in collagen or in a fibrin adhesive that polymerizes *in vivo* several seconds after injection, because both matrices have been reported to support tissue repair (Joosten et al., 1995; Herbert et al., 1998). We used the fibrin adhesive to test Y27632 because of favorable results with C3 (see below). Anterograde tracing with WGA-HRP of CST, a tract often used to study histological regeneration, was used to assess fiber growth in six groups of animals: animals treated with fibrin plus C3 (*n* = 13), collagen plus C3 (*n* = 12), fibrin plus Y27632 (*n* = 5), fibrin alone (*n* = 10), collagen alone (*n* = 7), and SCI with no treatment (*n* = 13) (Fig. 3). All sections were counterstained with neutral red to verify that lesions extended past the central canal (Fig. 2*J*). A quantitative analysis of lesion depth showed no significant differences between treated and control groups (one-way ANOVA). Without C3 or Y27632 treatment, transected CST axons retracted back from the site of lesion by ~300 μ m (Fig. 2*H*), although in animals treated with fibrin alone some regenerative sprouts did extend from the retracted bundle (Fig. 2*G*). Application of C3 to the injured spinal cord elicited extensive sprouting into the dorsal white matter and the lesion scar (Fig. 2*A,C,E*). Treated animals with Y27632

showed regenerative sprouting into the dorsal white matter and toward the lesion site (data not shown). To assess axons distal to the lesion site, the distance of the longest axon was measured. Axons were found up to 12 mm from the lesion site in C3-treated animals and up to 3 mm from the lesion site in Y27632-treated animals (Fig. 3), whereas buffer-treated animals showed retraction from the lesion site. Therefore, after treatment with C3 or Y27632, axons were found to extend past the lesion into the distal white matter. These axons have a twisted course of growth typical of regenerated axons (Figs. 2*D,F*). Although neutral red staining showed lesions extended past the central canal (Fig. 2*I*), these experiments alone cannot rule out the possibility that secondary damage was reduced after injury. This could arise in the damaged CNS because C3 has neuroprotective effects (Liu et al., 2001; Trapp et al., 2001), in addition to promoting growth on inhibitory substrates (Fig. 1).

Effect of C3 on the expression of GAP-43 mRNA in the motor cortex of spinal cord injured animals

After thoracic spinal cord lesion, only axons that regenerate long distances show upregulation of GAP-43 mRNA expression (Fernandes et al., 1999). To confirm the growth response and the long-distance regeneration after Rho inactivation, we examined the pattern of GAP-43 mRNA expression in the motor cortex (Fig. 4*A,B*) of animals 1 month after CST transection with or without treatment with C3. *In situ* hybridization using ³⁵S-labeled riboprobes on coronal brain sections revealed high levels of GAP-43 mRNA expression in neurons of the motor cortex of C3-treated animals (Fig. 4*D*), whereas untreated animals showed GAP-43 signal similar to background (Fig. 4*C*). Quantitation of grain cluster densities in motor cortex showed significant upregulation of GAP-43 mRNA (Fig. 4*E*). These results indicate that C3 treatments elicit changes in gene expression consistent with axon regeneration.

Behavioral testing

To test functional recovery after SCI and C3 or Y27632 treatment, we measured HL motor function using the BBB locomotor rating scale (Basso et al., 1995) (*n* = 37 animals). Because a toe clearance phase cannot be evaluated in recuperating mice, we modified the rating to a 17 point scale (see Materials and Methods). Twenty-four hours after surgery, control mice were paraplegic (Fig. 5*A*) and moved by pulling themselves forward with their forelimbs (Fig. 5*B*). Mice treated with C3 or Y27632 showed a remarkable recovery within 24 hr (Fig. 5*A*), already walking with weight support (Fig. 5*A,C*) (movie 1; available at www.jneurosci.org). Although this early recovery is too rapid to be explained by long-distance regeneration, possible mechanisms include local reorganization of central pattern generator circuitry (Giménez y Ribotta et al., 2000) that may include sprouting from undamaged ventral fibers or interneurons, pharmacological activation of neurotransmitter receptors (Rossignol et al., 2000), or neuroprotection (C. Dubreuil, M. Winton, F. Yang, P. Morley, L. McKerracher, unpublished observations). Mice that had received C3 or Y27632 treatment continued to recover over the 1 month period of observation and exhibited HL–FL coordination (Fig. 5*E,G*) (movie 2; available at www.jneurosci.org). By contrast, the average recovery plateau for untreated animals was limited to unstable walking without HL–FL coordination (Fig. 5*D,F*) (movie 2). Retransection of the spinal cord at 3 weeks (*n* = 8) eliminated any difference between the C3-treated (*n* = 5) and control (*n* = 3) animals (BBB at day 6, 7.6 vs 7.3, respectively).

