Increased levels of synaptic proteins involved in synaptic plasticity after chronic intraocular pressure elevation and modulation by brain-derived neurotrophic factor in a glaucoma animal model

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ABSTRACT
The dendrites of retinal ganglion cells (RGCs) synapse with the axon terminals of bipolar cells in the inner plexiform layer (IPL). Changes in the RGC dendrites and synapses between the bipolar cells in the inner retinal layer may critically alter the function of RGCs in glaucoma. The present study attempted to discover changes in the synapse using brain-derived neurotrophic factor (BDNF) after glaucoma induction by chronic intraocular pressure elevation in a rat model. Immunohistochemical staining revealed that the BDNF-injected group had a significant increase in the level of synaptophysin, which is a presynaptic vesicle protein, in the innermost IPL compared with the phosphate-buffered saline (PBS)-injected group. SMI-32, which is a marker of RGCs, was colocalized with synaptophysin in RGC dendrites, and this colocalization significantly increased in the BDNF-injected group. After the induction of glaucoma, the BDNF-injected group exhibited increases in the total number of ribbon synapses, as seen using electron microscopy. Expression of calcium/calmodulin-dependent protein kinase II (CaMKII), cAMP-response element binding protein (CREB) and F-actin, which are key molecules involved in synaptic changes were upregulated after BDNF injection. These initial findings show the capability of BDNF to induce beneficial synaptic changes in glaucoma.

KEY WORDS: Retinal ganglion cell, Neurodegeneration, Synapse, IOP, BDNF

INTRODUCTION
Glaucoma is a neurodegenerative disease for which the pathological hallmark is retinal ganglion cell (RGC) death (Quigley et al., 1981). A number of studies have reported that a chronic elevation in intraocular pressure (IOP), which is a clinical hallmark of glaucoma, induces axonal degeneration and the apoptosis of RGCs (Quigley, 1999; Quigley et al., 1995; Garcia-Valenzuela et al., 1995). However, there are synaptic degenerations and changes in the size and morphology of the soma and dendrites of RGCs before the initiation of axonal degeneration and RGC apoptosis (Shou et al., 2003; Weber et al., 1998). Dendrites of RGCs are thought to respond and show compensative changes after injury, such as increasing dendritic receptive fields and developing new branches (Schwab, 1996; Dancause et al., 2005; Papadopoulos et al., 2002). A previous study from our research group found that RGCs exhibit a dendritic response as well as synaptic changes following chronic IOP elevation (Park et al., 2014). In that study, we found an increase in synaptophysin, which is a presynaptic vesicle protein, and a change in the morphological characteristics of the synapses. These findings suggest that there are biological attempts to modulate synaptic plasticity after elevations in IOP, and therefore, the next step will be finding a method to enhance synaptic plasticity following chronic IOP elevation in glaucoma.

Brain-derived neurotrophic factor (BDNF) has been extensively investigated and is known to contribute to synaptic development and the formation of connections between neurons during development (Zhong et al., 2015; Leal et al., 2014; Lu, 2003). Although there are studies showing synaptic rearrangements and dendritic shrinkage after glaucoma induction, BDNF application exhibited a delay in dendritic retraction (Agostinone and Di Polo, 2015; Binley et al., 2016). There was a study showing the role of BDNF after exercise through an AMP-activated protein kinase pathway, which prevented synapse elimination, but there are few studies focusing on the synaptic elements after BDNF application in the retina (Chrysostomou et al., 2016). Calcium/calmodulin-dependent protein kinase II (CaMKII) and cAMP-response element binding protein (CREB) are reported to have a role in synaptic plasticity (Kotalessi and Blackwell, 2010; Maren and Baudry, 1995; Evans, 2007). Previous investigations showed CaMKII as a mediator of synaptic plasticity in hippocampal and other neurons, through activation of the CREB pathway (Bustos et al., 2017). Several attempts, including BDNF application, were made to modulate the CaMKII and CREB pathway to promote synaptic plasticity and neurite outgrowth (Yan et al., 2016; Clarkson et al., 2015). However, there are only a few studies that have investigated the CaMKII and CREB pathway in relation to synaptic changes in RGCs and its modulation by BDNF in glaucoma. Thus, the present study investigated the effects of BDNF on the modulation of RGC synapses and the CaMKII-CREB pathway after chronic elevation in IOP.

