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Original Article

The neuroprotective effect of staurosporine on mouse retinal ganglion cells after optic nerve injury

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Abstract: This study aimed to investigate the neuroprotective properity of staurosporine (STS) and identify the neuroprotective mechanism of staurosporine in mouse retina ganglion cell after optic nerve injured. Mice (C57BL/6) were anaesthetised with a mixture of 5 mg/kg xylazine hydrochloride and 40 mg/kg tiletamine/zolazepam (Zoletil®). Optic nerves of the mice were crushed (Templeton JP et al., 2012). With micro-forceps, the bulbar conjunctiva was grasped and retracted, rotating the globe nasally. The exposed optic nerve was grasped approximately 1-3 mm from the globe with Dumont #N7 cross-action forceps for 10 s. One day after crushing, intravitreal injections of STS (500 nM) were administered using a Narishige IM-300 air pressure regulator. For analysing the change in ganglion cell number, the mice were allowed to live for 30 days, after which they were killed and the ganglion cell survival was assessed. A significant and marked loss of fluorescent spots was found after 30 days, with fewer 4',6-diamidino-2-phenylindole (DAPI)-expressing retinal ganglion cells (RGCs) remaining in the injured and phosphate buffered saline (PBS)-injected group than those in non-injured PBS-injected controls. However, RGC cell numbers dramatically increased in the STS intravitreal injection group. Moreover, degradation of nerve fibre (NF) was markedly reduced in the STS injection group compared with the injured and PBS-injected group by inducing astrocyte expression of Bcl-2. Our data suggested that injection of STS into the vitreous may have a potential therapeutic effect in retinal diseases such as glaucoma.

Keywords: Optic nerve injury, staurosporine, neuroprotection, astrocyte, retinal ganglion cells, Bcl-2

Introduction

Optic nerve injury (ONI) is a well-established model of delayed retrograde retinal ganglion cell death, which triggers a process of degeneration in damaged axons as well as in fibres [1]. Associated retrograde degeneration leads to apoptosis of RGCs [2]. Several strategies have been attempted to increase their survival [3, 4]. Research into optic nerve regeneration has identified some key molecules, and to date, partial recovery of visual function following ONI has been achieved [5]. Some papers have reported the protective effects of the anti-apoptotic gene bcl-2 on axotomised RGCs [6]. Upregulated expression of bcl-2 has prevented neuronal death in several *in vitro* systems [7, 8]. Mice overexpressing bcl-2 have enlarged optic nerves, suggesting that RGC survival during development is enhanced [9].

The main biologiclal activity of STS is the inhibition of protein kinase C (PKC) activity, and it is a functional neurotrophin agonist, promoting neurite outgrowth in neuroblastoma and brain primary neuroal culture [10]. It is known to mimic the effect of nerve growth factor (NGF) in promoting neurite outgrowth and induces ganglion cell differentiation by stimulating urokinase-type plasminogen activator expression and activation in the developing chick retina [11, 12]. Although the retinal neuroprotective effects of several materials have been extensively examined [13-15], there are no reports indicating the involvement of STS in RGC protection. Investigation of ONI-induced specific

protein expression suggests that modification of expression may lead to new therapeutic approaches.

In this study, we herein investigated whether administration of STS provides neuroprotection to RGCs following ONI, and evaluated the possible related proteins underlying these effects.

Materials and methods

Animals and procedures for the optic nerve injury model

C57BL/6 mice (8-10 weeks, female, 18-20 g) were purchased from Hyochang Science (Daegu, South Korea). Mice were kept in a facility under the following environmental conditions throughout the study: room temperature 25 ± 1°C, relative humidity 60 ± 10%, and alternating 12 h light-dark cycles (8 a.m. to 8 p.m.). All experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of optic nerve injury model and experimental groups

Thirty-two C57BL/6 mice were randomly assigned to one of three groups: normal control (phosphate buffered saline (PBS)-injected non-ONI) (n=8), PBS-injected ONI (n=12), and STS-injected ONI groups (n=12). The optic nerve injury was induced as described by Templeton JP et al. [16]. By using micro-forceps, the edge of the conjunctiva next to the globe was grasped and retracted, rotating the globe nasally. When the optic nerve was visualised, it was grasped approximately 1-3 mm from the globe with Dumont #N7 cross-action forceps (cat. #RS-5027; Roboz) for 10 s, with pressure from the self-clamping action used to compress the nerve.

