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Promoting Optic Nerve Regeneration in Adult Mice with Pharmaceutical Approach

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Abstract

Our previous research has suggested that lack of Bcl-2-supported axonal growth mechanisms and the presence of glial scarring following injury are major impediments of optic nerve regeneration in postnatal mice. Mice overexpressing Bcl-2 and simultaneously carrying impairment in glial scar formation supported robust optic nerve regeneration in the postnatal stage. To develop a therapeutic strategy for optic nerve damage, the combined effects of chemicals that induce Bcl-2 expression and selectively eliminate mature astrocytes—scar forming cells—were examined in mice. Mood-stabilizer, lithium, has been shown to induce Bcl-2 expression and stimulate axonal outgrowth in retinal ganglion cells in culture and in vivo. Moreover, astrotoxin (alpha-amino adipate), a glutamate analogue, selectively kills astrocytes while has minimal effects on surrounding neurons. In the present study, we sought to determine whether concurrent applications of lithium and astrotoxin were sufficient to induce optic nerve regeneration in mice. Induction of Bcl-2 expression was detected in the ganglion cell layer (GCL) of mice that received a lithium diet in compared with control-treated group. Moreover, efficient elimination of astrocytes and glial scarring was observed in the optic nerve of mice treated with astrotoxin. Simultaneous application of lithium and astrotoxin, but not any of the drugs alone, induced robust optic nerve regeneration in adult mice. These findings further support that a combinatorial approach of concurrent activation of Bcl-2-supported growth mechanism and suppression of glial scarring is required for successful regeneration of the severed optic nerve in adult mice. They suggest a potential therapeutic strategy for treating optic nerve and CNS damage.

Keywords

Axon regeneration; Lithium; Astrocyte; Bcl-2; Optic nerve; L- α -amino adipate

Introduction

Unlike the peripheral nervous system, nerve pathways in the CNS of adult mammals are unable to regenerate once injured. Presence of a hostile glial environment and lack of intrinsic growth ability by CNS axons are thought to contribute primarily to this growth failure. Accumulating evidence points to a critical role of reactive astrocytes and their ability to form glial scars in the blockade of CNS axon regeneration [1,2]. The failure of neurite outgrowth from transplanted neurons in vivo is associated directly with the presence of reactive glial cells [3, 4]. However, neutralizing the inhibitory effects presented by the mature CNS environment usually allowed only limited amount of axonal regrowth in the adult [5,6]. Mature CNS

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neurons, including retinal ganglion cells (RGCs), also lack the intrinsic mechanisms that can support axonal re-elongation after injury. Thus, for regeneration to be successful, a combinatorial approach that enables simultaneous suppression of glial scarring and reactivation of the intrinsic growth mechanisms may be required.

Rodent RGCs lose their intrinsic capacity to extend axons before birth [7,8]. Previously, we have shown that overexpression of Bcl-2 in postnatal RGCs is sufficient to support the intrinsic growth mechanisms of axons and results in robust optic nerve re-elongation after injury in mice in the neonatal stage, when the CNS environment remains permissive for such growth [9]. Not surprising, after the CNS environment becomes mature and develops inhibitory mechanisms that further block axon regeneration, the regeneration failed again even in the presence of Bcl-2 [10–12]. Our previous studies have suggested that RGCs overexpressing Bcl-2 retain the intrinsic capacity to grow axon throughout life; however, the mature CNS environment develops growth inhibitory mechanisms around postnatal day 4 in mice that further blocks optic nerve regeneration in older Bcl-2 transgenic mice [9,13]. More-over, we have demonstrated that maturation of astrocytes and with it, their ability to form glial scars after injury, contributes critically to the growth inhibition presented by the CNS environment. Therefore, for regeneration to occur in adult mice, it is essential to use combined approaches that activate Bcl-2-supported growth mechanisms and suppress growth inhibitory mechanisms in the CNS environment.

