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Shp-2 regulates the TrkB receptor activity in the retinal ganglion cells under glaucomatous stress

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ABSTRACT

Tropomyosin-receptor-kinase B (TrkB receptor) activation plays an important role in the survival of retinal ganglion cells (RGCs). This study reports a novel finding that, SH2 domain-containing phosphatase-2 (Shp-2) binds to the TrkB receptor in RGCs and negatively regulates its activity under glaucomatous stress. This enhanced binding of TrkB and Shp2 is mediated through caveolin. Caveolin 1 and 3 undergo hyper-phosphorylation in RGCs under stress and bind to the Shp2 phosphatase. Shp2 undergoes activation under glaucomatous stress conditions in RGCs *in vivo* with a concurrent loss of TrkB activity. Inhibiting the Shp2 phosphatase restored TrkB activity in cells exposed to excitotoxic and oxidative stress. Collectively, these findings implicate a molecular basis of Shp2 mediated TrkB deactivation leading to RGC degeneration observed in glaucoma.

1. Introduction

Glaucoma is a chronic disease involving irreversible loss of retinal ganglion cells (RGCs). Mechanisms for cell damage are not well understood, with elevated intraocular pressure (IOP) being the major risk factor. Tropomyosin-receptor-kinase B (TrkB), a member of the Trk family of receptor tyrosine kinases, is well expressed in RGCs [1–3] and plays a major role in their preferential degeneration in glaucoma [4]. BDNF and NT4 are the high affinity ligands for the TrkB receptor and their binding stimulates dimerization and autophosphorylation of the TrkB receptor and stimulates the major downstream pro-survival signalling pathways involving PI3K, phospholipase C-γ1 and MAPK [5]. TrkB activation through exogenously administered BDNF can rescue RGCs transiently but higher doses or multiple applications fail to support their long term survival as RGCs eventually get desensitised to the protective effects of BDNF [4,6]. TrkB gene therapy and BDNF treatment have a protective effect on dying RGCs [7]. The precise mechanism underlying a change in sensitivity of TrkB to BDNF treatment is poorly understood. Receptor tyrosine kinases are subjected to phosphorylation and dephosphorylation events in tandem, for biological actions and to maintain a fine equilibrium in the downstream signalling events [8]. Therefore, simultaneous dephosphorylation and deactivation of TrkB by an interacting phosphatase may restrain the protective effects of BDNF.

Shp-2 is a ubiquitously expressed tyrosine phosphatase that acts as a regulator of tyrosine kinase receptor signalling [9]. It has also been implicated in the signalling of TrkA receptors [10]. Shp2 regulates the downstream signalling of TrkB in mesencephalic and cortical neurons [11]. In cerebral neuronal cultures, Shp2 negatively regulates TrkB autophosphorylation and activation through BDNF in a calcium dependent manner [12].

In this study, we reveal molecular evidence that the Shp2–TrkB interaction in RGCs is mediated through the adapter protein caveolin (cav). Shp2 also interacts with the adapter proteins Gab and FRS2/SNT [12]. Cav, a principal component of Caveolae, comprises cav1, 2 and 3 isoforms and is well expressed in the retina [13,14]. In this report, we uncovered a novel mechanism of TrkB receptor deactivation by Shp2 phosphatase, under glaucomatous stress conditions in RGCs *in vivo*.

2. Materials and methods

2.1. Animals

All procedures involving animals were conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes and the guidelines of the ARVO statement for the use of animals in ophthalmic and vision research. Albino Sprague–Dawley rats were obtained from Animal Research Centre, Perth and maintained in our vivarium in cyclic light (12 h on; 12 h off; ~300 lux). All animals were

Abbreviations: RGC, retinal ganglion cell; BDNF, brain derived neurotrophic factor; Cav, caveolin; AON, antisense oligonucleotide; ON, optic nerve; IOP, intra-ocular pressure

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maintained in an air-conditioned room with controlled temperature (21 ± 2 °C). The animals were anaesthetised with an intraperitoneal injection of ketamine (75 mg/kg) and medetomidine (0.5 mg/kg).

2.2. Optic nerve axotomy and intra-ocular injections

An optic nerve transection was performed at a distance of 2–3 mm from the globe using a surgical protocol described previously [15] and verified by the loss of visual evoked potentials [16–18]. A chronic RGC degeneration model was established by a chronic increase of intraocular pressure (IOP) in rats by microbead (Fluospheres, Molecular Probes, 10 μm) injections in the anterior chamber as reported previously [19,20]. Intraocular injections (3.6×10^6 microbeads/mL, 5 μL) were made every week until a sustained increase in IOP was observed and the contralateral eye injected with an equivalent volume of saline. All the eyes injected with microbeads experienced a sustained increase of 20.4 ± 1.4 mm Hg for 6 weeks from a baseline IOP of 11.1 ± 0.4 mm Hg (Icare-LAB tonometer), while the control eyes depicted no such elevation of IOP. The animals which did not depict a sustained IOP increase of at least 18 mm Hg or above were strictly discarded. Four consecutive IOP readings were obtained from each eye and the average number was taken as the IOP for that time point.