RESULTS
BDNF application reduces retinal stress and RGC apoptosis by inducing BDNF expression and phosphorylation of Akt in the retina after IOP elevation
One week prior to episcleral vein cauterization procedure, the experiment eyes of each rat received an intravitreal injection of BDNF (5 μg/10 μl) while the opposite eye received an intravitreal injection of phosphate-buffered saline (PBS) as control (Fig. 1A). Cauterization of the episcleral vein induced a sustained elevation of IOP throughout the entire 8-week experiment in the eyes that were
analyzed (Fig. 1B). More specifically, 1 week after the surgery, there was a gradual increase in IOP from a basal value of 10.2±1.96 mmHg to 29.5±2.12 mmHg. The average IOP in the cauterized eye over the 8-week experimental period was 27.3±2.19 mmHg; control eyes that underwent sham surgery maintained normal IOP throughout the experiment. The intravitreal injections of BDNF and PBS did not result in differences in IOP in either the sham or glaucoma surgery groups.
Fig. 1. Study scheme and results of BDNF application to RGC apoptosis and GFAP expression. (A) Schematic of the experimental time points. (B) Verifying elevations in intracocular pressure (IOP) in a chronic hypertension model of glaucoma. Changes in IOP after the cautерization procedure are shown. The IOPs of the control (sham-operated) and cauterized eyes were measured at 0, 1, 4 and 8 weeks after cautèrization. The IOP of the cauterized eyes remained elevated throughout the 8-week experimental period. The intravitreal injections of phosphate-buffered saline (PBS) or brain-derived neurotrophic factor (BDNF) did not influence IOP in either the control (sham-operated) or cauterized groups. For the IOP measurements, the PBS-injected group and the BDNF-injected group each included 3 animals at each time point; total n=24. (C) Confocal micrographs of the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay showing apoptotic retinal ganglion cells (RGCs) in the ganglion cell layer (GCL) at week 4 after cautèrization. There was a significant decrease in TUNEL-positive RGCs in the BDNF-injected group compared with the PBS-injected group. For the TUNEL assay, the PBS-injected group and the BDNF-injected group each included 6 retinas at baseline and week 4: 10 sections per retina were analyzed; total n=48. Scale bar: 50 μm. *P<0.05. (D) Confocal micrographs of flat-mounted retinas stained for Brn3a, a specific RGC marker, at week 4 after cautèrization. Brn3a-positive cells significantly decreased at 4 weeks after cautèrization compared to baseline, which was significantly preserved after BDNF injection. For the flat-mount retina stain, the baseline control, the PBS-injected group and the BDNF-injected group each included 4 retinas; 24 sections per retina were analyzed; total n=12. Scale bar: 50 μm. **P<0.05. (E) Confocal micrographs of cross-sectional retinas stained for glial fibrillary acidic protein (GFAP). The immunoreactivity for GFAP increased throughout the inner retinal layers after the induction of glaucoma until week 4, but significantly decreased in the BDNF-injected group compared with the PBS-injected group. For the GFAP staining, the PBS-injected group and the BDNF-injected group each included 6 retinas at each time point; 10 sections per retina were analyzed; total n=48. Scale bar: 50 μm. **P<0.05.

After confirming IOP elevation, retinal stress and RGC death were evaluated by a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay and immunostaining of glial fibrillary acidic protein (GFAP), respectively. TUNEL staining revealed increased RGC apoptosis at 4 weeks after cautèrization; however, the number of TUNEL-positive cells in the ganglion cell layer (GCL) significantly decreased after BDNF injection compared with the PBS-injected group (Fig. 1C). Additionally, Brn3a staining, a specific RGC marker, on flat-mounted retinas shows decreased Brn3a-positive cells in the GCL after 4 weeks of cautèrization compared to the control. However, the number of Brn3a-positive cells in the GCL was significantly increased after BDNF application (Fig. 1D). Immunoreactivity for GFAP was elevated in the GCL, inner plexiform layer (IPL), inner nuclear layer and outer plexiform layer after 1 and 4 weeks following cautèrization (Fig. 1E); however, increased GFAP expression was reduced throughout the inner retinal layers by intravitreal injections of BDNF after 1 and 4 weeks following cautèrization.