In the present study, we set out to test the effects of a pharmacological approach that enables induction of Bcl-2 expression and suppression of glial scarring in adult mice. While the genetic engineered mouse models provide an excellent system that serves as a proof of principle, ideally, therapy for optic nerve injury should be accomplished with application of small molecular drugs. Previously, we have shown that a long-standing mood-stabilizer, lithium, induces Bcl-2 expression in the mouse retina and promotes retinal axon regeneration in vitro via a Bcl-2-dependent mechanism [14]. Lithium has also been reported to induce Bcl-2 expression in other CNS neurons [15,16], suggesting that lithium may be used widely in the CNS as a potential strategy for inducing Bcl-2 expression. Astrotoxin—L- α -amino adipate, a glutamate analogue—is then known to selectively enter astrocytes and induce cytotoxicity when it poses minimal effects on surrounding neurons and myelin [17–19]. These data promoted us to ask whether the combined treatments of lithium, that induces Bcl-2, and astrotoxin, which eliminates mature astrocytes and suppresses glial scarring, supported optic nerve/CNS regeneration in adult mice. The results of this study may provide a therapeutic strategy for treating optic nerve and CNS damage in human.

Experimental Procedure

Surgical Procedure

All experimental procedures and use of animals were approved by Animal Care and Use Committee in the Schepens Eye Research Institute and adhered to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Adult C57BL/6J mice were anesthetized with a mixture of ketamine (12.5 mg/ml, Sigma) and xylazine (2.5 mg/ml, Sigma) (0.05 ml/10 g). Optic nerve was exposed intraorbitally and crushed for 3 s using fine forceps [20–22]. Immediately after nerve crush, a piece of gelfoam soaked with L- α -amino adipate (10 mg/ml, Sigma) was placed against the crushed site. Gelfoam soaked with phosphate buffered saline (PBS) was used as control. To ensure continued delivery of astrotoxin, the gelfoam was replaced every 3 days post-injury (DPI). Following recovery from the optic nerve surgery, mice were divided into two groups: one group received a diet supplemented with lithium (3.5 mg lithium carbonate/kg, Bio-Serv), and the other group received a control diet. Mice were sacrificed on 8 DPI, and the mouse serum was collected to determine for serum lithium concentration (Medtox Laboratories Inc).

Retrograde Labeling of RGCs

To study the neuroprotective effect of lithium, RGCs were pre-labeled by placing bilaterally a gelfoam soaked with 6% FluoroGold on the superior colliculus (SC) for 1 week before optic nerve crush. After sacrificing, whole-mount retinas were prepared. At least 12 non-overlapping areas of the retina were selected randomly and photographed. Surviving RGCs that were labeled with FluoroGold were counted, and the density of survival RGCs was determined.

Retinal Histology and Immunohistochemistry

The eyeball and optic nerve were dissected out, fixed in 4% paraformaldehyde for 1 h, cryoprotected, embedded in O.T.C (Tissue-Tek), and cryosectioned at 12 μ m. Tissue sections were then subjected to cresyl violet staining or immunohistochemistry. For immunofluorescence labeling, tissue sections were reacted with Cy-3 conjugated mouse anti-GFAP (1:1000, Sigma) or rabbit anti-GAP-43 (1:200, Chemicon). Signal of GAP-43 was then visualized by reacting with FITC-conjugated goat anti-rabbit IgG (1:250, Vector Lab.). To detect the expression of Bcl-2 protein, mouse eyeballs were dissected and embedded without fixation. Before immunostaining, retinal sections were fixed with acetone that was stored at -20°C for 15 min and incubated with Bcl-2 antibody (1:50, Transduction Lab.) followed by reaction with biotinylated anti-mouse IgG (1:100, Vector Lab.) and DTAF-conjugated Streptavidin (1:100, Jackson Lab.). The sections were then counter-stained with DAPI (1:150, Sigma) to reveal cell nuclei before they were mounted with Vectashield (Vector Lab.) and examined under epifluorescence microscope.

Quantitation of Axon Regeneration

Following staining with anti-GAP-43 antibody, the longest distance of axons extending past the crush site into the optic nerve was visualized and measured in optic nerve sections under epifluorescence microscope (Nikon TE300). At least four optic nerve sections were sampled from each nerve, and the measured lengths of regenerating axons were averaged. Statistical significance was determined by one-way ANOVA and Tukey–Kramer multiple comparison tests.