Intravitreal injection of STS and phosphate buffered saline

One day after the injury, 16 mice were anaesthetised using the same protocol as that for the ONI procedure and were administered intravitreal injections of either 2 µl STS (500 µM) (n=6) or a sham injection of 2 µl PBS solution (n=6) by using a Narishige IM-300 air pressure regulator. The normal control group (n=4) received an injection of 2 µl PBS solution in the same way.

Retinal flat mounting

Thirty days after the injury, all mice were euthanized and the eyes of the mice were harvested. Harvested globes were rinsed in PBS, then their optic nerves were cut and lenses were removed. Retina and retinal pigment epithelium (RPE) were carefully separated with fine forceps and a soft brush. After harvesting the retina, four small cuts were made to assist in mounting the retina flat on nitrocellulose (NC) membrane. Whole and flat-mounted retinal tissues were fixed in 4% paraformaldehyde (PFA) in PBS for 2 h at 4°C.

Immunofluorescence (IF) staining of flat-mounted retinas

For immunofluorescence staining, retinal tissues were washed three times with PBS for 30 min each time. After removal from the NC membrane, tissues were transferred to glass slides. Staining was performed as described in detail before [17].

Imaging the whole mounted retina for cell counts

Images were captured using a Zeiss fluorescence microscope (Axio Vision 4; Carl Zeiss, Jena, Germany). An area of each retina, 100 µm from optic nerve disc, was selected and imaged. From the captured photos, we randomly selected five photos from each specimen and measured ganglion cell number (DAPI-positive cells) automatically using ImageJ software. Total ganglion cell numbers were taken as the average of three of the measured results, excepting the minimum and maximum values from the five images.

Immunofluorescence staining of vertically-sectioned retina

For immunofluorescence staining, sectioned retinal tissue slides were deparaffinised and then rehydrated through an ethanol series. Staining was performed as described in detail before [17]. The specimens were then incubated overnight with monoclonal or polyclonal antibodies against Tuj-1, GFAP and Bcl-2 (diluted 1:50-100).