For the anti-sense oligonucleotides (AON) a 33-gauge needle was used for injections (5 μL) at a 45° angle 2 mm behind the corneoscleral limbus into the vitreous body without touching the lens. After the injection, the needle was left in place for another minute to allow dispersion of the oligonucleotides into the vitreous. The experimental eyes were injected with AON, and the contralateral eyes with scrambled oligonucleotides.

2.3. Knock down of caveolin expression

Caveolin expression was knocked down in the RGCs *in vivo* using intravitreal injections of phosphorthioate modified cav mRNA AONs [21]. AONs can penetrate through the cell membrane and arrest the translation of specific mRNA, leading to the inhibition of biosynthesis of the corresponding protein [21]. The treatment of AONs with polyethyleneimine (PEI) compactly packs them into fine nano particles which further helps them cross the membrane barrier in the mammalian cells [22–25]. Rats were intravitreally injected either with cav AON (every week) or a scrambled control treated with PEI resulting in knock down of cav in retina compared to controls (Fig. 6). AONs were generated corresponding to the homologous region of cav1, 2 and 3 to knock down expression of all cav isoforms. The AONs were also used to knock down the expression of cav isoforms in primary RGC culture. Cav AON: 5′-mC*mC*mG*mC*mA*mA*T*C*A*C*A*T*C*T*T*C*A*A*A*G*mU*mC*mA*mA*mU*mC-3′. Scrambled: 5′-mC*mC*mC*mA*mU*mA*A*C*A*T*T*C*G*A*A*C*A*A*G*T*mC*mC*mA*mU*mU*mC-3′. (*-Phosphorthioate; m-2′ O-methyl RNA base — IDT Technologies, Australia). The sequence of AONs corresponded to cav1 α-195–220 (AF439778.1), cav1 β-102–127 (AF439779.1), cav-2 150–175 (AF439780.1), cav-3 114–139 (U31968.1) mRNA sequence (EMBL-EBI).

2.4. Isolation of retinal ganglion cells and culture

Following rat retinal dissociation, the cellular suspension was treated with $CD^{90.1}$ MicroBeads which led to selective magnetic labelling of the RGCs. The labelled RGCs were pre-enriched as per manufacturer's instructions (Miltenyi Biotec GmbH) and passed through columns subjected to strong magnetic field and eluted [26,27]. Brn3a (Abcam), Thy-1 (Cell Signalling) and GFAP (Sigma) antibodies were used to follow the enrichment of RGCs from the whole retinal cells and quantified. RGCs were either used directly or maintained in culture (Neurobasal-A media with 2% B-27, GIBCO) in 20 μg/mL poly-D-lysine (Sigma) coated plates and transfected using Lipofectamine RNAiMAX (Invitrogen). The cells were collected for experiments approximately 24 h after transfection. RGC-5 cells were maintained in DMEM medium containing 10% (vol/vol) foetal bovine serum at 37 °C in 5% CO_2. Approximately 2.0×10^5 cells were seeded in each culture dish 6–12 h before treatment. Subsequently, cells were treated either with Glu (25 μM) + H_2O_2 (2 μM) or Shp2 inhibitor (PHPS1; 5 μM) or both of these for 24 h followed by lysing the cells and subjecting them to western blotting.

2.5. Western blotting and immunoprecipitations

RGCs were lysed in lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 1 mM EDTA) containing (10 μg/ml aprotinin, 10 μM leupeptin, 1 mM PMSF) and (1 mM $NaVO_3$, 100 mM NaF, 1 mM Na_2MoO_4, 10 mM $Na_4P_2O_7$). The proteins were resolved by 10% SDS-PAGE and transferred to PVDF membrane as explained previously [28] and the blots developed using ECL kit (Pierce). Immunoprecipitation was carried out according to the method described earlier [29] and immunoprecipitates subjected to assay procedures or immunoblot analysis with indicated antibodies in the respective figures. Band intensities were normalised to the total amount of protein immunoprecipitated in each case and quantified in the linear range of detection using ImageJ software (NIH, USA).

2.6. Shp-2 phosphatase assay

Shp2 enzyme assays were performed following immunoprecipitation, using pNPP (1 mg/mL) as substrate in buffer containing 50 mM imidazole (pH 7.5) and 5 mM dithiothreitol as described previously [30] and absorbance was measured at 410 nm using Flex Station 3 microplate reader.

2.7. TrkB tyrosine kinase assay

The TrkB kinase assays were performed following immunoprecipitations using anti-TrkB antibody (Santa Cruz, CA, 0.5 μg) by modifications of the methods reported previously [31,32]. The kinase reaction was performed at room temperature in kinase assay buffer (50 mM HEPES, pH 7.4, 12 mM $MgCl_2$, and 5 mM $MnCl_2$) containing 100 μM ATP and 3 mg/mL poly-Glu:Tyr peptide (Cell Signalling) for 30 min. The soluble peptide (100 μL) was then coated on the microplates overnight as reported previously [33] and the plate was washed three times with PBS containing 0.05% Tween-20 followed by adding 100 μL of pY100 (Cell Signalling, 1:500) antibody and then HRP-linked secondary antibody. The plate was re-incubated at 37 °C for 1 h and washed. Finally, 100 μL of tetramethylbenzidine (Sigma) was added and incubated at room temperature for 30 min and A_{650} measured using Flex Station 3 microplate reader.