Semi-quantitative RT-PCR

The procedure of RT-PCR was described previously [14]. In brief, total retinal RNAs were extracted from adult wild-type mice that consumed a regular diet or a diet containing lithium. Retinal RNAs isolated from Bcl-2 transgenic mice driven under the promoter of neural specific enolase promoter [23] were used as positive control. To clean off potentially contaminating genomic DNA, RNAs were treated with DNA-free (Ambion) before they were subjected to reverse transcription. One microgram total RNA was subjected to reverse transcription in a 20 μ l reaction mixture containing 4 μ l RT buffer, 1 μ g oligo-dT primer, 0.5 mM of each of the dNTPs, 10 mM dithiothreitol, and 5 U RNase inhibitor (Invitrogen). The reaction was performed at 42°C for 45 min with 1 U of reverse transcriptase (Superscript II; Invitrogen) and terminated by incubating at 75°C for 15 min. APCR mixture with specific primers was then heated at 94°C for 5 min. The PCR was carried out with denaturing at 94°C for 45 s, annealing at 55°C for 30 s and extension at 72°C for 45 s for 20–30 cycles (20 cycles for amplifying a house-keeping gene glyceraldehydes-3-phosphate dehydrogenase (G3PDH) or 30 cycles for amplifying Bcl-2 signal). The reaction was finally stopped at 72°C for 7 min. The relative amounts of cDNA were normalized by comparing the level of PCR amplification for an internal control gene G3PDH (forward primer: agaacatcatccctgcaccc; reverse primer: agccgtattcattgtcatacc). Thus, each PCR reaction contained equivalent amounts of cDNAs. The relative amount of target gene, mouse Bcl-2 (forward primer: agcattgcggaggaagtaga; reverse primer: tagccctctgtgacagctt), was then amplified and compared. PCR products were resolved

by electrophoresis using 2% agarose gels and photographed with a Kodak DC120 digital camera (Eastman Kodak; Rochester, NY).

Results

Induction of Bcl-2 Expression in the Mouse Retina by Lithium

Previously, we have reported that lithium induces Bcl-2 expression in cultured RGCs. To determine whether lithium induces Bcl-2 expression in adult retinal neurons *in vivo*, we fed mice with food chow that contained lithium. The serum concentration of lithium measured from mice consumed a diet containing lithium ($n = 9$) was significantly higher (0.80 ± 0.15 mEq/l) than those consumed a control diet ($n = 3$; <0.1 mEq/l) (Fig. 1A). This concentration is within the well-established safety and therapeutic profile of lithium [24]. Moreover, the results of semi-quantitative RT-PCR also indicated that mice receiving lithium-containing diet exhibited a strong induction of Bcl-2 mRNA in their retinas in compared with mice received a regular diet, in which their retinas revealed an undetectable level of Bcl-2 mRNA (Fig. 1B). To determine the type of cells that upregulated Bcl-2 in the mouse retina, we performed immunohistochemistry staining for Bcl-2 protein. Expression of Bcl-2 protein was detected in the ganglion cell layer (GCL) of mice consumed a lithium-containing diet but not in the retinas of mice consumed a control diet (Fig. 2). Therefore, lithium can induce Bcl-2 expression in the GCL of adult mouse retina *in vivo*.

Robust Regeneration of the Optic Nerve Induced by Lithium and Astrotoxin

As it has been reported, optic nerve regenerative failure reflects both the lack of Bcl-2-supported intrinsic growth mechanism in RGCs and the presence of glial scarring that presents inhibitory mechanisms for such growth [13]. We proposed that successful regeneration of severed optic nerve in adult could be achieved pharmacologically via concurrent induction of Bcl-2 expression and elimination of mature astrocytes—scar forming cells. To test this, we evaluated the effect of lithium, when were used alone or simultaneously with astrotoxin, on optic nerve regeneration in adult mice.

