Lithium Chloride Reinforces the Regeneration-Promoting Effect of Chondroitinase ABC on Rubrospinal Neurons after Spinal Cord Injury

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ABSTRACT

After spinal cord injury, enzymatic digestion of chondroitin sulfate proteoglycans promotes axonal regeneration of central nervous system neurons across the lesion scar. We examined whether chondroitinase ABC (ChABC) promotes the axonal regeneration of rubrospinal tract (RST) neurons following injury to the spinal cord. The effect of a GSK-3β inhibitor, lithium chloride (LiCl), on the regeneration of axotomized RST neurons was also assessed. Adult rats received a unilateral hemisection at the seventh cervical spinal cord segment (C7). Four weeks after different treatments, regeneration of RST axons across the lesion scar was examined by injection of Fluoro-Gold at spinal segment T2, and locomotor recovery was studied by a test of forelimb usage. Injured RST axons did not regenerate spontaneously after spinal cord injury, and intraperitoneal injection of LiCl alone did not promote the regeneration of RST axons. Administration of ChABC at the lesion site enhanced the regeneration of RST axons by 20%. Combined treatment of LiCl together with ChABC significantly increased the regeneration of RST axons to 42%. Animals receiving combined treatment used both forelimbs together more often than animals that received sham or single treatment. Immunoblotting and immunohistochemical analysis revealed that LiCl induced the expression of inactive GSK-3β as well as the upregulation of Bcl-2 in injured RST neurons. These results indicate that *in vivo*, LiCl inhibits GSK-3β and reinforces the regeneration-promoting function of ChABC through a Bcl-2-dependent mechanism. Combined use of LiCl together with ChABC could be a novel treatment for spinal cord injury.

Key words: axonal regeneration; behavioral analysis; chondroitin sulfate; lithium; rubrospinal tract; spinal cord injury

INTRODUCTION

NEURONS IN THE ADULT mammalian spinal cord fail to regenerate axons after injury, resulting in permanent functional deficits. Modulation of the extrinsic environment and modeling of the intrinsic response are both em- ployed as strategies for promoting regeneration and recovery of injured central nervous system (CNS) axons. Specific examples include grafts of peripheral nerves (Richardson et al., 1982), fetal CNS tissue (Himes et al., 1994; Diener and Bregman, 1998), or genetically modified nonneuronal cells (Jin et al., 2002); neutralization of

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growth-inhibitory molecules (Caroni and Schwab, 1988; Schnell and Schwab, 1990; Thallmair et al., 1998); application of neurotrophic factors (Kobayashi et al., 1997; Shibayama et al., 1998); and overexpression of growth-promoting genes (Mason et al., 2002).

Injury to the spinal cord elicits robust glial reactions and subsequent formation of gliotic scar (Schwab et al., 2001; Liesi and Kauppila, 2002). Chondroitin sulfate proteoglycans (CSPGs) are one of the inhibitory components of the gliotic scar that hinder the regeneration of severed axons (Dou and Levine, 1994; Bovolenta et al., 1997; Fidler et al., 1999). Following spinal cord injury, upregulation of CSPGs has been found in the gliotic scar surrounding the lesion site (Wang et al., 1997). We and others have recently demonstrated that in vivo, enzymatic degradation of CSPGs at the lesion site can significantly promote the regeneration of severed axons across the gliotic scar in injured brain and spinal cord (Yick et al., 2000, 2003; Moon et al., 2001; Bradbury et al., 2002).

Lithium has been used for the treatment and prophylaxis of mania and depression in humans for almost half a century. Increasing numbers of studies have indicated that lithium has neuroprotective effects (Nonaka et al., 1998; Chen and Chuang, 1999; Wei et al., 2001). However, the molecular mechanisms underlying the actions of lithium have not been fully elucidated. Recent studies have shown that lithium exerts effects via a number of intracellular signaling pathways, including upregulation of Bcl-2 and direct inhibition of glycogen synthase kinase-3β (GSK-3β) (Chen et al., 1999; Manji et al., 2000). It has been suggested that lithium-induced Bcl-2 upregulation plays a crucial role in neuroprotection against glutamate excitotoxicity (Chen and Chuang, 1999). Bcl-2 also supports the intrinsic growth potential of injured axons (Chierzi et al., 1999; Holm and Isacson, 1999). GSK-3β is a key downstream target of the PI3-kinase/Akt signaling pathway that regulates apoptosis (Pap and Cooper, 1998; Crowder and Freeman, 2000). Neuronal apoptosis induced by trophic factor withdrawal is associated with an increase in GSK-3β activity (Hetman et al., 2000).

A severed rubrospinal tract (RST) can regenerate its axons under appropriate conditions (e.g. following application of neurotrophic factors; Xu and Martin, 1991; Liu et al., 1999; Jin et al., 2002). The RST is a descending pathway that has an almost complete contralateral trajectory, allowing unambiguous interpretations of the axonal tracing results (Brown, 1974). In this study, we show that enzymatic degradation of CSPGs by chondroitinase ABC (ChABC) can promote the axonal regeneration of RST after spinal cord injury. Interestingly, we demonstrate for the first time that the use of lithium chloride (LiCl), a GSK-3β inhibitor, can markedly reinforce the regeneration-promoting effect of ChABC on axotomized

RST neurons, resulting in a significant recovery of forelimb function.

MATERIALS AND METHODS

Surgical Procedures

Adult 9-week-old Sprague-Dawley rats (200–250 g) were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg). Under an operating microscope, a dorsal laminectomy was carried out and the seventh cervical spinal cord segment (C7) was identified. After opening the dura mater, a right hemisection was performed at this level, using a pair of spring scissors.