Western blot analysis revealed that, compared with the PBS-injected group, the BDNF-injected group had significantly higher levels of BDNF in the retina at baseline and 1 week after cautèrization (Fig. 2A), which was 1 week and 2 weeks after the BDNF injection, respectively. Synaptophysin and phosphorylated Akt (p-Akt) protein levels were significantly elevated at baseline (1 week after the injection) and week 1 (2 weeks after injection) in the BDNF-injected group compared to the PBS-injection group. Thereafter, synaptophysin and p-Akt protein level decreased to basal level at week 4 compared to the PBS-injected group in the western blot analysis.

The immunoreactivity of p-Akt increased in the innermost IPL and peaked at 1 week after cautèrization (Fig. 2B). Comparison between the BDNF- and PBS-injected groups shows that BDNF injection significantly increased expression of p-Akt in the innermost IPL at 1, 4 and 8 weeks after cautèrization.

Increased number of presynaptic vesicles co-stained with RGC dendrites after the application of BDNF

The presynaptic vesicle proteins were assessed by immunostaining for synaptophysin. Immunoreactivity of synaptophysin and co-labeling with protein kinase C-alpha (PKCα), which is a marker of bipolar cells, revealed that increased expression of synaptophysin occurred within the bipolar cells in the innermost IPL (Fig. 3A). The location was similar to the p-Akt expression in the innermost IPL. After BDNF injection, the co-labeling of synaptophysin and PKCα significantly increased in the IPL compared to the PBS-injection group after 1 week of cautèrization.

To investigate dendritic morphology and presynaptic vesicle protein expression, flat-mount preparations of the retinas were analyzed via an immunostaining procedure for markers of RGCs. From the flat-mounted retina, six z-stack images of 0.5-μm intervals were averaged, resulting in a scan of 2.5 μm thickness starting from the GCL surface. SMI-32, which is a marker of neurofilaments, stains both the soma and dendrites of the RGCs. In the PBS-injected group, the colocalization between SMI-32 and synaptophysin significantly increased at 1 and 4 weeks after cautèrization compared to baseline in the GCL (Fig. 3B). Compared with the PBS-injected group, the colocalization between SMI-32 and synaptophysin further increased in the BDNF-injected group at 1 and 4 weeks after cautèrization. The dendritic morphology of the RGCs in the BDNF-injected group shows an increased number of dendritic branches compared to the PBS-injected group at baseline and 1 week after cautèrization (Fig. 3B, magnified images).

The application of BDNF increased the number of ribbon synapses and expression of synaptic spines

The number of ribbon synapses in the IPL was quantified in 10 fields per 6 retinal sections from 6 eyes in each group using transmission electron microscopy (Fig. 4A). The numbers were 6.5/50 μm² and 2.1/50 μm² at baseline and 4 weeks after cautèrization, respectively, in the PBS-injected group, and 5.2/50 μm² and 4.5/50 μm² at baseline and 4 weeks after cautèrization, respectively, in the BDNF-injected group. The number of ribbon synapses were significantly increased in the BDNF-injected group at 4 weeks after cautèrization compared to the PBS-injected group.

F-actin (filamentous actin) is the final product of activated CaMKII and CREB, and composes the synaptic spine of dendrites. Immunostaining with anti-F-actin and -SMI-32 in retinal whole mounts focused on the GCL shows that BDNF injection increases F-actin expression around the dendrites of RGCs compared to the PBS-injection group at 4 and 8 weeks after cautèrization (Fig. 4B).

Changes in the molecular pathway involved in synaptic plasticity after application of BDNF