Immediately after optic nerve crush, a piece of gelform soaked in astrotoxin solution or PBS was placed against the crush site. Both PBS and astrotoxin-treated mice were then divided into two groups: one group received a diet containing lithium, and the other received a control diet. Mice were sacrificed on day 8 after nerve crush to assess for axon regeneration. The crush site of the optic nerve was identified by a dense area of degenerating cells and a narrow outline of the optic nerve following staining of nerve sections with cresyl violet (Fig. 3A, E, I). Regenerating axons were revealed by immunostaining with primary antibody against GAP-43.

As expected, no regenerative response was observed in control mice that consumed a control diet and received PBS treatment ($n = 6$). Most RGC axons degenerated eventually. Similarly, in mice receiving lithium-containing diet alone, without the application of astrotoxin or with administration of gelfoams soaked in PBS ($n = 11$, Fig. 3A–D), no sign of regeneration was observed. In both control and lithium-treated groups, many severed retinal axons expressing GAP-43 were seen to terminate anterior to the crushed site of the severed optic nerve (Fig. 3B), suggesting an inhibitory environment for axon elongation. Expression of GFAP, a marker of astrocytes, was detected along the entire nerve (Fig. 3C). In the other group, mice receiving astrotoxin alone but consumed a control diet ($n = 3$, Fig. 3E–H), again no signs of axon regeneration were detected (Fig. 4F) although reactive astrocytes (GFAP-negative) was completely deleted posterior to the crushed site (Fig. 3G). Only in mice received both lithium and astrotoxin simultaneously ($n = 6$, Fig. 3I–L), severed retinal axons re-grew robustly into the GFAP-negative zone of optic nerve (Fig. 3L). A significant number of growth cone-like structures of regenerating axons were observed posterior to the crushed site (Fig. 3M, N).

To quantify axon regeneration and measure the length of regenerating axons immunostaining of anti-GAP-43 anti-body was used to label regenerating axons. Although mice receiving lithium or astrotoxin treatment alone extended longer axons than the control-treated group, no significant differences in distance of axon regeneration were detected among these groups ($P > 0.05$) (Fig. 4). In contrast, mice simultaneously receiving lithium and astrotoxin extended numerous axons posterior to the crush site. Such regenerated axons were significantly longer than that of the other groups (Fig. 4, $P < 0.001$). The data indicate that, for optic nerve regeneration to occur in the adult, a combined strategy enabling simultaneous expression of Bcl-2 and removal of glial scars is required.

Effect of Lithium on RGC Survival

To determine whether lithium promotes optic nerve regeneration through enhancing RGC survival, we assessed quantitatively RGC survival by placing a retrograde tracer FG in the superior colliculus to label RGCs. Eight days after optic nerve crush, surviving RGCs were counted in retinal whole mount preparation. In the retinas of control mice, the density of FG-labeled RGCs ($2,170 \pm 140/\text{mm}^2$) was similar to that in mice consumed a lithium diet ($2,134 \pm 128/\text{mm}^2$). Optic nerve crush resulted in a decrease in the number of survival RGCs, but no significant difference in the number of surviving RGCs between the lithium-treated ($404 \pm 64/\text{mm}^2$) and control group ($327 \pm 66/\text{mm}^2$) was detected (Fig. 5, $P > 0.05$). The result suggests that the effect of lithium to promote optic nerve regeneration is not a result of increased RGC survival in these mice.

Discussion

We here showed that supplement of lithium induces Bcl-2 expression in RGCs of adult mouse retina *in vivo*. Moreover, application of astrotoxin successfully eliminates astrocytes around the injury site of the optic nerve and provides a CNS environment that is permissive for axon regeneration. Mice received concurrent treatment of lithium and astrotoxin revealed robust optic nerve regeneration after injury, while treatment of lithium or astrotoxin alone is not sufficient to promote optic nerve regeneration.

Our data indicate that administration of astrotoxin in the CNS *in vivo* selectively ablates astrocytes without adverse effect on other cell types. This observation is consistent with that has been reported by others showing target binding and toxicity of astrotoxin to astrocytes but not other cell types, including neurons, oligodendrocytes, microglia and endothelial cells [17,19]. Astrocytic scar [25] has long been suspected to present growth inhibitors for CNS axon regeneration. In particular, chondroitin sulfate proteoglycans (CSPG) produced by reactive astrocytes, primary scar forming cells, has been shown to block CNS axon regeneration [25,26]. Removal of glial cells including astrocytes and oligodendrocytes by a neurotoxic agent or ethidium bromide improved, to some extent, axonal regeneration in the CNS [27]. In the present study, our results suggested that astrotoxin may be used safely as a potential treatment for removal of glial scars and promoting nerve regeneration. The results further demonstrate that astroglial cells present a key impediment to prevent optic nerve regeneration in adult mice.