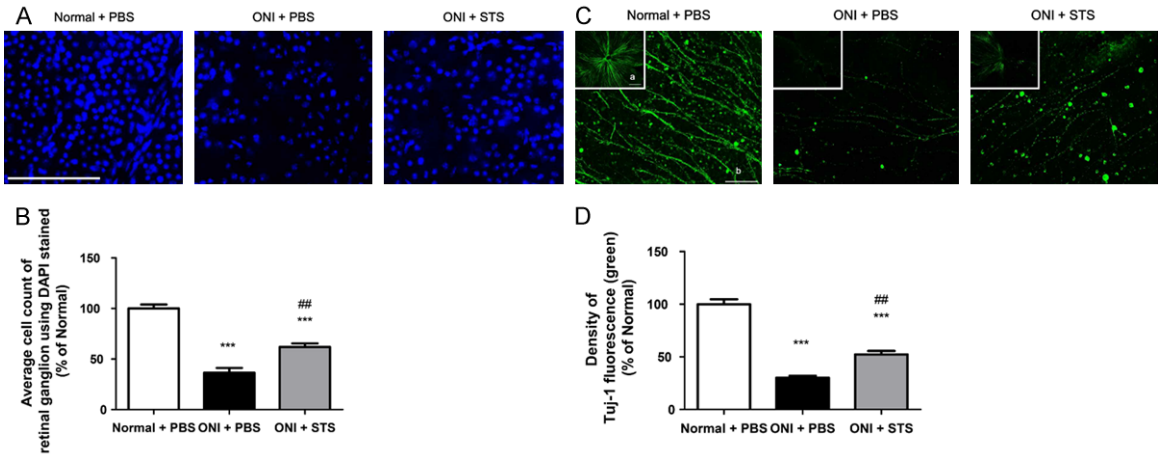

Figure 1. STS-mediated RGC survival and blockade of RGC nerve fibres degradation after ONI at 30 days. A. DAPI staining of flat-mounted retinal tissue at 30 days after harvesting fromPBS-injected non-ONI control, PBS-injected ONI, STS-injected ONI groups. Scale bar: 50 μm. B. Quantitative analysis of GC cell numbers in DAPI-stained flat-mounted retinal tissues. The graph shows the average cell count. C. Tuj-1 staining of flat-mounted retinal tissue at 30 days after ONI in non-injured and PBS-injected control, injured and PBS-injected, injured and STS-injected subjects. Small images were observed at ×40 magnification, Scale bar (a): 500 μm. High magnification shows representative images from smaller images. Scale bar (b): 100 μm. D. Quantitative analysis of fluorescence density in Tuj-1 stained flat-mounted retinal tissues. PBS-injected non-ONI control retina was treated as 100%. Data are presented as means ± SEM. Differences were considered statistically significant as follows: $^{***}P<0.001$ vs. PBS-injected non-ONI control retina, $^{\#\#}P<0.01$ vs. PBS-injected ONI retina.

Statistical analysis

Data were evaluated by one-way analysis of variance (ANOVA) with Turkey's test. The analyses were performed using GraphPad PRISM® software (GraphPad PRISM software Inc., Version 5.02, CA, USA). Results are expressed as means ± standard error of the mean (SEM), and *p*-values of <0.05 were considered significant.

Results

STS promotes RGC survival following optic nerve injury

PBS-injected non-ONI control mouse retina had an average RGC cell number of 151.75 ± 12.01 in captured photos. In comparison with the control, we found that by 30 days after the ONI, there was substantial reduction in the number of retinal ganglion cells. PBS-injected ONI retinas had an approximate 63.59% loss of RGC (remaining RGC 36.4 ± 9.55%; $P<0.001$). In contrast, STS injection significantly promoted RGC survival by an approximate 25.54% compared with PBS injection (remaining RGC approximately 61.94 ± 6.91%; P<0.01). The above results prove that STS can act as a neuroprotectant for ganglion cells that would otherwise die from ONI-induced neurotoxicity.

STS prevents RGC nerve fibre degradation

We also investigated whether STS prevents RGC nerve fibre degradation in ONI retina with whole-mount immunohistochemistry using antibody raised against Tuj-1, a marker for RGCs and neurons (**Figure 1C** and **1D**). In PBS-injected non-ONI retina, Tuj-1 positive nerve fibres exhibited a linear trajectory from the optic disc to the medial region of the retina (**Figure 1C** small panel). Radial nerve fibres appear from the medial to the peripheral retina and they spread throughout the whole retinal surface. However, in PBS-injected ONI retina, Tuj-1-positive nerve fibres underwent degenerative events. The few remaining nerve fibres after ONI and PBS injection were found in the medial region. Tuj-1-positive values decreased to 30.27 ± 2.85% compared with PBS-injected non-ONI retina ($P<0.001$). By comparison, STS injection significantly upregulated Tuj-1 protein expression. The reduction was suppressed to 47.65 ± 5.65% ($P<0.01$). Taken together, our results suggest that STS injection following retinal injury serves a neuroprotectant function.