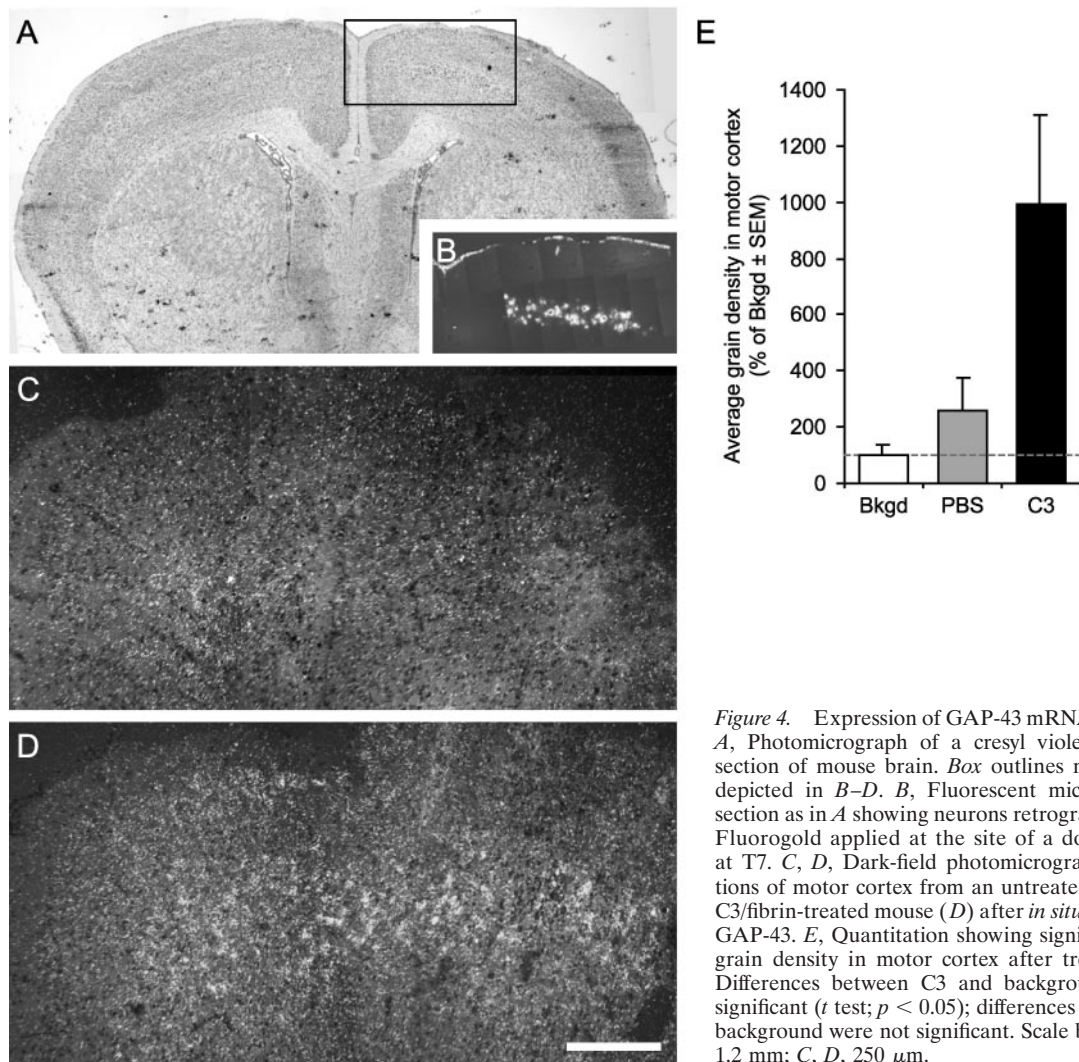


Figure 4. Expression of GAP-43 mRNA in motor cortex. *A*, Photomicrograph of a cresyl violet-stained coronal section of mouse brain. *Box* outlines motor cortex area depicted in *B–D*. *B*, Fluorescent micrograph of same section as in *A* showing neurons retrogradely labeled with Fluorogold applied at the site of a dorsal hemisection at T7. *C*, *D*, Dark-field photomicrographs showing sections of motor cortex from an untreated mouse (*C*) or a C3/fibrin-treated mouse (*D*) after *in situ* hybridization for GAP-43. *E*, Quantitation showing significantly increased grain density in motor cortex after treatment with C3. Differences between C3 and background (*Bkgd*) were significant (*t* test; $p < 0.05$); differences between PBS and background were not significant. Scale bar: *A*, 1.8 mm; *B*, 1.2 mm; *C*, *D*, 250 μ m.

ROK but also to protein kinase N (Amano et al., 1996), rhotekin, and other targets (Reid et al., 1996). Therefore, for CNS repair, Rho appears to be a more efficient target than ROK.