Following spinal cord hemisection, animals were divided into four groups. In the first group, animals received a piece of gelfoam soaked with 0.1 M phosphate-buffered saline (PBS), placed on top of the lesion site, together with a daily intraperitoneal injection of 1.0 mL PBS to serve as the sham control. In the second group, animals received a gelfoam soaked with PBS, placed on top of the lesion site, together with daily intraperitoneal injections of 1.0 mL of 85 mg/kg LiCl. In the third group of animals, a small piece of gelfoam soaked with 10 μL of 2.5 units/mL of *Proteus vulgaris* ChABC lyase (Seikagaku Corporation) was placed on top of the lesion site. The animals also received daily intraperitoneal injections of 1.0 mL PBS. In the fourth group, animals received a combined treatment of daily intraperitoneal injections of 85 mg/kg LiCl together with a piece of gelfoam soaked with 10 μL of 2.5 units/mL of ChABC placed on top of the lesion site. We have shown that intraperitoneal injection of 85 mg/kg LiCl can protect retinal ganglion cells in a rat glaucoma model (unpublished observations). In addition, ChABC at 2.5 units/mL enhances the axonal regrowth of Clarke's neurons after spinal cord injury (Yick et al., 2000, 2003). After operation, animals were allowed to survive for 1 month and were then further subdivided into subgroups for either axonal tracing ($n = 5$) or behavioral analysis ($n = 4$). Two additional groups of animals received daily intraperitoneal injection of either PBS or 85 mg/kg LiCl and were used for immunohistochemistry and Western blot analysis. Animals for immunohistochemistry and Western blot were allowed to survive for either 1 or 5 days ($n = 8$ for each time point, $n = 4$ for immunohistochemistry, and $n = 4$ for Western blot).

Retrograde Labeling of Regenerated RST Neurons

Fluoro-Gold (FG, 6% w/v, Fluorochrome) was used to label the RST neurons that had regenerated their axons

933

across the injury scar and that reentered the caudal spinal cord. Two days before the end of the postinjury survival period (1 month), animals were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg). A dorsal laminectomy was then carried out, and a second thoracic (T2) spinal segment was identified. FG at a volume of 0.5 μL was manually injected into the right T2 spinal cord using a Hamilton syringe. Because of the diffusibility of FG in the vicinity of the injection site and because a relatively large amount of FG (0.5 μL) was used, this procedure actually labeled RST neurons in both sides.

The RST is a descending pathway in the superficial dorsolateral white matter of the spinal cord that has almost complete (>99%) contralateral axonal projection without spared axons and collateral sprouting (Brown, 1974). Right hemisection at the C7 spinal cord severed axons from the left red nucleus. Except for rare ipsilateral projections (<1% of total RST neurons), injured RST neurons in the left midbrain with axons regenerating beyond the hemisection site to T2 were labeled by FG. Following C7 spinal cord hemisection, the ipsilateral intact side of the midbrain was used as an internal control.

Tissue Preparation

After the postinjury survival periods, animals were anesthetized with a lethal dose of ketamine and xylazine and were perfused intracardially with normal saline, followed by 400 mL of fixative containing 4% paraformaldehyde in 0.1 M PBS. The brain and spinal cord were removed, postfixed with paraformaldehyde overnight, and then placed in 30% phosphate-buffered sucrose. Brain and spinal cord tissues were cut into 30-μm sections on a cryostat and mounted onto gelatin-coated slides.

Western Blot and Immunohistochemistry

The effect of LiCl on the phosphorylation state of GSK-3β and the expression of Bcl-2 in RST neurons was examined both 1 and 5 days after the surgical operation. For Western blotting, the primary antibodies used were the polyclonal anti-p-GSK-3β-serine-9 (Cell Signaling Technology, Beverly, MA), the monoclonal anti-GSK-3β (Transduction Laboratories, Lexington, KY), and the monoclonal anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA). The injured and the contralateral intact side of the red nucleus were rapidly dissected and freshly frozen at the end of the postoperative survival periods. Samples of four rats per group were pooled, and the tissue was immediately placed in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 4 mM EDTA, pH 7.4) containing protease inhibitor cocktails (Sigma, St. Louis, MO), and was homogenized in a Vibra-Cell (Sonic and

Materials, Newtown, CT) homogenizer. The homogenate was centrifuged at 14,000 $\times$ g for 40 min at 4°C. The protein content was assayed by a Coomassie Brilliant Blue Protein Assay Reagent (Bio-Rad Laboratories, Richmond, CA). Total protein of 50 μg was loaded onto 12.5% polyacrylamide gel with 3.0% stacking gel for electrophoresis. Proteins were transferred to nitrocellulose membrane (Protran, Schleicher & Schuell, Keene, NH) at 4°C overnight. The blots were rinsed in Tris-buffered saline (TBS, pH 7.5) containing 0.1% Tween 20 (TBST) and were then blocked in TBST containing 5% nonfat milk powder and 2% bovine serum albumin. These blots were then incubated with the primary antibody for 1 h. The signals were visualized by species-specific horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and a chemiluminescent substrate (Amersham Biosciences, Arlington Heights, IL).

For immunohistochemistry, the primary antibodies used were the polyclonal anti-p-GSK-3β-serine-9 and the monoclonal anti-Bcl-2. Perfusion-fixed serial cross sections from the midbrain were preblocked with 2% normal serum with 0.1% Triton X-100 (Sigma) and then incubated at 4°C for 48 h with either anti-p-GSK-3β Ser9 (1:100) or anti-Bcl-2 (1:200) diluted in Tris-buffered saline (TBS, pH 7.4) containing 2% normal serum with 0.1% Triton X-100. The signal of the primary antibody was detected by TRITC-conjugated goat anti-rabbit-IgG (1:200 diluted in TBS) at room temperature for 1 h. After rinsing in TBS, sections were coverslipped in fluorescent mounting medium (Dako, Carpenteria, CA). Control sections that did not receive the primary antibody were used to distinguish between specific and nonspecific staining.