2.8. Statistical analysis

Data were analysed and graphed using GraphPad Prism software (GraphPad Software, CA). All values are mean ± SD from given *n* sizes and compared by Student's *t* test for unpaired data. The significance was set at $p<0.05$.

3. Results

3.1. Enhanced interaction of TrkB with Shp2 in RGCs under stress conditions

An acute and a chronic RGC degeneration models were established with either complete optic nerve (ON) axotomy or repeat microbead administration into the anterior chamber leading to chronic increase of IOP respectively. The RGCs were isolated from the rat retinas subjected to optic nerve axotomy (1 week) and those exposed to chronically increased IOP (4 weeks) [26,27]. The increase of RGC markers Thy-1 and Brn3a and loss of major contaminant Müller glial cell marker GFAP indicated an enrichment of the RGCs (Fig. 1A). Thy-1 expression in the whole rat retina was 0.75 ± 0.3% ($p<0.003$) of the expression observed in the

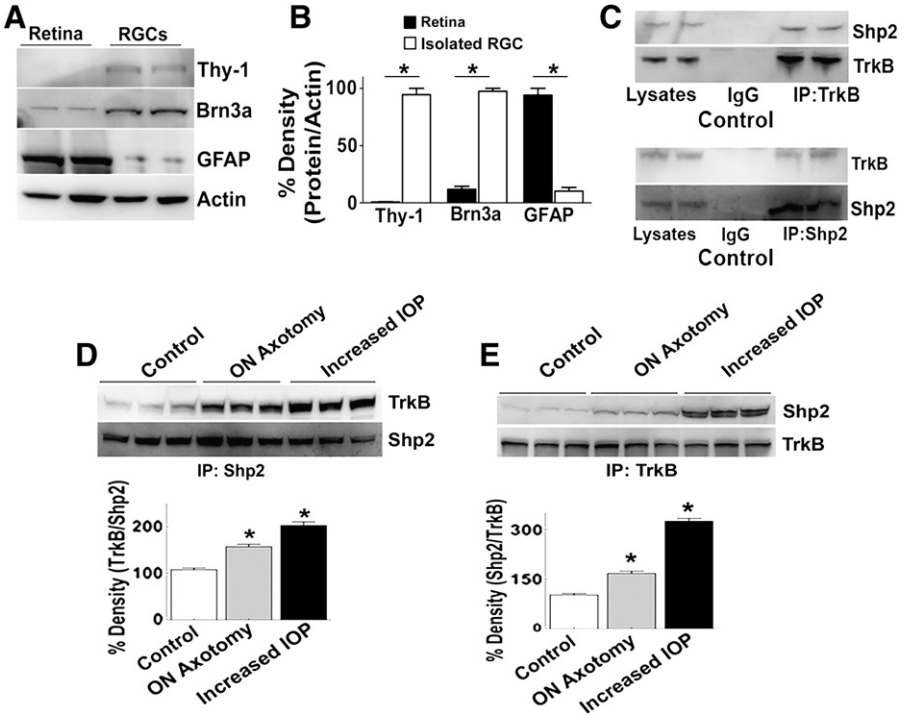

Fig. 1. TrkB interacts with Shp2 in RGCs and this interaction is enhanced under stress conditions. A. Enrichment of the RGC fraction indicated by Thy-1 and Brn3a and loss of standard contaminating Muller glial cell marker, GFAP. B. Densitometric analysis of the band intensities in (A) to quantify the enrichment of RGCs. C. Immunoprecipitations of TrkB and Shp2 establish that the two proteins reciprocally interact in rat RGCs *in vivo*. Whole cell lysates and non-immune IgG were used as controls. D, E. An augmented TrkB and Shp2 co-immunoprecipitation is observed in RGCs isolated from retinas exposed to stress following ON axotomy and exposure to chronically increased IOP. Densitometric analysis of TrkB binding to Shp2 and *vice versa*. Data are mean ± SD, n = 3 each, *p<0.001.

isolated RGCs, Brn3a expression was 11.8 ± 3.7% (p<0.002) in retina compared to that of the isolated RGCs while in contrast, GFAP expression in the isolated RGC fraction was depleted to 10.2 ± 4.5% (p<0.007) of that detected in the retina. Our results indicate that a very significant degree of RGC purity was achieved.

RGCs isolated from saline injected retinas were used as controls. The RGCs were lysed and subjected to immunoprecipitations using specific TrkB and Shp2 antibodies. Our studies indicate that TrkB and Shp2 reciprocally co-immunoprecipitate each other (Fig. 1B) and this association is significantly enhanced under both ON axotomy and increased IOP stress conditions (Fig. 1C, D). There was no non-specific binding to the non-immune IgGs. A much stronger interaction (2–3 folds) was observed in RGCs isolated from chronically increased intra-ocular pressure exposed retinas. Since glutamate excitotoxicity and oxidative stress are major factors implicated in RGC degeneration in glaucoma [34], we sought to determine the effect of these stress conditions on TrkB–Shp2 interactions. The primary culture of rat RGCs as well as the RGC-5 cell line were subjected to mild excitotoxic and oxidative stress conditions by incubating the cells with Glu and H_2O_2 (Glu, 25 μM, H_2O_2, 2 μM) for 24 h followed by lysing the cells and subjecting them to Western blotting. A 2–3 fold increase in the reciprocal interaction between these two proteins was observed in the rat RGCs while a 4–5 fold increase was observed in the RGC-5 cells (Fig. 2A, B).