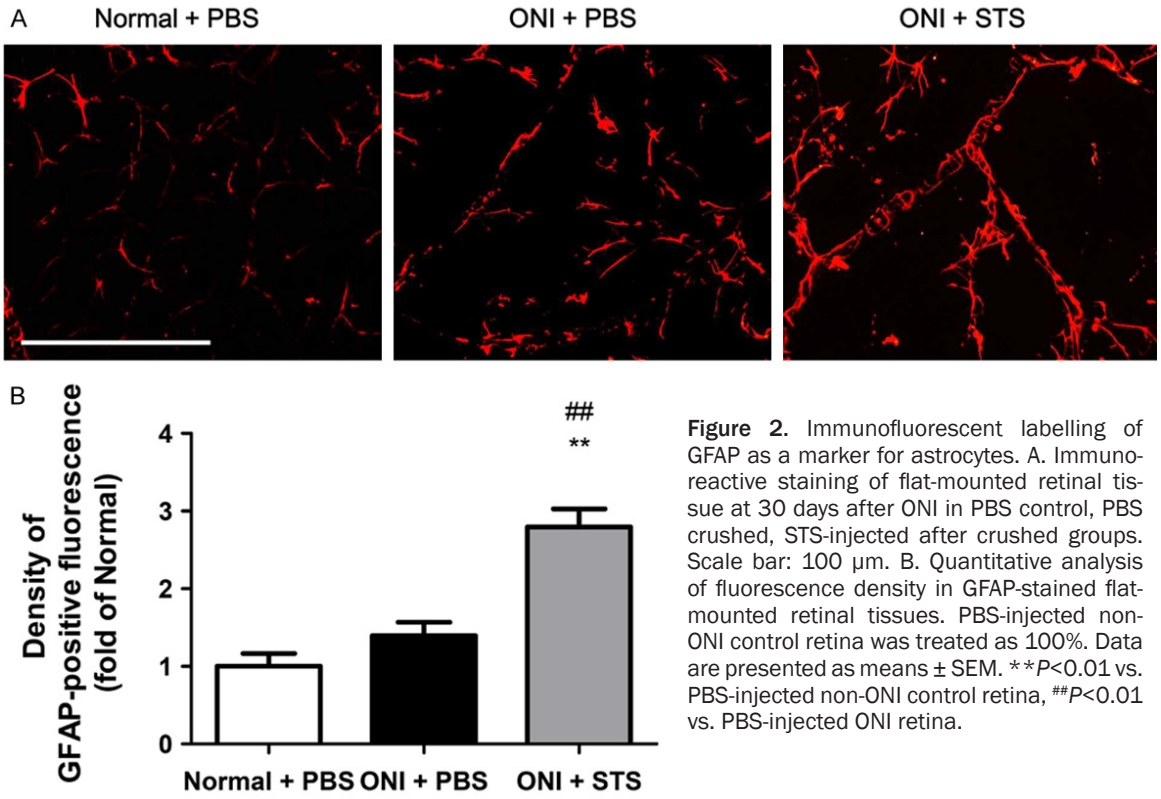

Figure 2. Immunofluorescent labelling of GFAP as a marker for astrocytes. A. Immunoreactive staining of flat-mounted retinal tissue at 30 days after ONI in PBS control, PBS crushed, STS-injected after crushed groups. Scale bar: 100 μm. B. Quantitative analysis of fluorescence density in GFAP-stained flat-mounted retinal tissues. PBS-injected non-ONI control retina was treated as 100%. Data are presented as means ± SEM. $^{**}P<0.01$ vs. PBS-injected non-ONI control retina, $^{\#\#}P<0.01$ vs. PBS-injected ONI retina.

STS injection increases GFAP expression across the entire retina after ONI

Expression of GFAP serves as a marker of developmental processes as well as an indicator of gliosis in response to injury [18]. We determined whether STS regulates activation of GFAP along the vitreal surface in flat-mounted retinal tissue using immunoreactive staining for GFAP (**Figure 2**). In PBS-injected non-ONI and ONI retinas, GFAP-positive astrocytes were ubiquitously scattered throughout the retina, but GFAP was expressed at low levels in astrocytes. By comparison, in STS-injected ONI retinas, GFAP-positive astrocytes were distinctly and strongly activated. This result suggests that the increase in GFAP-positive cells was induced by STS injection.

STS injection promotes altered expression of GFAP in IPL, OPL and GCL and Tuj-1 in GCL after ONI

The retinal tissue from each eye was paraffin-embedded and vertically sectioned. PBS-injected non-ONI retina showed no glial cell (GFAP-positive) activation, as indicated by the very small amount of GFAP immunoreactivity in the ganglion cell layer (GCL) only (**Figure 3** upper panels). In contrast, PBS-injected ONI retina exhibited a dramatic increase in the proportion of GFAP-immunoreactive cells (**Figure 3A** middle panels and **3B**). Moreover, GFAP immunoreactivity was observed in Müller glia spanning a narrow band in the inner plexiform layer (IPL) [19] and across the entire thickness of the neural retina. GFAP expression was also detected in rare astrocytes along the vitreal surface of the retina. The reduction in the number of ganglion cells in the GCL was paralleled by an increase in the expression of GFAP, a marker for retinal damage (**Figure 3A** middle panels) [19], in the Müller glial cells in the IPL. In comparison with PBS injection, STS injection reduced GFAP immunoreactivity in the IPL and increased GFAP expression along the vitreal surface of the retina in the GCL (**Figure 3A** lower panels and **3B**) in ONI eyes. Tuj-1 expression also markedly reduced in PBS-injected ONI, but Tuj-1 positive cells remained in the GCL in STS-injected ONI retina (**Figure 3A** and **3B**). These results suggest that damage to the GCL by ONI is prevented by STS injection.