To find out the molecular pathway involved in the changes in synapses after IOP elevation and BDNF application, western blot analysis for proteins of N-methyl-D-aspartate receptor (NMDAR)1 and 2B, phosphorylated CaMKII (p-CaMKII) and phosphorylated CREB (p-CREB) were performed (Fig. 5). After BDNF injection, a significant increase in NMDAR1 and 2B, CaMKII, and p-CREB were found in the glaucomatous retina at week 4 compared to the PBS-injected group. Immunostainings for p-CaMKII and p-CREB were significantly increased in the RGCs, which were stained with optineurin as a ganglion cell marker, after BDNF injection compared to the PBS-injected group throughout the experimental periods (Fig. 6A,6B). The number of co-labelling RGCs with...
p-CaMKII and optineurin (OPN) significantly increased at all experimental time points in the BDNF-injected group compared with the PBS-injected group. The pixel area of p-CaMKII in the GCL was significantly increased in the BDNF-injection group compared to the PBS-injected group at baseline and 1 week after cauterization (Fig. 6A). The number of cells stained with p-CREB in the GCL showed a significant increase after BDNF-injection at 1, 4 and 8 weeks after cauterization (Fig. 6B).
Changes in the dendritic structure and morphology of RGCs occur prior to cell death in the glaucomatous eye; therefore, the present study aimed to investigate dendritic changes and synapses in greater detail. Early changes in RGC dendritic structure may have critical consequences for synaptic efficacy and may underlie functional deficits prior to RGC loss in patients with glaucoma (Weber and Harman, 2005). Our research group previously identified changes in synaptic vesicle proteins and the characteristics of the ribbon synapse after the induction of glaucoma (Park et al., 2014). In the present study, analyses of synaptophysin revealed that presynaptic proteins in the bipolar cells in the IPL gradually increased throughout the 8-week experimental period. Although the total number of synapses decreased due to significant RGC loss, there appeared to be attempts to increase the number of synaptic proteins and structures in the inner layers of the retina after chronic IOP elevation. Using electron microscopy, the present study identified immature and newly formed ribbon synapses in the inner retinal layers, which suggests that there is a compensatory mechanism for the restoration of synaptic connections between RGCs and bipolar cells following RGC apoptosis. The present findings indicate that this process can be modulated by the application of BDNF.

The present study also found that, in addition to synaptic changes, BDNF also modulated alterations in dendritic structure. In animal models of glaucoma using elevated IOP, the dendritic structure of the RGCs are either maintained or gain greater dendritic complexity (in a few select RGCs) compared with the control eyes (Kalesnykas et al., 2012). In the present study, the application of BDNF resulted in an increased number and thickening of the dendritic branches at each time point relative to the PBS-injected group. These results suggest that BDNF can modulate the morphological plasticity of RGC dendrites (Morgan et al., 2006). F-actin, which is the component of dendritic spines, increased around the RGC dendrites, indicating synaptic enhancement or synaptic plasticity. A number of studies have shown that BDNF is vital for maintaining the health of RGCs in the retina and for protecting RGCs from insult-induced apoptosis (Unoki and LaVail, 1994; Peinado-Ramon et al., 1996). Among the wide variety of neurotrophic factors, BDNF is considered to be the most potent survival factor for injured RGCs (Jelsma et al., 1993; Mansour-Robaey et al., 1994; Perez and Caminos, 1995) because it promotes neuronal survival in cell cultures (Thanos and Vanselow, 1989; Johnson et al., 1986), stimulates the growth of neurites from regenerating RGCs (Cohen-Cory and Fraser, 1995), and significantly reduces axotomy-induced damage of the optic nerve and RGCs in vivo (Carmignoto et al., 1989; Mey and Thanos, 1993). Additionally, BDNF plays a role in the regulation of pre- and post-synaptic proteins, the enhancement of synaptic transmission, and increases in synaptic vesicle docking in the developing brain (Pascual et al., 2001; Patterson et al., 1996; Kang and Schuman, 1995). Furthermore, the BDNF protein and its downstream tyrosine receptor kinase B receptor signaling factors, including Akt, have been consistently reported to contribute to synapse formation during development (Jia et al., 2008). Endogenous BDNF could be stimulated after glaucoma induction. As shown in our western blot analysis, there is an increase in BDNF expression at week 1 after glaucoma induction in the PBS-injection group. The synaptic plasticity after application of BDNF could be a result of both endogenous and exogenous BDNF.

In the present study, there was an increase in the level of synaptic vesicle proteins and docked synaptic vesicles after the BDNF injection. Furthermore, the innermost IPL exhibited an increase in phosphorylated Akt levels after the early phase of the BDNF injection, which may be explained by the role of BDNF in synapse enhancement (Kim and Park, 2005; Kim et al., 2007). Expression of CaMKII and CREB increased in the RGCs after BDNF injection until 4 weeks after IOP elevation. This represents an additional potential therapeutic application for BDNF via its enhancement of synaptic plasticity in the adult retina. In particular, NMDAR1 and 2B were increased after 4 weeks of IOP elevation in the BDNF-injected group. NMDAR contributes to synaptic plasticity that involves CaMKII and CREB phosphorylation and is critical for synaptic transmission (Riccio and Ginty, 2002; Lau and Zuki, 2007). From our study, BDNF increases pathways involving NMDAR and its downstream pathways to maintain synapses and increase synaptic plasticity of the RGCs.
induction until 8 weeks after glaucoma induction. This pattern was also shown in the present study and BDNF injection further increased synaptophysin expression at baseline and 1 week after glaucoma induction, which did not last at week 4. Repeated injections of BDNF or another type of delivery system may prolong the effects of this neurotrophic factor and should be further investigated to determine its application as a neuroenhancement therapy in glaucoma due to its ability to strengthen the synapse in the inner retina.