3.2. Shp-2 regulates the TrkB activity in stressed RGCs

The RGCs isolated from control, ON axotomised and increased IOP exposed retinas were lysed and equal amounts of proteins (10 μg) were subjected to Western blotting, to analyse the expression of TrkB, BDNF and Shp2 in these cells (Fig. 3A). Quantification of the densitometric analysis of the band intensities normalised to the actin indicated that TrkB levels increased significantly 1 week after ON axotomy. The increase in TrkB expression in increased IOP exposed RGCs was not observed to be significant. A two fold increase in BDNF levels was observed upon ON axotomy. A significant increase in BDNF levels was also observed under increased IOP. No significant changes were observed in the Shp2 expression levels in either of these conditions. The TrkB immunoprecipitates

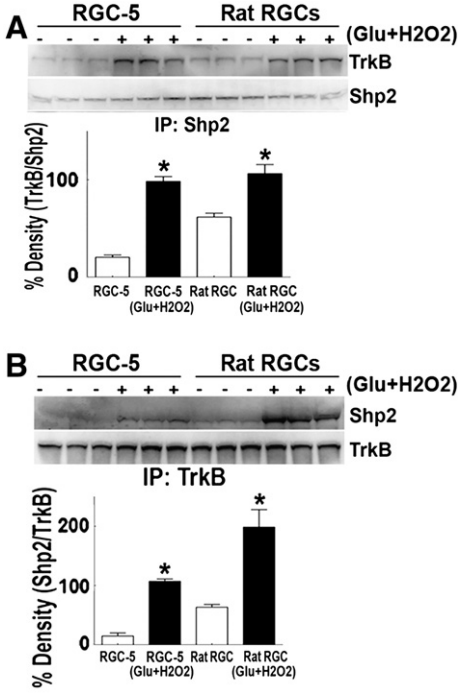

Fig. 2. TrkB–Shp2 interaction is enhanced in RGCs under excitotoxic and oxidative stress conditions. The RGC-5 cell line as well as the primary rat RGCs maintained in culture were subjected to Glu + H_2O_2 treatment for 24 h. A. Shp2 and B. TrkB immunoprecipitations revealed their reciprocal interactions with each other. The binding was significantly enhanced upon exposure to stress conditions. Densitometric analysis of TrkB binding to Shp2 and *vice versa*. Data are mean ± SD, n = 3 each, *p<0.001.

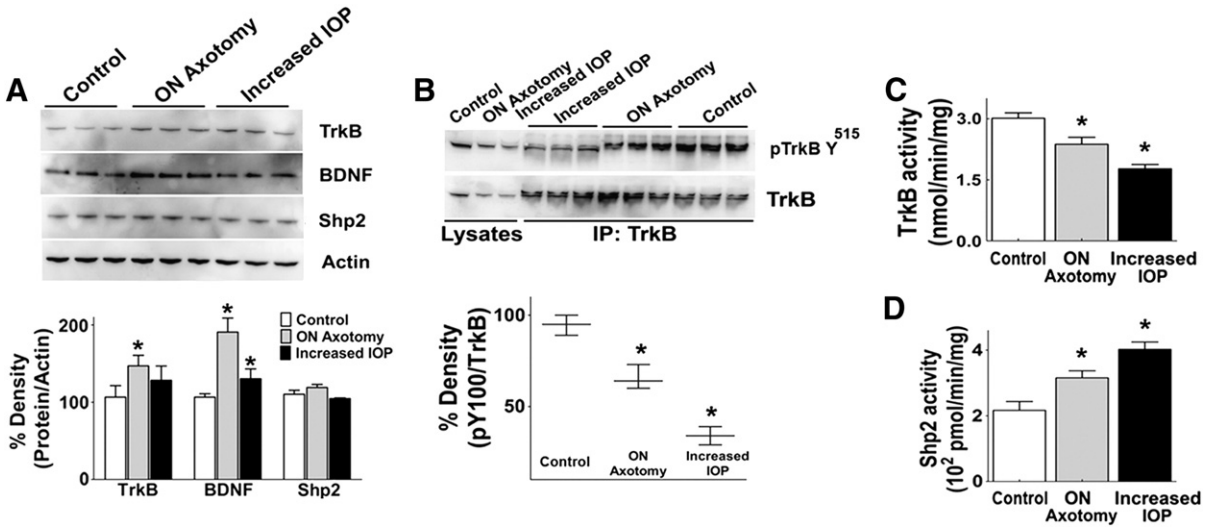

Fig. 3. The TrkB kinase and Shp2 phosphatase activities are altered in RGCs under stress. A. Protein expression of TrkB, BDNF and Shp2 in the rat retinal ganglion cells under control, ON axotomy and increased IOP conditions. Actin was used as the loading control in each case. Densitometric analysis of the band intensities relative to the actin expression in each case is plotted (*$p<0.05$). B. Immunoprecipitation of TrkB from rat RGCs followed by probing with pTrkB Y^{515} antibody reveals a loss of TrkB phosphorylation under stress conditions. Densitometric analysis in the linear range of detection revealed a significantly greater loss in TrkB phosphorylation in increased IOP conditions compared to the controls as well as ON axotomised retinas (*$p<0.03$). Immunoprecipitations of C. TrkB and D. Shp2 from RGC lysates (50 μg) followed by respective enzyme assays demonstrate a decline in TrkB activity and a corresponding increase in Shp2 activity in RGCs exposed to ON axotomy and chronic IOP stress (*$p<0.001$). Data are mean ± SD, n = 3 each experiment.