Image Analysis, Cell Quantification, and Statistics

All images were captured using a SPOT color charge-coupled device camera (Diagnostic Instruments, Durham, NC) attached to a Leica fluorescent microscope. For the quantification of regenerated RST neurons, the number of FG-labeled RST neurons was counted in every other section throughout the rostrocaudal extent of the red nucleus (Kobayashi et al., 1997; Liu et al., 1999). Only the neurons with visible nuclei were counted, to avoid overestimation of the number of FG-labeled RST neurons. Counting was performed blind to the treatment of the animals. The number of FG-labeled RST neurons on the lesion side was expressed as a percentage of the total number of FG-labeled neurons on the contralateral intact side. For the survival of RST neurons after treatments, sections were counterstained with neutral red. Only the cells that were large in size and that possessed a stellate shape were counted as surviving neurons. Data were compared

among groups using one-way ANOVA followed by a Tukey-Kramer multiple comparisons test.

Behavioral Analysis

The use of forelimbs during spontaneous vertical exploration was examined 1 month after different treatments as described (Liu et al., 1999), with minor modifications. Rats were placed for 5 min in a clear Plexiglas cylinder (15 cm in diameter and 30 cm high) that encourages use of the forelimbs for vertical exploration. The following behaviors were scored: independent use of the left (unimpaired) or right (impaired) forelimbs for contacting the wall of the cylinder, and simultaneous use of both forelimbs to contact the wall of the cylinder. The assessments were made by two researchers blinded to the treatments. The vertical exploration behavior was expressed in terms of percentage use of unimpaired forelimb relative to the total number of impaired, unimpaired, and both limb use; percentage use of impaired forelimb relative to the total number of impaired, unimpaired, and both limb use; and percentage use of both forelimbs relative to the total number of impaired, unimpaired, and both limb use. The differences between groups were tested by one-way ANOVA followed by Bonferroni post hoc analysis.

RESULTS

Regeneration of RST after ChABC Treatment

One month after spinal cord hemisection, the regeneration of RST axons was studied using the FG retrograde tracing technique. On the lesioned side of red nucleus, almost no FG-labeled RST neurons were found in the sham control animals ($2.3 \pm 0.9\%$) 1 month after the operation (Figs. 1 and 2). The few scattered FG-labeled RST neurons on the lesion side represented those that had ipsilateral axonal projections without being axotomized. After local application of 2.5 units/mL ChABC on top of the spinal cord hemisection site, there were $22.1 \pm 4.6\%$ of the RST neurons on the lesion side labeled by FG. We did not found any observable differences on the number of large, stellate-shaped RST neurons on the injured side between the PBS- and ChABC-treated groups (data not shown). This result indicates that enzymatic degradation of CSPGs can promote the axonal regeneration of RST neurons beyond the lesion site after spinal cord injury.

Effect of LiCl and ChABC on RST Regeneration

After hemisection of the spinal cord, daily intraperitoneal injection of 85 μg/kg LiCl for 1 month did not alter the percentage of FG-labeled RST neurons as compared with the sham control ($p > 0.05$) (Figs. 1 and 2).

However, combined treatment of application of ChABC at the lesion site together with daily injection of LiCl significantly increased the percentage of FG-labeled RST neurons to $41.5 \pm 6.0\%$ ($p < 0.001$ compared with all the other treatment groups). No observable differences were found in the number of large, stellate-shaped RST neurons on the injured side between different treatment groups (data not shown).

To examine whether there were FG-labeled axons in the spinal cord lesion scar, horizontal sections from C7 were cut 1 month after spinal cord hemisection. In all groups, the spinal cord lesion cavity was filled with scar tissue without cystic spaces. There were no FG-labeled axons within the lesion site in either the sham or the LiCl-treated animals (Fig. 3A,B). In animals treated with ChABC alone or with LiCl together with ChABC, FG-labeled axons approximately 100-200 μm in length were found in the lesion scar (Fig. 3C,D). The morphology of the axons within the scar was similar in these two treatment groups.

Phosphorylation of GSK-3β after LiCl Treatment

Following spinal cord injury, Western blot analysis on lysates of the lesioned red nucleus revealed that the level of serine-9-phosphorylated GSK-3β increased 1 and 5 days after LiCl treatment (Fig. 4). The immunoreactivity of p-GSK-3β-serine-9 was barely detectable in the sham control. The signal of the phosphorylation-independent antibody, anti-GSK-3β, remained stable in all groups. These results indicate that LiCl leads to the inactivation of GSK-3β via phosphorylation.

To localize the expression of inactive GSK-3β, tissue sections from the midbrain were immunostained with the p-GSK-3β-serine-9 antibody. Inconsistent with the Western blotting result, intense immunoreactivity of the p-GSK-3β-serine-9 was found on the injured side of the red nucleus 1 and 5 days after LiCl treatment (Fig. 5). The p-GSK-3β-serine-9 immunoreactivity was mainly observed in the cytoplasm of the RST neurons. There were no p-GSK-3β-serine-9 immunopositive neurons found in the intact contralateral side of the red nucleus in LiCl-treated animals, nor in the injured side of the red nucleus of the sham control animals. These results indicate that daily intraperitoneal injection of LiCl inactivates GSK-3β by serine-9 phosphorylation in the lesioned RST neurons after spinal cord injury.