Our results indicate that elimination of astroglial cells alone is not sufficient to support axonal regeneration; rather, a combined treatment of astrotoxin and lithium is required to stimulate optic nerve regeneration in adult mice. Lithium, a long-standing mood-stabilizing drug [28], has been shown to have many functions including induction of Bcl-2 expression from CNS neurons [16], promotion of neurite outgrowth [14,28] and neuronal survival [29], and stimulation of the proliferation of neuronal stem cells [30]. In the present study, lithium's effects on supporting optic nerve regrowth in mice is likely through inducing Bcl-2 expression and with it, promoting the intrinsic regenerative capacity of RGC axons. In consistent with our previous observation in Bcl-2 transgenic mice [9,13,14], activating the intrinsic growth

mechanisms of RGC axons alone, by inducing Bcl-2 expression, does not suffice optic nerve regeneration in adult mice. Two parallel mechanisms work concurrently that prevent the regeneration of CNS axons in the adult: the lack of Bcl-2-supported intrinsic mechanism and the presence of inhibitory environment in the mature CNS. Therefore, suppressing glial scarring by ablation of astrocytes or inducing Bcl-2 expression in the adult retina with lithium alone is unable to stimulate optic nerve regeneration in adult mice; concurrent administration lithium and astrotoxin is needed. These results further demonstrated that activation of Bcl-2-support growth mechanism and removal of reactive astrocytes are two essential elements of CNS axonal regeneration.

The exact mechanisms through which lithium promotes the intrinsic growth capacity of RGC axons remain obscure. Our previous report suggests that induction of Bcl-2 expression by lithium is required for its effect on promoting RGC axonal regrowth, at least when it was examined in culture [14]. The present study further indicated that short-term administration of lithium is sufficient to induce the expression of Bcl-2 in the adult retina in vivo. This is in agreement with the other reports showing that short-term delivery of lithium in vivo was sufficient to reduce neuronal damage following an insult of ischemic/ reperfusion [31]. While lithium induced the expression of Bcl-2, an anti-apoptotic molecule, in the ganglion cell layer of the adult retina, no significant improvement of RGC survival was observed in lithium-treated group as compared to that of the control group (Fig. 5). Although there appeared to be a tendency of ~20% increase in the number of RGCs in lithium-treated retina as compared to that of the control retina, the difference was not statistically significant. In part, this may be caused by the limited sample size as well as by that short-term administration of lithium induced Bcl-2 expression only in a small population of RGCs. Therefore, based on these observations, we shall not completely rule out the possibility that lithium induces Bcl-2 expression that enhances both the survival and axon regenerative potential of RGCs.

The molecular pathways through which Bcl-2 supports the intrinsic growth capacity of CNS axons have only begun to be understood. We found that Bcl-2, residing in the endoplasmic reticulum, regulates intracellular Ca^{2+} signaling that activates cAMP response element binding protein (CREB) and stimulates the regenerative response of CNS axons [32]. It has been shown that lithium can stimulate the phosphatidylinositol-3-kinase/Akt signaling cascade which up-regulates Bcl-2 expression [33] and inhibits the expression of p53, an established negative regulator of expression of Bcl-2 [15,34]. Moreover, lithium may also promote axon regeneration by directly increasing the DNA binding activity of CREB [35] and decreasing the activity of glycogen synthase kinase-3 (GSK-3) which suppresses CREB activation [36,37]. Taken together, our data suggest a possibility of using combined therapy of lithium and astrotoxin that manipulate both the intrinsic properties and the mature CNS environment to promote nerve regeneration. Although finding ways that target astrotoxin to the CNS, passing through the blood brain barrier, remains as a challenge, it has been suggested that once reached targets, regenerating axons have the capacity to establish functional connections in the adult CNS. Previous research using peripheral nerve grafts revealed that regenerated RGC axons preserve some capacities to form well-differentiated synapses and distinguish between target and non-target neurons [38,39]. In view of the safety profiles of lithium and astrotoxin in vivo, these results support further clinical studies for using such novel combined strategy for treating devastating illnesses such as glaucoma or other CNS damage that involves axon degeneration.