from RGCs isolated from the control, ON axotomised and increased IOP exposed retinas indicate a sharp decline in the Tyr^{515} phosphorylation of the TrkB receptor using pTrkB Y^{515} antibody (1:1000, Abcam). The loss of phosphorylation was higher in the increased IOP compared to the ON axotomised retinas. Quantification indicated that the phosphorylation was reduced to 65% of the original intensity in ON axotomised and 25% of the original in increased IOP exposed RGCs respectively (Fig. 3B). TrkB immunoprecipitates were also used to assess the tyrosine kinase activity of the TrkB receptor in control RGC lysates and those exposed to the above two stress conditions. Corresponding to the TrkB phosphorylation we observed a decline in TrkB tyrosine kinase activity in the RGC stress models *in vivo*. The TrkB activity was reduced by 20 and 50% respectively in the ON axotomy and increased IOP stress conditions (Fig. 3C). Conversely, an increase in the activity of Shp2 phosphatase (1.4 and 2 fold respectively) was observed in the RGC stress models (Fig. 3D). The Shp2 assays were performed on the immunoprecipitates using equal amounts of Shp2 antibody. Similarly, a decline in the TrkB Y^{515} phosphorylation and tyrosine kinase activity was observed in the RGC-5 cells exposed to Glu + H_2O_2 mediated excitotoxic and oxidative stress conditions. The phosphorylation analysis and kinase assays were carried out on the immunoprecipitates using equal amounts of TrkB antibody. The inhibition of Shp-2 phosphatase using Shp-2 inhibitor (Santa Cruz, CA) in culture resulted in a sustained maintenance of normal TrkB phosphorylation and kinase activity even under the indicated stress conditions (Fig. 4B,C). No effect of Shp2 inhibition on the TrkB phosphorylation/activity was however observed under the normal culture conditions. The expression of TrkB in each case is shown (Fig. 4B). The *in vivo* Shp2 inhibition in RGC-5 cells was established by the loss of Erk phosphorylation and maintenance of Akt phosphorylation [35]. There was also no effect on the Shp2 expression (Fig. 4A). Erk 1/2 (1:1000, Cell Signalling), and Actin (1:1000, Sigma) were used as the loading controls.

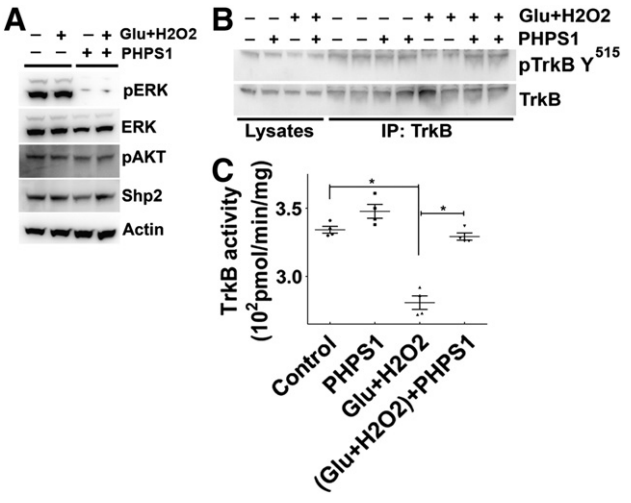

Fig. 4. Shp2 inhibition results in restoration of TrkB activity under the stress conditions. RGC-5 cells were incubated with Shp2 inhibitor PHPS1 (5 μM, Santa Cruz) for 24 h with or without Glu + H_2O_2 followed by washing the cells with PBS. A. Loss of Erk phosphorylation (Phospho-Erk1/2, 1:1000, Cell Signalling) but not the Akt indicated the effectiveness of the inhibitor. B. TrkB immunoprecipitates from cell lysates (100 μg) were immunoblotted and probed with phospho-TrkB Y^{515} and TrkB antibodies and (C) assayed for TrkB kinase activity indicating an increase in TrkB tyrosine kinase activity upon Shp2 inhibition under the indicated stress conditions. The protein expression of TrkB in total cellular lysate is shown in each case. (Data are mean ± SD, n = 4, $p<0.002$).