Expression of Bcl-2 after LiCl Treatment

It has been reported that Bcl-2 protects neurons from apoptotic cell death as well as enhances axonal regeneration after CNS injury (Chen et al., 1997; Yukawa et al., 2002; Huang et al., 2003). Here we have sought to ex-

lesion side   control side
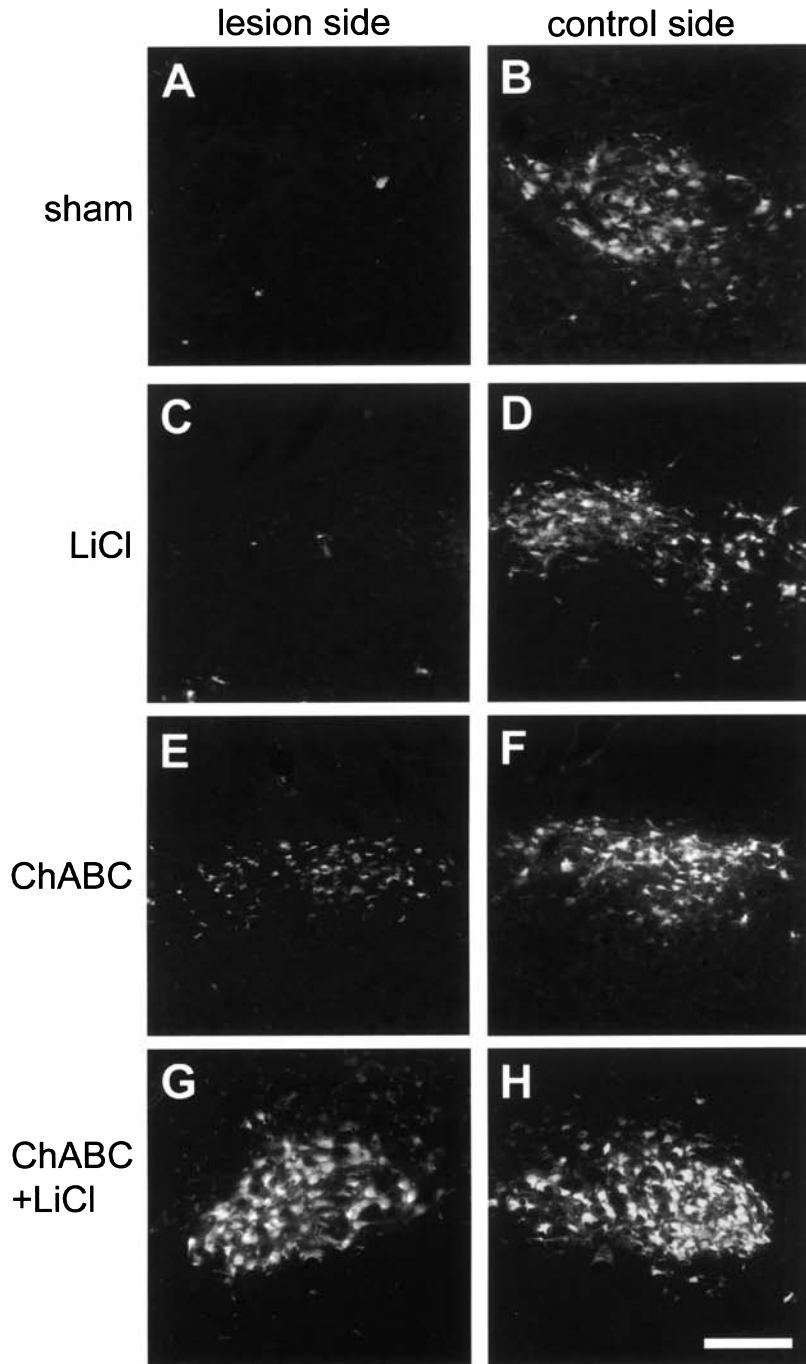
FIG. 1. Photomicrographs of midbrain cross sections showing Fluoro-Gold (FG)–labeled rubrospinal tract (RST) neurons. The animals received a right hemisection of the spinal cord at C7. The postoperative survival period was 1 month. The RST neurons in both the control side (right) and the lesion side (left) were retrogradely labeled by injection of FG at T2 spinal cord. In the sham group (**A,B**) and the LiCl-treated group (**C,D**), there are virtually no RST neurons labeled with FG on the lesion side. (**E,F**) In chondroitinase ABC-treated animals, few FG-labeled RST neurons are found on the lesion side. (**G,H**) In animals treated with LiCl together with chondroitinase ABC, numerous RST neurons are labeled with FG on the left lesion side. Bar = 200 μm.
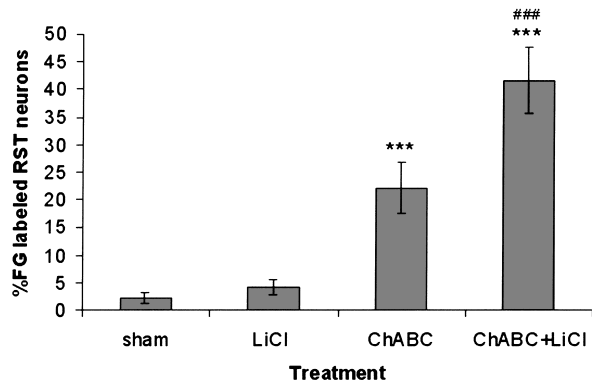
FIG. 2. Percentages of rubrospinal tract (RST) neurons labeled with Fluoro-Gold (FG) after different treatments. The animals received a right hemisection of the spinal cord at C7. The postoperative survival period was 1 month. In the sham and LiCl-treated animals, virtually no RST neurons are labeled by FG. Local delivery of chondroitinase ABC (ChABC) increases the percentage of FG-labeled RST neurons to 22% (sham compared with ChABC ***$p < 0.001$). In animals treated with LiCl together with ChABC, there are 42% of the RST neurons labeled with FG (sham compared with LiCl+ChABC ***$p < 0.001$; ChABC compared with LiCl+ChABC ###$p < 0.001$). Data are given as percentages of FG-labeled RST neurons on the lesion side against the number of FG-labeled RST neurons on the contralateral intact side, mean ± SEM.