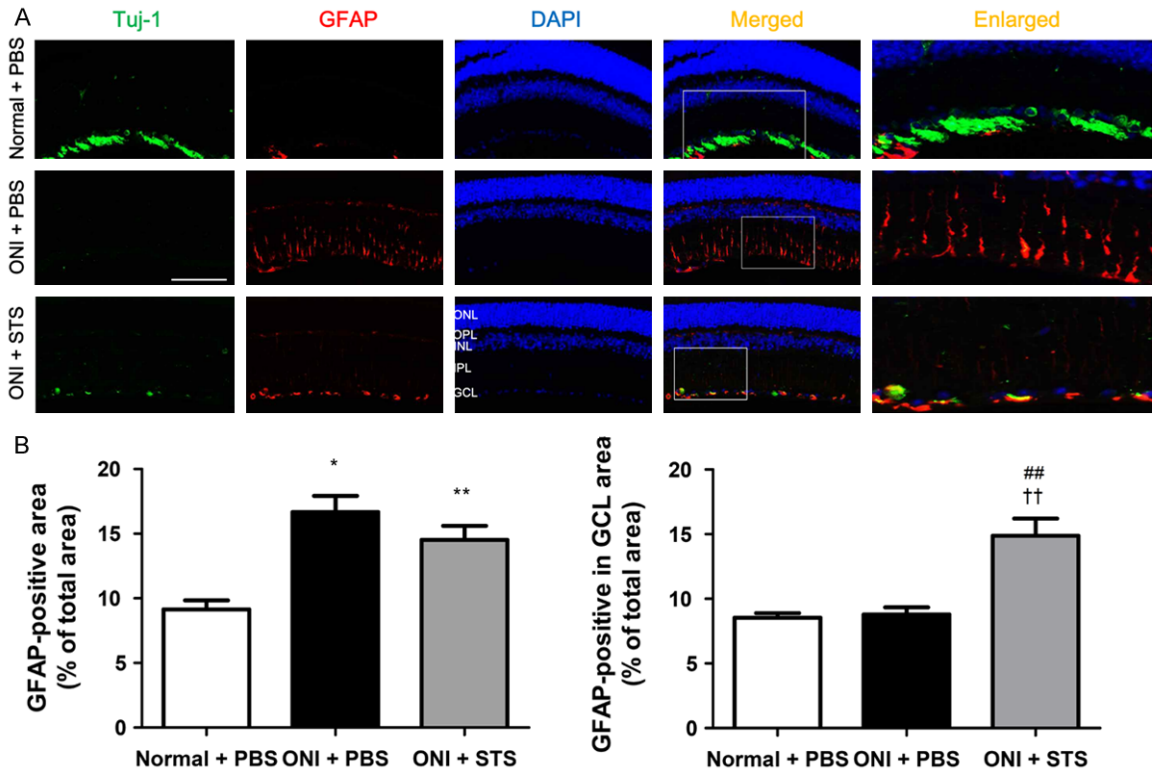

Figure 3. Change in Müller glial cell and astrocyte activation after injection of PBS or STS in ONI. Paraffin-embedded retinal tissues were vertically sectioned. A. Tuj-1, as a marker of post-mitotic neuronal precursors, GFAP, as a marker of Müller glial cells and astrocytes, and DAPI, as a nuclear counterstain were used. Right panels are higher magnification of the boxes shown in the left panels. Scale bar: 100 μm. B. Quantitative analysis of GFAP immunostaining. At least five fields were captured for each condition. GFAP-positive area in each field was measured as percentage of total area, applying the same threshold parameters to all images. Data are presented as means ± SEM. **P*<0.05 vs. PBS-injected non-ONI control retina, ***P*<0.01 vs. PBS-injected ONI retina. ††*P*<0.05 vs. PBS-injected non-ONI control retina.