3.3. Caveolin interacts with Shp2 and undergoes hyperphosphorylation in RGCs under stress conditions

The Shp2 interactions with cav isoforms were investigated in RGCs under normal and glaucomatous stress conditions. Shp2 immunoprecipitations using equal amounts of Shp2 antibody followed by probing the immunoblots with cav-1 (Cell Signalling), 2 (Epitomics) and 3 (Cell Signalling) (1:1000) specific antibodies revealed their isoform specific interactions with Shp2. While cav-1 and cav-3 depict significantly several fold increased binding to Shp2 under RGC stress, a reduced Shp2–cav-2 interaction was observed under these conditions. RGC lysates indicate the expression of all these proteins under both normal and stress conditions (Fig. 5A). Further, the immunoprecipitations of cav1 and cav3 isoforms using equal amounts of specific antibodies followed by probing the immunoblots with pY100 antibody revealed that both cav-1 and cav-3 undergo hyperphosphorylation upon ON axotomy and under chronic IOP increase compared to the controls. Densitometric analysis revealed that cav-1 phosphorylation is enhanced 4 folds in RGCs under stress while cav-3 depicts more variability

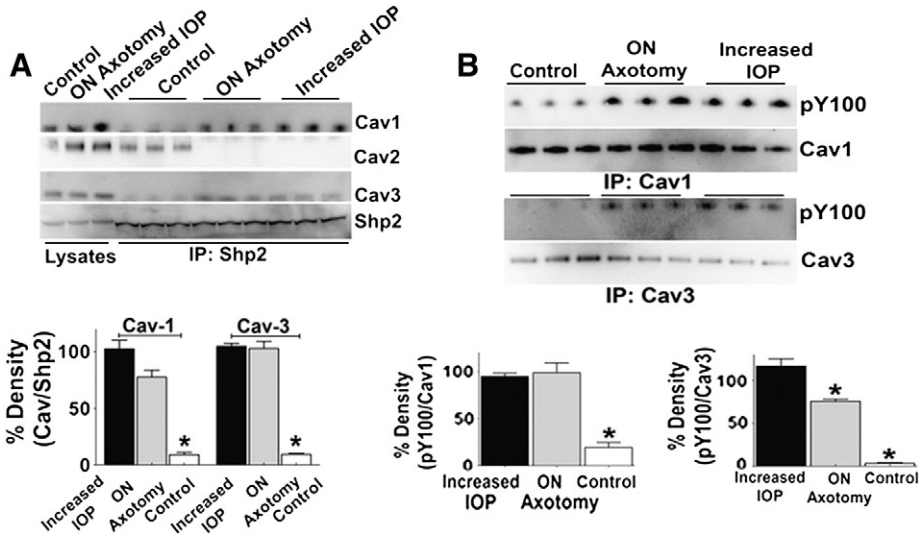

Fig. 5. Caveolin undergoes hyperphosphorylation and binds to Shp-2 in RGCs under glaucomatous stress. A. Shp2 immunoprecipitations indicate that cav1 and 3 but not cav2 depict an enhanced binding with Shp2 in RGCs from retinas exposed to ON axotomy or chronically increased IOP. Densitometric analysis revealed a significantly greater binding in the RGC stress models compared to the controls as indicated. Cav immunoprecipitation under chronically increased IOP conditions was set as 100% ($p<0.001$). B. Cav1 and 3 immunoprecipitations followed by probing with pY100 antibodies demonstrate that both cav1 and cav3 undergo hyper-phosphorylation in ON axotomy and chronically increased IOP conditions. The band intensities were analysed for quantification in the linear range of detection ($p<0.002$). The comparable expression of proteins in total cellular lysate is shown in each case (10 μg protein). Data are mean ± SD, n = 3 each experiment.

in its phosphorylation status in response to the type of stress faced by the RGCs (Fig. 5B).

3.4. Shp2–TrkB interaction is mediated through caveolin

Expression of cav-1, 2 and 3 isoforms was knocked down in the rat retinas and primary culture of RGCs using AONs as shown (Figs. 6A, 7A). The quantification of the band intensities indicated an efficient and significant knock down of all the cav isoforms *in vivo* as well as under the culture conditions (Figs. 6A, 7A). The 26 base chimeric sequence was 100% complementary to the mRNA sequences of cav-1 α and β isoforms, 70% complementary to the cav-2 and 89% complementary to the cav-3. RGC lysates were subjected to immunoprecipitation using specific TrkB and Shp2 antibodies (Fig. 6B). Densitometric quantification of the results indicate that the basal level of TrkB–Shp2 interaction was completely lost in the cav knock down RGCs. The expression of the two proteins was observed under both the control and cav knock down conditions. In another set of experiments where primary RGCs maintained under defined culture conditions were exposed to mild excitotoxic and oxidative stress indicated previously, we further observed a loss of the interaction of TrkB and Shp2 under cav knock down conditions. An interesting observation was a partial restoration of the Shp2–TrkB interaction upon exposure of the cav knock down cells to mild excitotoxic and oxidative stress using Glu + H_2O_2 further indicating an increased binding of the two proteins in stress conditions (Fig. 7B). RGCs maintained under normal conditions and ones exposed to stress conditions alone were used as controls.