The remarkable improvement in function within 1 d of spinal cord lesion and treatment with C3 or Y27632 may be caused by increased neuroprotection in the lesioned spinal cord. Neuroprotection by itself is important for improved functional recovery after SCI (Pencalet et al., 1993; Gaviria et al., 2000), and a growing volume of literature suggests that the application of C3 to ischemic CNS tissue has neuroprotective effects (Laufs et al., 2000; Trapp et al., 2001). Moreover, inactivation of Rho in spinal cord reduces the number of apoptotic cells (C. Dubreuil, M. Winton, F. Yang, P. Morley, and L. McKerracher, unpublished observations). It has been demonstrated that the application of C3 after middle cerebral artery occlusion reduces infarct volumes (Trapp et al., 2001). Therefore, Rho signaling pathway is a good target to both prevent cell death and stimulate regeneration. The ability of C3 and Y27632 to block unwanted effects of Rho activation, cell death, and neurite retraction are likely to contribute importantly to improved outcome after SCI. It is also possible that C3 and Y27632 treatments affected other cells, such as leukocytes; immediate improvement in functional recovery after SCI has been observed 24 hr after treatment with gabaxate mesilate, a protease inhibitor that inhibits activation of leukocytes

(Taoka et al., 1997). Therefore, the short-term effects that we observe after treatment with C3 or Y27632 are likely caused by the ability of these compounds to limit the cell damage that occurs immediately after injury.

It should be kept in mind that mice show important differences from rats in their response to spinal cord injury, most notably the absence of necrotic cavitation (Steward et al., 1999). In our experiments, we used an over-hemisection of the spinal cord to test whether Rho or ROK inactivation was able to promote repair. Many strategies that work well to promote regeneration after hemisection are not effective after complete transection of the spinal cord. It has been shown that sparing of ventrolateral fibers may translate into improved locomotor performance (Brustein and Rossignol, 1998) because these fibers are important in the initiation and control of spinal central pattern generators (for review, see Rossignol et al., 2000). Sprouting of uninjured collaterals (Weidner et al., 2001) or sprouting of fibers that are part of the circuitry of the spinal cord (Giménez y Ribotta, 2000) are likely to contribute importantly to repair. It was demonstrated recently that reorganization of spared pathways also contributes to functional recovery (Raineteau et al., 2001). Thus, inactivation of Rho may help stimulate and enhance the spontaneous repair process that leads to limited recovery after SCI, in addition to its effects on regeneration.