amine whether LiCl treatment enhances the expression of Bcl-2 in RST neurons after injury of the spinal cord. Immunoblot analysis on lysates of the injured red nucleus revealed that the expression level of Bcl-2 increased 5 days after LiCl treatment (Fig. 4). The expression of Bcl-2 was undetectable in the normal and sham control.
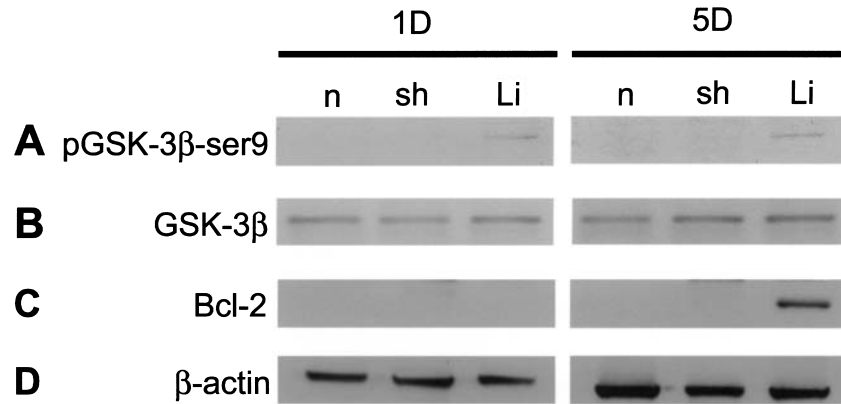
After spinal cord hemisection, cryosections from midbrains harvested at different time points were immunostained with an antibody against Bcl-2. In the absence of LiCl, no Bcl-2-expressing neurons were found on both the contralateral control and injured side of the red nucleus at all the time points examined. Treatment with LiCl resulted in an intense Bcl-2 immunoreactivity observed on the injured side of the red nucleus—but not on the intact control side—5 days after the operation (Fig. 6). These results indicate that Bcl-2 is involved in the additive regeneration-promoting effect of LiCl on axotomized RST neurons after treatment with ChABC.

Behavioral Analysis

To examine the recovery of function, a Plexiglas cylinder was used to test forelimb use during spontaneous vertical exploration 1 month after different treatments. The use of forelimbs was expressed by the percentage of wall
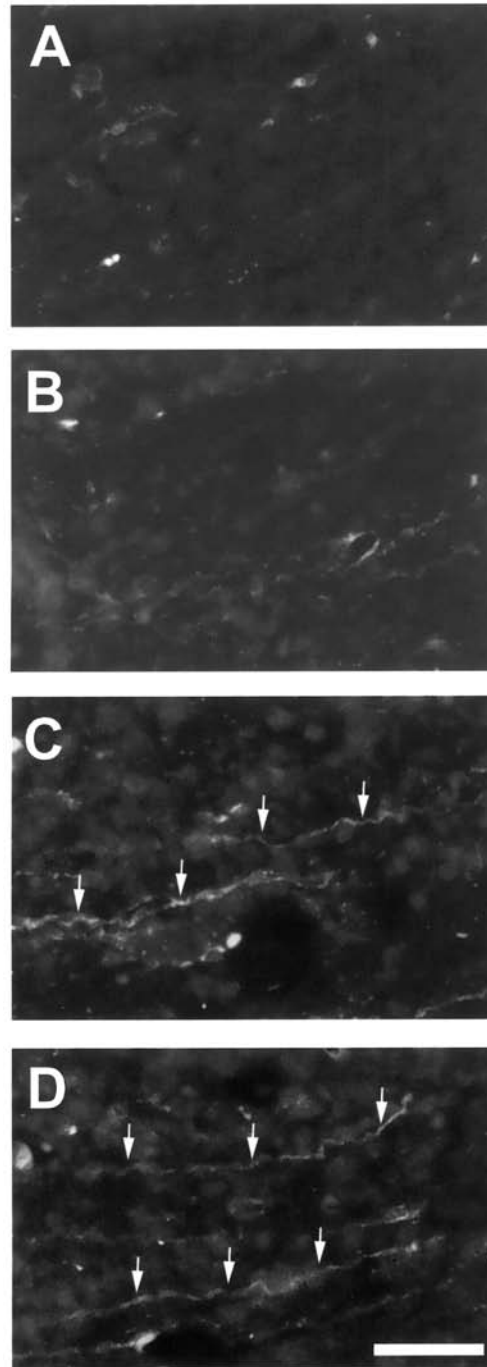

FIG. 3. Horizontal sections showing the Fluoro-Gold (FG)-labeled axons within the site of spinal cord hemisection. In the sham (**A**) or LiCl-treated animals (**B**), no FG-labeled axons are seen within the lesion scar. In the animals treated with chondroitinase ABC (**C**) or LiCl together with chondroitinase ABC (**D**), a few FG-labeled axons are observed in the hemisection site being approximately 100–200 μm long (arrows) in the focal plane. Bar = 50 μm.