4. Discussion

The prolonged dephosphorylation of TrkB hampers the axonal regeneration and other neuroprotective effects of BDNF and NT4 [36]. Our experiments establish that TrkB and Shp2 bind each other under normal physiological conditions in RGCs *in vivo*. The major finding of this study is that TrkB tyrosine kinase activity in the RGCs under stress

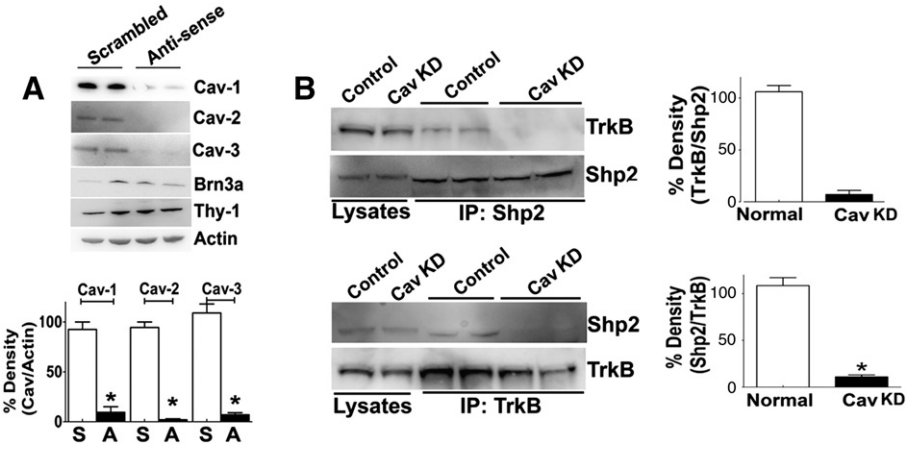

Fig. 6. TrkB–Shp2 interaction in retinal ganglion cells is dependent on the protein caveolin. A. Intravitreal administration of cav antisense (A) oligonucleotides (AONs) results in knock down (KD) of expression of cav 1, 2 and 3 isoforms in RGCs *in vivo*. Scrambled oligonucleotide (S) injections were used as controls (n = 20). Expression of ganglion cell markers Brn3a and Thy-1 is shown. Actin was used as loading control. Densitometric analysis of the band intensities is shown for each of the isoform and compared with scrambled injections. B. Reciprocal immunoprecipitations of Shp2 and TrkB reveal that their mutual interaction was lost in RGCs isolated from cav knock down retinas. The expression of each of the proteins is shown total cellular lysates. Densitometric analysis of the band intensities indicated that there was almost a complete loss of interaction between the two proteins upon cav knock down. Data are mean ± SD, $p<0.001$.

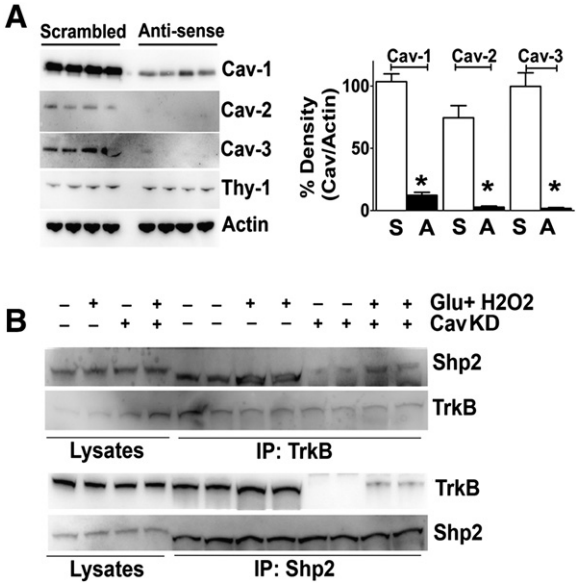

Fig. 7. Stress exposure to caveolin knock down RGCs results in restoration of TrkB–Shp2 interactions. A. RGCs isolated from rat retinas were treated with cav AONs to ablate cav expression. Treatment with scrambled oligos was used as control. Expression of RGC marker Thy-1 is shown. Actin was used as loading control. Densitometric analysis of the knock down of various cav isoforms is plotted. Data are mean ± SD, n = 4 each, $p<0.001$. B. Primary cultures of rat RGCs were either exposed to mild excitotoxic and oxidative stress in normal or cav knock down conditions. RGCs under normal and cav knock down conditions were used as controls. Reciprocal immunoprecipitations reveal that while TrkB–Shp2 interaction is enhanced under the indicated stress conditions, it is vanished upon cav ablation and partly restored when cav ablated RGCs are exposed to stress.

conditions is regulated through Shp2, highlighting an atypical TrkB–Shp2 interaction in a pathological process. Even though TrkB signalling can be stimulated by the BDNF/NT4 trophic factors, it will be short-lived in such cases as TrkB is simultaneously being dephosphorylated and deactivated by the associated phosphatase Shp2. Shp2 plays a role in establishing the neuronal fate in retinal development and MAP kinase signalling in Muller cells indicating it to be an important molecule in the retina [37].