**Conclusions**

The present study demonstrated that the application of BDNF increased the expression of synaptic vesicle proteins in the inner retina after the induction of glaucoma. Additionally, BDNF increased the total number of synapses between the RGCs and bipolar cells in the glaucomatous retina. Involved pathways were p-Akt, CaMKII and CREB, which increased F-actin in the RGC dendrites. These initial findings regarding the capability of BDNF...
Fig. 3. Effects of BDNF application to expression of synaptophysin and dendritic structures. (A) Confocal micrographs of retinal sections double-stained for synaptophysin, which is a marker of synaptic vesicles, and protein kinase C-alpha (PKCα), which is a marker of bipolar cells, 1 week after cautery. In the PBS-injected group, synaptophysin was expressed in the innermost inner plexiform layer (IPL) and the outer plexiform layer (OPL) at week 4 after the induction of glaucoma; these levels increased after the injection of BDNF. Furthermore, the expression of synaptophysin and the co-staining between synaptophysin and PKCα were both increased in the innermost IPL in the BDNF-injected group compared with the PBS-injected group. Magnified confocal micrographs of the innermost IPL showed that the co-stained area significantly increased in the BDNF-injected group compared with the PBS-injected group. For the synaptophysin staining and quantification, the PBS-injected group and the BDNF-injected group each included 6 retinas at baseline and week 1; 10 sections per retina were analyzed; total n=18. Scale bars: 50 μm. (B) Confocal micrographs of flat-mounted retinas double-stained for synaptophysin, which is a marker of synaptic vesicles, and SMI-32, which is an RGC marker, focused on the border of the ganglion cell layer (GCL) and the IPL. SMI-32 staining was observed in both the soma and dendrites of the RGCs. Synaptophysin immunoreactivity increased at weeks 1 and 4 after the induction of glaucoma compared with week 0 (control). After the application of BDNF, SMI-32-positive RGCs increased at weeks 0 and 1 along with increased synaptophysin immunoreactivity at weeks 1 and 4. Additionally, the SMI-32 immunoreactivity revealed increased and thickened RGC dendrites at weeks 0, 1 and 4 after the BDNF injection. For the flat-mount synaptophysin staining and quantification, the PBS-injected group and the BDNF-injected group each included 6 retinas at baseline, week 1 and week 4; 10 sections per retina were analyzed; total n=36. Scale bars: 50 μm.

After the surgery, chloramphenicol eye drops and oxytetracycline ointment were applied to the eyes. Only the eyes that did not suffer scleral burns with subsequent necrosis or any surgical complications were used for the present study. IOP was measured directly and carefully with a tonometer (Tono-Pen) after topical anesthetization with a proparacaine hydrochloride ophthalmic solution (Alcane; Alcon Laboratories, Fort Worth, TX, USA); the animals were kept as calm as possible to minimize any effects on the IOP readings. The experimental analyses were performed at 1, 4 and 8 weeks after the cauteryation, and any eyes that did not show sustained IOP throughout the 8-week experimental period were excluded from the analyses.

**Intravitreal injections**

Prior to the surgery, the rats were anesthetized with intraperitoneal injection of tiletamine-zolazepam (50 mg/kg; Zoletil-Virbac) and xylazine hydrochloride (15 mg/kg; Rompun, Bayer). Next, the pupils were dilated with eye drops containing 0.5% tropicamide and 2.5% phenylephrine hydrochloride, and the ocular surface was anesthetized with a topical application of 0.5% proparacaine hydrochloride (Alcane). One week prior to the episcleral vein cauteryation, a microinjection needle was used to deliver either 5 μg/10 μl of BDNF or 10 μl of PBS intravitreally.

**Tissue preparation**

For the immunohistochemical analyses at each time point, the eyes were quickly enucleated and dissected. The posterior eye cups were placed in chilled fixative (4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4). Then, the isolated retinas were immersed in the same fixative for 2 h at 4°C. After several washes, the fixed retinas were cryoprotected in 30% sucrose containing 0.1 M PB for 6 h at 4°C and then stored in the same buffer at −70°C. For the electron microscopy analyses, the retinal tissues were fixed in glutaraldehyde. For the western blot analysis, the retinal tissues were quickly dissected, frozen in liquid nitrogen and stored at −70°C.