FIG. 4. Phosphorylation of GSK-3β and upregulation of Bcl-2 after treatment with LiCl. Fifty micrograms of total proteins from the lesion side of the red nucleus were analyzed by Western blot at 1 and 5 days after spinal cord hemisection. (A) There was no p-GSK-3β-serine 9 immunoreactivity detected in normal (n) and sham (sh) animals. Treatment with LiCl increases the phosphorylation of GSK-3β at serine-9 by 1 day, persisting up to 5 days, after spinal cord hemisection. (B) The expression level of phosphorylation-independent GSK-3β was stable throughout the experiment. (C) No Bcl-2 immunoreactivity was detected in normal and sham animals. LiCl treatment increased the expression of Bcl-2 by 5 days after spinal cord hemisection. (D) β-actin staining was used as an internal control.
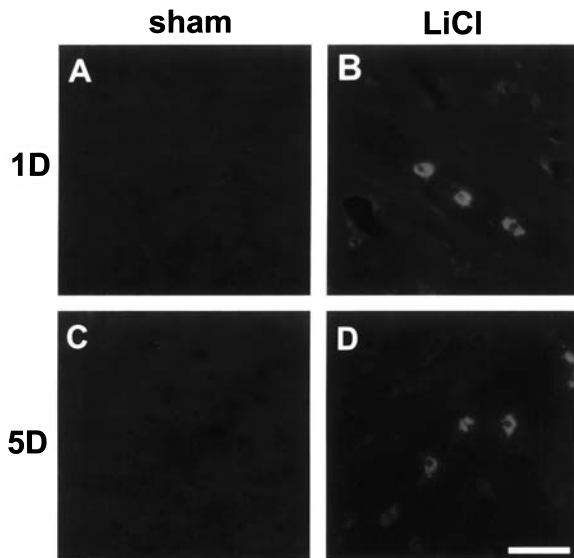

FIG. 5. Photomicrographs of midbrain cross sections showing the expression of the phosphorylated serine-9 of GSK-3β in rubrospinal tract neurons on the lesion side. After hemisection of the C7 spinal cord, the animals received daily intraperitoneal injections of LiCl. (A,C) In the sham group, no p-GSK-3β-serine-9 immunoreactive rubrospinal tract neurons are found by 1 and 5 days after the operation. (B,D) In LiCl-treated animals, neurons showing positive immunoreactivity for p-GSK-3β-serine-9 are found in the RST nucleus by 1 and 5 days. Bar = 50 μm.
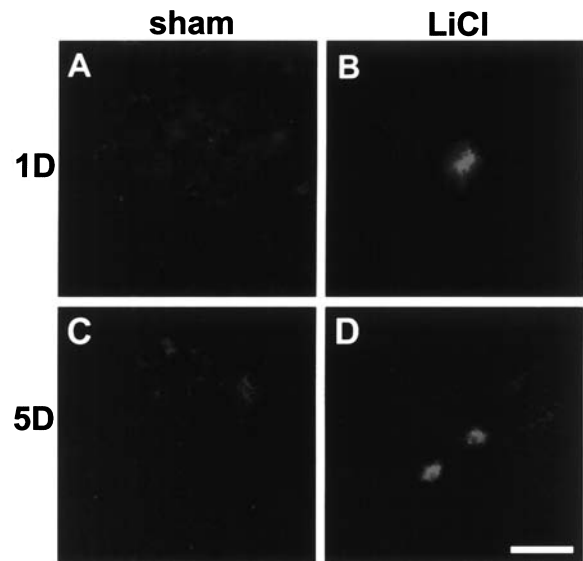
FIG. 6. Photomicrographs of midbrain cross sections showing the expression of the Bcl-2 in rubrospinal tract neurons on the lesion side. After hemisection of the C7 spinal cord, the animals received daily intraperitoneal injections of LiCl. (A,C) In the sham group, no Bcl-2 immunoreactive RST neurons are found by 1 and 5 days after the operation. (B,D) In LiCl-treated animals, neurons showing positive immunoreactivity for Bcl-2 are found in the RST nucleus by 1 and 5 days. Bar = 50 μm.
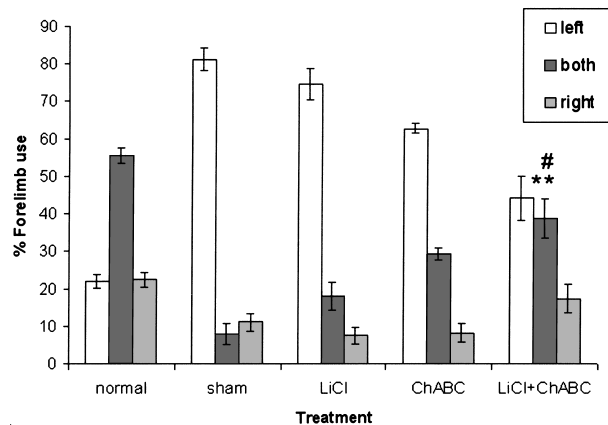
FIG. 7. Behavioral analysis of forelimb function in vertical exploration test. Normal animals use a single forelimb or both forelimbs together for vertical exploration. One month after spinal cord hemisection, animals in the sham and LiCl-treated groups rarely use their right (impaired) forelimb. Chondroitinase ABC (ChABC)-treated animals use both forelimbs more frequently for exploration. Animals treated with LiCl together with ChABC showed significant use of both forelimbs compared to the sham and single treated animals. Data are mean ± SEM. Sham compared with ChABC **$p < 0.01$. ChABC compared with LiCl+ChABC #$p < 0.05$.

the other treatment groups (17.3 ± 3.8%; sham compared with LiCl+ChABC, $p < 0.01$; LiCl compared with LiCl+ChABC, $p < 0.01$; ChABC compared with LiCl+ChABC, $p < 0.05$). These results demonstrate that treatment with ChABC, but not LiCl alone, partially promotes the recovery of function after spinal cord injury. Most interestingly, combined treatment of LiCl together with ChABC significantly increases the extent of recovery as compared with ChABC treatment alone.