TrkB phosphorylation at Tyr^{515} is important for the binding of Shc adaptor protein and downstream signalling events [38]. A loss of TrkB phosphorylation was observed under the stress conditions. Parallel to the loss of phosphorylation of the TrkB receptor at Y^{515}, the TrkB tyrosine kinase activity was down-regulated in both of the RGC stress models in retina (Fig. 3B). The Shp2-TrkB association was observed both *in vivo* (Fig. 1) and in the RGC neuronal culture under glaucomatous stress conditions (Fig. 2). The association was much more prominent in the case of chronic stress associated with the increase of IOP compared to the acute RGC distress model of ON axotomy (Fig. 1). TrkB expression was found to be enhanced in RGCs 1 week after ON axotomy corresponding to the previous report indicating an initial upregulation of TrkB mRNA [39]. No significant alteration in TrkB expression under the increased IOP was observed (Fig. 3A). We also observed an increased BDNF expression under both the acute as well as chronic stress models of RGCs at the indicated time points, similar to previous reports [40]. However, the TrkB phosphorylation was still observed to be decreased in both of the models, indicating the involvement of other regulatory mechanisms. A concurrent upregulation of Shp2 activity was observed upon stress induction (Fig. 3D). The expression of Shp2 protein was however unchanged under both stress conditions. Similar results were obtained in the RGC-5 cells where stress induction significantly decreased the TrkB activity, while inhibiting Shp2 under stress conditions restored the TrkB activity close to its original levels in the normal cells (Fig. 4). These experiments indicate that retinal stress triggers an activation of the Shp2 phosphatase which binds with the TrkB receptor and eventually lead to its deactivation. Inhibition of shp-2 activity in the normal cells however, did not significantly enhance the TrkB activity indicating that the Shp-2 undertakes a regulatory role for TrkB predominantly under the stress conditions (Fig. 4). The *Shp2* deletion in cerebral neuronal cultures alleviates TrkB inhibition and stimulates cell survival after treatment with glutamate [12]. Genetic ablation of Shp2 abolished Erk phosphorylation in the neural retina with pErk staining predominantly co-localising in the Muller cell bodies in the ganglion cell layer (GCL) and inner nuclear layer (INL) of retina leading to widespread retinal degeneration [37]. We observed similar loss of Erk phosphorylation in the RGC-5 cells upon pharmacological inhibition of Shp2 (Fig. 4). Shp2 also plays a role in the activation of Erk signalling in the cortical neurons and several of the human neuroblastoma cell-lines [11,41]. Further, Shp2 binding to the TrkB receptors is dependent on the protein caveolin. Cav-$1^{-/-}$ mice depict an enhanced Erk phosphorylation in the hippocampus [42]. Consistent with our *in vivo* findings in the RGCs, previous studies indicate that cav-1 undergoes hyperphosphorylation under the oxidative and osmotic cellular stress in fibroblast cultures [43,44]. In the trabecular meshwork cell lines obtained from glaucoma patients, a decline in cav-1 phosphorylation and expression upon treatment dexamethasone was observed [45], indicating that cav phosphorylation in glaucoma may be specific to RGCs. The effects of phosphorylation of cav isoforms on the Erk signalling in RGCs are not known. Cav plays a role in the Shp2 activation in astrocytes exposed to the oxidative stress [46]. It is shown to play a role in the maintenance of blood retinal barrier permeability, retinal homeostasis and outer retinal function [47,48]. The *cav* gene locus also appears to play an important role in glaucoma pathogenesis [49,50]. We observed a hyper-phosphorylation of cav1 and 3 in RGCs exposed to glaucomatous stress and a corresponding Shp2–cav interaction and Shp2 activation leading to the TrkB dephosphorylation. We observed an association of Shp2 with all the cav isoforms in the RGCs, although the association was more prominent for cav-1 and cav-3 under the stress conditions (Fig. 5A). The cav2–Shp2 interactions were surprisingly lost under the RGC stress conditions indicating that cav2 behaves differently compared to other isoforms. Corresponding to this interaction, we observed an enhanced tyrosine phosphorylation of the cav-1 and cav-3 (Fig. 5B). This experiment indicates that cav activates Shp2 which in turn dephosphorylates the TrkB receptor. Cav may bind to the SH2 domains of Shp2 thereby relieving the auto-inhibition of the Shp2 and revealing its previously latent phosphatase activity [51]. It is possible that cav acts as a signalling platform and recruits a pool of Shp2 in close proximity to the TrkB receptor, which translates into the increased dephosphorylation of the TrkB and downregulation of its signalling. The essential role of this interaction was further confirmed by the transient ablation of cav in RGCs *in vivo* (Fig. 6A) as well as in the isolated rat RGCs (Fig. 7A). Knock down of all cav isoforms to avoid mutual complementation, resulted in complete loss of the Shp2 and TrkB association (Fig. 6B). Since cav2 is unstable in the absence of cav1 [52], cav1 knock down further adds to the cav2 ablation. The association was partly restored when the cav ablated cells were exposed to stress further indicating that Shp2-TrkB association is enhanced under these conditions (Fig. 7B). The results corresponded to those observed by inhibiting Shp2 under the stress conditions (Fig. 4B,C).

This mechanism indicates that TrkB activity is tightly regulated by Shp2 in caveolin dependent manner in stressed RGCs, and could form the molecular basis of why TrkB eventually fails to respond to the protective effects of BDNF in glaucoma. It will be interesting to determine how this TrkB dephosphorylation is influenced by the Shp2 in other neurodegenerative disorders. This study therefore, suggests Shp2 as a promising drug target to protect TrkB activation and concomitantly provide neuroprotection to dying RGCs in glaucoma.

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