**TUNEL assay**

The presence of apoptotic cells was evaluated using TUNEL staining. For this procedure, the retinas were dissected from the choroid and the central portion of the superior nasal quadrant (1.5 mm from the optic disc) was trimmed into small pieces. Next, cryosections of the retina (50 μm) were immersed in 4% paraformaldehyde and washed with PBS. The tissue was stained using the TUNEL method according to the manufacturer’s protocol (In Situ Cell Death Detection Kit; Roche Applied Science, Indianapolis, IN, USA). The next day, the sections were washed several times in PBS, incubated with goat anti-rabbit Alexa® Fluor 546 (Molecular Probes, Eugene, OR, USA), washed again several times in PBS, immersed in 0.1 M PB for 30 min, and then mounted using VECTASHIELD® Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI; H-1206; Vector Laboratories, Burlingame, CA, USA). The sections were washed, coverslipped and examined using confocal laser scanning microscopy (Zeiss, Germany).

For the quantification of TUNEL-positive cells, positive immunohistochemically stained cells in the GCL were counted in each retinal section (40× magnified cross-sectional images of the retina). TUNEL assay was performed in controls and at week 4 in both sham-operated controls and cauterized eyes (total of n=24). In each group, 6 retinas from 6 eyes were analyzed with 10 retinal sections per retina. Mean value of the count from 10 retinal sections was used in the statistical analysis.

**Transmission electron microscopy**

The electron microscopy analyses were conducted using retinal sections from vein-cauterized and sham-operated subjects 4 weeks after the surgery (6 retinas per group); 10 fields of each eye were examined. Retinal sections (100 μm) were cut with a vibratome, post-fixed with 4% glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.4) for 1 h, and then fixed with 1% osmium tetroxide in 0.1 mol/l cacodylate buffer for 2 h. After a rinse with distilled water, the sections were treated overnight with 1% aqueous uranyl acetate, dehydrated in increasing concentrations of ethanol solutions up to 100%, further dehydrated with dry acetone, and then embedded using Durcupan™ ACM. Ultrathin sections (0.1 μm) were cut and mounted on Formvar-coated slot grids, stained with 3% lead citrate and examined with a Zeiss transmission electron microscope (Zeiss). The ribbon synapses in the
IPL between RGCs and bipolar cells were counted in 50 micrographs (2500 μm² total) for every 10 fields in each retina. For the quantification, the average of counts from 10 fields in 6 retinas were used in the statistical analysis.

**Immunohistochemistry**

The retinal expression of synaptophysin was assessed to evaluate the presence of synaptic vesicles. For the fluorescence staining, the samples were pre-embedded in 3% agar in deionized water and then sliced with a...
vibratome (50 μm) and washed several times in PBS. The sections were incubated in 10% normal donkey serum in PBS for 1 h at room temperature to block nonspecific binding activity and then incubated in anti-rabbit synaptophysin (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After several washes with PBS, the sections were incubated with goat anti-rabbit Alexa® Fluor 488 (Molecular Probes). For the double-labeling studies, the sections were incubated with either anti-mouse PKCζ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), SMI-32 (Covance, Emeryville, CA, USA), OPN (Santa Cruz Biotechnology), p-Akt (Cell Signaling Technology, Boston, MA, USA), p-Calmodulin II (Santa Cruz Biotechnology), p-CREB (Cell Signaling Technology), F-actin (Cell Signaling Technology), NMDAR1 (Cell Signaling Technology) and NMDAR2B (Cell Signaling Technology) in 0.1 M PBS containing 0.5% Triton X-100 overnight at 4°C. The sections were then rinsed with 0.1 M PBS for 30 min and incubated with goat anti-mouse Alexa® Fluor 488 (Molecular Probes) for 90 min at room temperature. After further washing in 0.1 M PB for 30 min, the sections were mounted using VECTASHIELD® Mounting Medium with DAPI (H-1200; Vector Laboratories). The slides were washed, covered with coverslips and examined by confocal laser scanning microscopy (Zeiss LSM 510, Carl Zeiss Co. Ltd). For flat-mounted retinas, images were taken starting from the surface of the GCL. Averages of 6 z-stack images of 0.5-μm intervals were taken, resulting in a 2.5-μm thickness scan starting from the GCL surface.