DISCUSSION

Enzymatic degradation of CSPGs promotes the axonal regeneration of neurons across the lesion scar following CNS injuries. Lithium has been shown to protect neurons and enhance axonal regrowth potential via GSK-3β and Bcl-2 signaling pathways (Bournat et al., 2000; Huang et al., 2003). In this study, we provide *in vivo* evidence that application of ChABC at the lesion site promotes the axonal regeneration of RST neurons after injury of the spinal cord. Most important, combined treatment of LiCl together with ChABC further enhances the regeneration of RST neurons following this injury. This combined treatment can improve the forelimb function in a vertical exploration test. Western blot and immunohistochemical analyses reveal that LiCl treatment induces the expression of the p-GSK-3β-serine-9 as well as the upregulation of Bcl-2 in severed RST neurons. These results indicate that in axotomized RST neurons, lithium reinforces the regeneration-promoting effect of ChABC via a mechanism involving the inactivation of GSK-3β and upregulation of Bcl-2. Our study confirms previously published data indicating that enzymatic removal of CSPGs promotes axonal regrowth of injured neurons and that Bcl-2 is involved in the neuroprotective effect of lithium.

Previous strategies for promoting the axonal regeneration of injured RST have been focused on the use of neurotrophic factors. In a model of chronic spinal cord injury, continuous administration of BDNF at the midbrain reverses massive atrophy and promotes regeneration of rubrospinal axons into a peripheral nerve implanted at the lesion site (Kwon et al., 2002). Following hemisection of the spinal cord, transplants of fibroblasts genetically modified to express BDNF prevent atrophy, enhance axonal regeneration of rubrospinal neurons, and result in partial recovery of forelimb function (Liu et al., 1999, 2002; Jin et al., 2002). It has been suggested that neurotrophic factors promote the regeneration of axotomized rubrospinal neurons by stimulating the expression of GAP-43 and Tα1-tubulin (Kobayashi et al., 1997; Fernandes et al., 1999).

exploration that was initiated by right (impaired) or left (normal) forelimb alone or by both forelimbs together (Fig. 7). Normal rats explored the cylinder wall using either a single forelimb (about 45%) or both forelimbs together (about 55%). Animals receiving hemisection at C7 rarely used the forelimb ipsilateral to the injury (sham group, 7.7 ± 3.0%), leading to asymmetry of forelimb use. Animals treated with LiCl used both forelimbs slightly more often, but the difference was not significant from the sham group (18.1 ± 3.7%; sham compared with LiCl, $p > 0.05$). In the ChABC-treated group, rats used both forelimbs more frequently than did the sham group (29.1 ± 1.9%; sham compared with ChABC, $p < 0.01$), resulting in more symmetrical forelimb use. However, combined treatment of LiCl together with ChABC significantly promoted the use of both forelimbs as compared with all other treatment groups (38.7 ± 5.2%; sham compared with LiCl+ChABC, $p < 0.01$; LiCl compared with LiCl+ChABC, $p < 0.01$; ChABC compared with LiCl+ChABC, $p < 0.05$). Rats receiving the combined treatment used both forelimbs together (38.7 ± 5.2%) as frequently as they used their unimpaired forelimb alone (44.1 ± 5.9%; both forelimbs compared with unaffected limb, $p > 0.05$). In addition, the animals receiving the combined treatment used their impaired forelimb alone to explore the wall more frequently compared with all

We have previously shown that enzymatic degradation of CSPGs at the injury scar can enhance the regeneration of the ascending Clarke's neurons after spinal cord hemisection (Yick et al., 2000). Removing the glycosaminoglycan chains of CSPGs also promotes the regeneration of severed axons in nigrostriatal and corticospinal tracts (Moon et al., 2001; Bradbury et al., 2002). In this study, we demonstrate that CSPGs at the injury scar are important inhibitory factors for axon regeneration of severed rubrospinal neurons. Taken together, these *in vivo* studies provide evidence that CSPGs are critical inhibitory molecules against axonal regeneration in the injured CNS. We are investigating whether multiple treatment with ChABC would be more effective in promoting axonal regeneration after spinal cord injury.

Although its precise mechanism of action remains unknown, lithium is a commonly used drug for the treatment of mania and depression (Post et al., 1992). An *in vitro* study has demonstrated that lithium supports the cell survival and axonal regeneration of retinal ganglion neurons (Huang et al., 2003). Long-term exposure to lithium significantly protects cultured cerebellar, cerebral cortical, and hippocampal neurons against glutamate-induced apoptotic neuronal death (Nonaka et al., 1998). In a rat model of Huntington's disease, subcutaneous administration of lithium reduces the size of quinolinic acid-induced lesion and protects striatal neurons from apoptotic death (Wei et al., 2001). Long-term lithium treatment also protects cerebellar granule cell neurons from β-amyloid peptide-induced death (Wei et al., 2000). Lithium can prevent potassium efflux-induced neuronal cell apoptosis, with the induction of caspase-3 expression (Li and El-Mallahk, 2000). Our results show that treatment of LiCl alone fails to promote the regeneration of severed RST axons. Interestingly, LiCl seems to potentiate the neuroregenerative function of ChABC, resulting in an additive effect that promotes axonal regeneration of injured RST neurons. One of the possibilities is that LiCl protects the injured RST neurons from death so that more axons can regenerate across the growth-permissive lesion scar after ChABC treatment. It is also possible that lithium binds to CSPGs and hyaluronic acid and changes the water-binding capacity of GAGs, resulting in a synergistic effect of LiCl and ChABC on axonal regeneration.