STS induces Bcl-2 expression in Müller cells and astrocytes after ONI

To determine whether is Bcl-2 expressed during GFAP activation, we performed double-staining with Bcl-2 and GFAP antibodies. As shown in **Figure 4**, in PBS-injected non-ONI retina, Bcl-2 was only expressed in the outer plexiform layer (OPL), and was not detected in the GCL or other areas. In both PBS- and STS-injected ONI retina, Bcl-2 expression was markedly increased in the GCL. Moreover, Bcl-2 protein was co-localized with GFAP in the GCL. In comparison with STS-injected ONI retina, Bcl-2 protein was hardly detected at all in the GCL of PBS-injected ONI retina. To determine whether Bcl-2 and Tuj-1 expression are correlated, we also performed double-staining with Bcl-2 and Tuj-1 antibodies. Bcl-2 expression was not co-localized with Tuj-1 (data not shown).

Discussion

Optic neuropathies and neurodegenerative disease, of which glaucoma is the most common, are characterised by axonal degeneration in the optic nerve and apoptotic death of retinal ganglion cell somas, leading to irreversible vision loss [20, 21]. Understanding the processes and mechanisms of neuronal death and neuronal survival is a prerequisite for the development of neuroprotective measures. The murine optic nerve injury model is useful to study the process of RGC death and survival [22].

While searching for new treatments for retinal diseases, we have tried to address the neuroprotective effect of STS on RGCs. In research, STS is commonly used to induce apoptosis in several cell types [23-27] and it is a potent activator of neuronal, glial, and neurosphere differ-

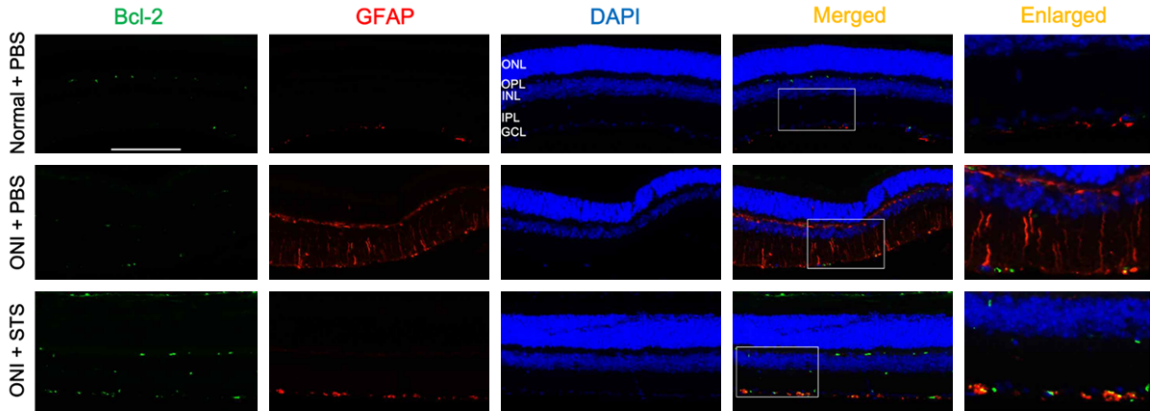

Figure 4. Expression of Bcl-2 protein in astrocytes after injection of PBS or STS in ONI. Paraffin-embedded retinal tissues were vertically sectioned. Bcl-2, as anti-apoptotic protein, GFAP, as a marker of Müller glial cells and astrocytes, and DAPI, as a nuclear counterstain were used. Right panels are higher magnification of the boxes shown in the left panels. Scale bar: 100 μm.

entiation in murine embryonic stem cells [28]. Moreover, STS reportedly has a variety of effects on neuronal cell lines PC-12 and RGC-5 [11, 29]. However, there are no reports on the role of STS in RGC protection after optic nerve crush injury.