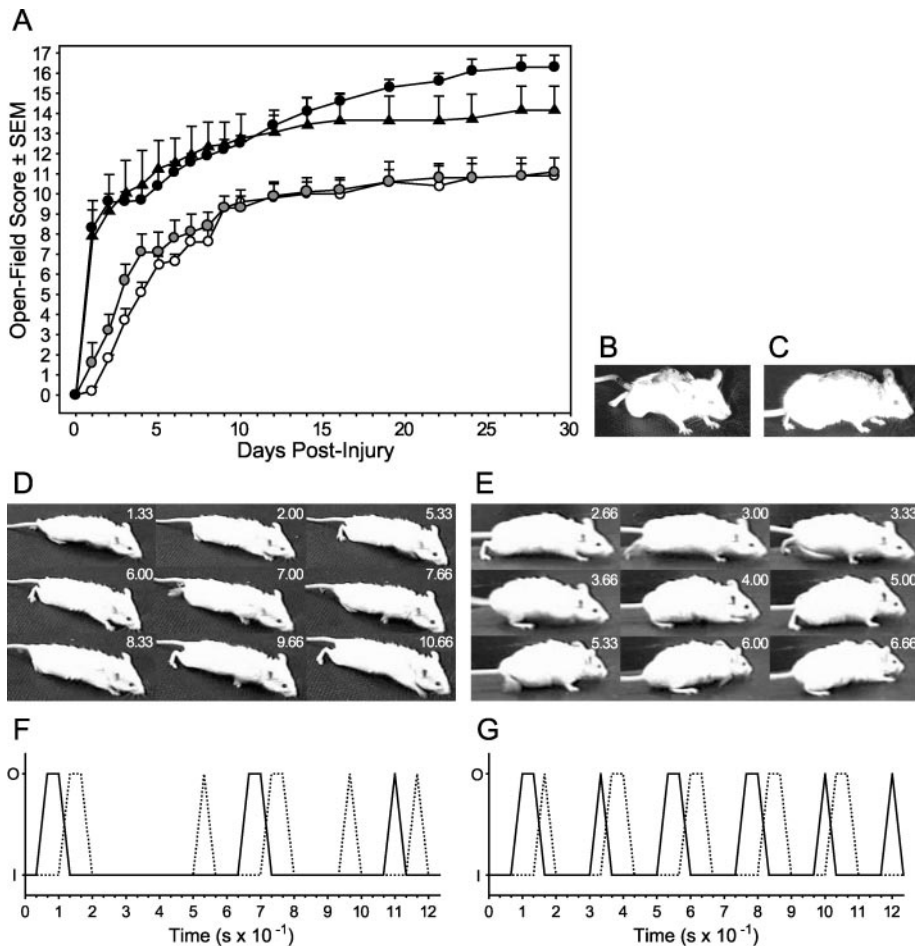


Figure 5. Analysis of functional recovery. *A*, Modified BBB scores of C3-treated (black circles; $n = 11$), Y27632-treated (black triangles; $n = 11$), and untreated (open circles; $n = 10$) mice to evaluate recovery of locomotion during the month after dorsal over-hemisection. At 24 hr, 1 week, 2 weeks, and 1 month, differences between groups of animals were evaluated by the Mann–Whitney U test. p values were similar at all four time points: C3-treated versus fibrin-treated, $p < 0.001$; Y27632-treated versus fibrin-treated, $p < 0.05$; C3-treated versus Y27632-treated, NS; fibrin-treated versus untreated, NS. *B*, Photograph of a spinal cord-injured mouse 24 hr after injury; HL cannot support body weight. *C*, Photograph of a C3/fibrin-treated mouse 24 hr after injury; body weight is supported by HL. *D*, *E*, Selected videoframes of representative untreated and C3/fibrin-treated mice, respectively, to show differences in recovery of HL–FL coordination 30 d after lesion. Although C3-treated mice alternate front paw and foot placements properly, untreated mice do not show one-to-one correspondence between HL and FL stepping. *Numbers* refer to elapsed time in tenths of seconds. *F*, *G*, For the mice depicted in *D* and *E*, respectively, HL–FL coordination is represented graphically. The position of the right hindpaw (solid line) and the right front paw (dotted line) on (I) or off (O) the ground was noted for each 1 of 38 sequential videoframes. The untreated mouse in *D* moves its forelimb twice before each HL step. NS, Not significant.

To study the effects of C3 and Y27632 on axons *in vivo*, we chose to study the CST because it is one of the best characterized tracts for studies of axon regeneration in the spinal cord. Moreover, the CST can be anterogradely labeled, and in mice, most of the fibers within the CST are located just above the central canal in the dorsal spinal cord. The dorsal over-hemisection that we used for our studies would eliminate not only the CST fibers but also other dorsal and lateral descending pathways while sparing the ventral pathways essential for locomotion. We demonstrated with our *in vitro* studies that treatment with C3 or Y27632 can stimulate axon growth on inhibitory substrates. *In vivo*, we observed sprouting and long-distance regeneration. It is well documented that reorganization of collateral CST fibers occurs after SCI (Weidner et al., 2001). Also, dendritic remodeling of neurons can be enhanced by inactivation of Rho (Ruchhoeft et al., 1999). Thus, inactivation of Rho, which is known to affect both axons and dendrites, is likely to enhance spontaneous plasticity of axons and dendrites, leading to functional remodeling of spinal cord circuitry.

The BBB open-field locomotor test cannot be correlated with the regeneration of specific tracts. Although the late recovery of HL–FL coordination that we observe at 1 month is consistent with regeneration of cut fibers, we cannot completely rule out the possibility of protective sparing, as reported with other strategies that promote repair after CNS injury (Lazarov-Spiegler et al., 1996; Hauben et al., 2000). However, our observation that GAP-43 is upregulated in the motor cortex is consistent with the interpretation that anterogradely labeled CST fibers past the

injury site represent regenerated fibers. Increased GAP-43 expression correlates with regeneration of rubrospinal neurons, and projection neurons do not express GAP-43 after thoracic injury alone (Ferandes et al., 1999). Therefore the upregulation of GAP-43 expression is a good indicator for long-distance regeneration. We observed long-term improvements in BBB outcomes, even in animals in which substantial CST labeling was not observed. Therefore, many factors (neuroprotection, remodeling, and regeneration) and many fiber tracts (dorsal and ventral) are likely to contribute to functional recovery after SCI and treatment with Rho or Y27632. Notwithstanding the possibility of multiple mechanisms, long-distance regeneration is likely to have been important for the improved function at later stages. These treatments that potentiate spontaneous functional recovery in open-field tests give hope that effective treatment for spinal cord injury will be developed in the foreseeable future. The treatment that we have developed to promote functional recovery and axonal regeneration after SCI is simple: an inhibitor of Rho signaling pathway injected at the lesion site in a tissue adhesive. These studies show the potential for a new, straightforward treatment to reduce functional impairment after SCI.

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