We have found that the number of surviving neurons decreases in the injured red nucleus both in the sham controls and following lithium treatment (data not shown). This result is different from a previous study, which showed that axotomized RST neurons survive for 1 year with regrowth by applying brain-derived neurotrophic factor (Kwon et al., 2002). It is possible that the dose of LiCl we used can increase the expression of Bcl-2 but is not optimal for promoting the survival of injured RST neurons. We cannot rule out the possibility, too, that the number of surviving neurons is underestimated because only large and morphologically normal neurons are counted, and not the cells with massive atrophy. Further studies are required to determine whether LiCl can promote the survival of axotomized RST neurons after spinal cord injury.

Here, we demonstrate that the number of FG-labeled RST neurons is significantly increased after the combined treatment of LiCl together with ChABC. The number of RST neurons that are labeled for p-GSK-3β-serine 9 or for Bcl-2 is limited. This result indicates that in addition to inactivation of GSK-3β and upregulation of Bcl-2, other mechanisms may be involved in the synergistic effect of LiCl and ChABC.

There is growing evidence that GSK-3β signaling is involved in neurodegeneration by modulating neuronal apoptosis. GSK-3β is activated by phosphorylation at Tyr216 kinase domain and is inactivated through phosphorylation of serine-9 (Kaylor and Orr, 2002). The executive phase of apoptosis is associated with a selective phosphorylation of serine-9 together with dephosphorylation of tyrosine-216 in GSK-3β (Bhat et al., 2000; Elyaman et al., 2002). Lithium is a selective inhibitor of GSK-3β, which may in turn regulate transcriptional factors such as nuclear factor kappa-B (Bournat et al., 2000) and cyclic AMP response element binding protein (Grimes and Jope, 2001). The activity of GSK-3β is negatively regulated by signal transduction cascades that protect neurons against apoptosis, including the PI-3 kinase-Akt pathways (Hetman et al., 2000). By using a neuronal apoptosis model in neonatal rats, it has been demonstrated that LiCl can prevent GSK-3β–mediated Tau phosphorylation in response to a NMDA receptor antagonist (Elyaman et al., 2002). Our Western blot and immunohistochemical analyses provide *in vivo* evidence that after spinal cord hemisection, LiCl treatment inhibits GSK-3β by phosphorylation at serine-9 in the red nucleus.

In addition to GSK-3β inhibition, it has been recently demonstrated that lithium regulates the expression of antiapoptotic proteins like Bcl-2. Bcl-2 is an antiapoptotic gene that inhibits Bax-mediated release of cytochrome *c* or apoptosis-inducing factors from mitochondria, thereby preventing caspase activation of cell death. In a culture model, lithium increases the expression of Bcl-2 mRNA, as well as promotes both the survival and axonal regeneration of retinal ganglion cells (Huang et al., 2003). In a rat model of Huntington's disease, lithium treatment increases Bcl-2 protein levels and suppresses excitotoxin-induced striatal lesions (Wei et al., 2001). Under glutamate excitotoxicity, long-term treatment of cultured cerebellar granule cells with lithium upregulates the

mRNA and protein levels of Bcl-2 remarkably (Chen and Chuang, 1999). In this study, immunoblotting and immunohistochemical analyses reveal that Bcl-2 is upregulated in the injured red nucleus after LiCl treatment. It is possible that LiCl reinforces the neuroregenerative-promoting effect of ChABC by mediating the upregulation of Bcl-2 expression in injured RST neurons. However, further studies are required to examine the temporal relationship between Bcl-2 upregulation and RST axonal regeneration after spinal cord injury.

In our study, administration of LiCl alone did not promote RST regeneration, but it enhanced the regeneration-promoting function of ChABC on injured RST. It has been demonstrated that the role of lithium on enhancing neuronal survival may involve inhibition of NMDA receptors, downregulation of proapoptotic genes such as *p53* and *Bax*, increased expression of the neuroprotective protein Bcl-2, and activation of the cell survival kinase Akt. However, the role of LiCl in the promotion of axonal regeneration is unclear. Bcl-2 plays a key role in promoting regeneration of injured axons in the CNS (Chen et al., 1997). Lithium enhances the axonal regeneration of retinal ganglion cells through a Bcl-2–dependent mechanism (Huang et al., 2003). Further studies are required to understand the precise mechanism of lithium-promoted axonal regeneration in the injured CNS.

On the basis of observations of vertical exploring activity with forelimbs in a cylinder, our behavioral result shows that treatment with ChABC reduces the asymmetry of forelimb use after injury of the spinal cord. Interestingly, combined treatment of LiCl together with ChABC further relieves the asymmetry of forelimb use compared with ChABC treatment alone. Treatment with LiCl alone does not improve forelimb function. These results are consistent with our histological data, showing that the number of regenerating RST neurons is increased in the animals with the combined treatment compared with ChABC application alone.

This study provides *in vivo* evidence that application of ChABC promotes the axonal regeneration of RST neurons after spinal cord injury. Most interestingly, combined treatment of ChABC together with LiCl results in an additive effect on promoting RST axonal regeneration. The combined treatment enhances the recovery of forelimb function compared with a single application of ChABC alone. Treatment with LiCl inhibits the activity of GSK-3β and upregulates the expression of Bcl-2 in axotomized RST neurons. These results indicate that LiCl reinforces the neuroregeneration-promoting function of ChABC via inhibiting GSK-3β and upregulating Bcl-2. To improve the regenerative response after CNS injury, it will be important to develop intervention that alters the CSPG components of the glial scar as well as stimulates the intrinsic regrowth capability of the injured neurons. Combined use of lithium together with ChABC may be a novel strategy for the treatment of spinal cord injury.

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