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Fig. 1. Intake of lithium-containing diet increases lithium concentration in serum and upregulates Bcl-2 expression

(A) Graph showing a significant increased in lithium concentration in lithium-treated mice than the control wild-type (wt) mice consumed control diet (Student's *t*-test, $P < 0.00001$). Values are mean $\pm$ s.d., (B) RT-PCR results show the induction of Bcl-2 mRNA in the retinas of lithium-treated mice. Note the Bcl-2 mRNA expresses in the retinas derived from lithium-treated wt mice and the positive control Bcl-2 transgenic (tg) mice but not in the wt mice that consumed a control diet. The expression of house-keeping gene, G3PDH, is served as an internal control

Fig. 2. Localization of lithium induced Bcl-2 expression in adult mouse retina

Epifluorescence photomicrographs of retinal sections derived from mice fed with control diet (A–C) or lithium-containing diet (D–F). DAPI staining localizes the nuclear layers in retina (B, E). Arrow marks the co-localization of Bcl-2 protein in ganglion cell layer (GCL). The insert is an enlarged image from the arrowhead pointed cells (F). Note there is undetectable signal of Bcl-2 protein in the control retina (A–C). Scale = 10 μ m

Fig. 3. Simultaneously administration of lithium and astrotoxin promote robust regeneration of the severed optic nerves in adult wt mice

Montages of photomicrographs showing optic nerve sections in adult wt mice 8 days after optic nerve crush (A–L). *First row*: Cresyl violet staining identifies the crushed site of optic nerve and marked by asterisk. *Second row*: GAP-43 staining reveals the length regenerating axons along the crushed optic nerve. Extensive GAP-43 positive axons regenerate posterior to the crush site derived from the mice receiving lithium and astrotoxin simultaneously (J) but no regenerating axons were observed posterior to the crushed site in the other groups (B and F). *Third row*: Strong GFAP staining extends along the crushed optic nerve. In the groups of mice receiving astrotoxin, there is a track of GFAP-negative area along the optic nerve (G and K). In the group of mice receiving lithium and astrotoxin simultaneously, the overlay shows that the regenerating axons fulfill the GFAP-negative area, which is astrocyte-ablated, of the crushed optic nerve (I). Scale = 250 μ m. *Fourth row*: Growth cone-like structure of regenerating axons in the crushed optic nerve. In the mice received lithium-containing diet and astrotoxin simultaneously, many GAP-43 labeled regenerating axons with growth cone-like structure (arrow marked) exhibits at their expanding tip (M and N). Scale = 5 μ m

Fig. 4. A graph showing the length of regenerating axons in the crushed optic nerve

The length of regenerating axons in the mice simultaneously receiving lithium (Li) and astrotoxin (AA) is significantly longer than that of the other groups ($P < 0.001$). The numbers of regenerating axons measured in the groups of control, Li, AA and Li + AA are 8.17 ± 4.09 , 84.4 ± 28.2 , 52.2 ± 48.9 and 435 ± 68.2 , respectively. Values are mean $\pm$ SEM

Fig. 5. Lithium-containing diet exerts no effect on prevention of RGCs death

(A) The graph shows the density of surviving RGCs, which is FluoroGold (FG) pre-labeled, in normal retinas (N) and retinas with optic nerve lesion (L) in the wt mice. There is no significant difference between the corresponding groups with or without lithium-treated ($P > 0.05$). Photomicrographs showing the surviving FG-labeled RGCs in normal retina (B) and lesioned retina (C) derived from lithium-treated mice. Arrow marks the microglia with a feature of elongated cell body and fine processes. Scale = 50 μ m. Li -ve, mice received control food chow; Li +ve, mice received lithium-containing food chow