Using a previously described ONI model [16], we found that STS attenuated RGC loss (**Figure 1**). In this study, we suggested that STS is a very potent protector of ONI-induced ganglion cell death in mice. We demonstrated the neuroprotective effect of intravitreal injection of STS four weeks after ONI. Death of RGCs in our study occurred gradually (data not shown). By 30 days after the injury, the RGC population was reduced to 63.6% of the normal ($P<0.001$). In flat-mounted retinal tissue, STS blocked degradation of RGC nerve fibres (**Figure 2**). The reduction was to 47.65 ± 5.65% compared to 69.72 ± 2.85% after ONI and PBS injection ($P<0.005$). Although the suppression of RGC death was only 22.07%, this reduction would be significant in many ocular diseases if reproduced in a clinical situation. Namely, our results suggest that STS may provide a novel approach for the treatment of neuronal degenerative diseases.

Optic nerve crush injuries induce changes in the surrounding microenvironment, producing complex signals that contribute to Müller cell and astrocyte activation. In response to diverse insults such as glaucoma, ischemia, optic neuritis, optic nerve crush, and optic nerve transection, quiescent Müller glial cells enter an active state. Other studies, both *in vitro* and *in vivo*, suggest that Müller cell activation may be neuroprotective [30, 31]. Activated astrocytes also provide a permissive substrate for axonal regrowth [32] and can facilitate the integration of transplanted neural stem cells [33]. Modulating Müller glial cell and astrocyte activation is thought to be an effective therapeutic strategy for improving neuronal survival and reversing the loss of neuronal function. It is this astrocyte activation by STS injection, which is protective of neuronal axons. Reactive astrocytes were once thought to be detrimental cells responsible for neuronal demise, but recent studies have changed this view, and they are now considered to be potential endogenous repair agents [34-37]. Targeted activation of astrocytesis a potential neuroprotective strategy [38].

Several neurotransmitters regulate the release of neuroprotective factors from astrocytes. From these various neuroprotective candidates, we focused and examined the B-cell lymphoma 2 (Bcl-2) protein after ONI. Bcl-2 is an important anti-apoptotic factor, and increasing attention has focused on its role in astrocyte activation. Survival of retinal ganglion cells 1 month after intracranial crush of the optic nerve was found to be 100% in adult Bcl-2 mice and 44% in matched wild-type mice [39]. The efficacy of Bcl-2 in promoting neuronal survival *in vivo* has been evaluated in a transgenic mouse overexpressing the bcl-2 gene in neurons [9]; 2 months after transection of the optic

nerve, 63% of retinal ganglion cells survived in the bcl-2 mouse, whereas only 5% survived in the wild type. In the present study, to determine whether Bcl-2 expression increases in GFAP activation, we investigated Bcl-2 expression in STS-injected retina after ONI. Bcl-2 expression was scarcely detected in non-ONI-PBS-injected retina (**Figure 4**). By comparison of non-ONI-PBS-with ONI-PBS-injected eyes, Bcl-2 positive was found to be co-localised with GFAP (**Figure 4**), butnot Tuj-1 (data not shown) in the GCL of ONI-STS-injected retina. We suggest that the neuroprotective effects of STS in our experimental model are mediated in part by stimulation of Bcl-2.

Taken together, our findings provide new insights into one potential mechanism for STS's protective actions and glial cell regulation in neurological disease, where increased neuronal degeneration has been implicated after injury of nerves. These data demonstrate for the first time the neuroprotective effect of STS and its mechanism of action in ONI.

There are some limitations of this study. We did not examine the effect of STS over time prior to 30 days (e.g., after 1, 3, 7 days and 2 and 3 weeks). Another limitation is that we did not identify the other neuronal survival-promoting factors released by astrocyte activation after ONI-STS injection. Although much remains to be understood on a molecular level, the current data point to STS as a new and promising candidate for therapeutic interventions in the future.

In conclusion, STS is protective of ganglion cell death after ONI. Our study supports the theory that STS is likely to suppress neurodegeneration in the injured optic nerve by increasing the expression of Bcl-2 in astrocytes and by regulating glial cells. These findings suggest promising strategies for the treatment of optic nerve diseases.

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Disclosure of conflict of interest

None.

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