For the quantification of immunohistochemical images, images of the target protein were converted into a binary slab through the mean threshold algorithm of ImageJ software (http://rsb.info.nih.gov/ij/index.html), which automatically computes the threshold value as the mean of the local grayscale distribution. Each binarized 8 bit image was converted into a red, green, blue color model and then split into 3 channels (red, green and blue). After assigning white pixels as target protein and black pixels as background, the expression level of target protein in the GCL was calculated using ImageJ. For the quantification, 6 retinas from 6 eyes were analyzed with 10 retinal sections per each group. The mean value of the count from 10 retinal sections was used in the statistical analysis.

**Brd3a staining and quantification from flat-mount retina**

Immediately after sacrifice, the superior side of each eye was marked for orientation, and both eyes were enucleated. The anterior segments were removed and the posterior segments were fixed in 4% paraformaldehyde in 0.1 M PB, pH 7.4, for 30 min. The retina was then isolated, divided into 4 equal quadrants and flat-mounted on slides. The retinas were incubated in 0.5% Triton X-100 and then with 10% normal donkey serum in PBS overnight at 4°C. After several PBS washes, tissues were incubated with anti-mouse Brd3a (Santa Cruz Biotechnology) overnight at 4°C. After several washes with PBS, the sections were incubated with goat anti-rabbit Alexa® Fluor 546 (Molecular Probes). Brd3a-positive RGCs were counted as previously reported (Levkovitch-Verbin et al. 2003). Briefly, each retinal quadrant was divided into central, middle and peripheral regions (1, 2 and 3 mm from the optic disc, respectively), and microscopic fields measuring 200×250 μm² were selected. Labeled ganglion cells were counted at 200× magnification in 4 central regions, 8 middle regions and 12 peripheral regions in the 4 quadrants of the retina. Corresponding regions from each retina of experimental and control groups were used for counting.

**Western blot analysis**

Both the control and experimental retinas were homogenized in a radioimmunoassay precipitation assay (RIPA) buffer consisting of 1% Triton X-100, 5% sodium dodecyl sulfate (SDS), 5% deoxycholic acid, 0.5 M Tris-HCl (pH 7.5), 10% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 200 mM sodium orthovanadate and 200 mM sodium fluoride. The tissue extracts were incubated for 10 min on ice and clarified by centrifugation at 10,000 g for 25 min at 4°C. Total protein and clarified by centrifugation at 10,000 g for 25 min at 4°C.
levels in the retinal extracts were measured using a standard bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). The retinal extracts (40 μg of total protein) were then resuspended in 5× sample buffer (60 mM Tris-HCl, pH 7.4, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% Bromophenol Blue) at a 4:1 ratio, boiled for 5 min and resolved using an SDS-polyacrylamide gel electrophoresis (PAGE) procedure.

The proteins were then transferred onto a nitrocellulose membrane and the blots were stained with Ponceau S (Sigma-Aldrich, St Louis, MO, USA) to visualize the protein bands, and ensure equal protein loading and uniform transfer. The blots were washed and blocked for 45 min with 5% non-dried skim milk in Tris-buffered saline with Tween 20 (TBST; 20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.1% Tween 20) and then probed for 24 h using

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**Fig. 6. Effects of BDNF application to expression of CaMKII and CREB.**

(A) Confocal micrographs of retinal sections stained for p-CaMKII and optineurin (OPN), which is a ganglion cell marker. The double-stained cells in the GCL are marked with white arrows. BDNF-injected retinas showed a significant increase in double-stained cells in the GCL at all experimental time points. The pixel areas for p-CaMKII in the GCL showed significant upregulated expression at baseline and 1 week after cauterization. For the immunohistochemical staining, the PBS-injected group and the BDNF-injected group each included 6 rats at each time point; total n=36. Scale bar: 50 μm. *P<0.05.

(B) Confocal micrographs of retinal sections stained for p-CREB and OPN. The double-stained cells in the GCL are marked with white arrows. After cauterization, expression of CREB decreased in the GCL at all experimental time points compared with the baseline. However, a significant increase in expression of CREB was found after BDNF injection and the double-stained cells with CREB and OPN in the GCL significantly increased at baseline, 1 week and 4 weeks after cauterization. For both p-CaMKII and p-CREB staining, the PBS-injected group and the BDNF-injected group each included 6 retinas at each time point; total n=48. Scale bar: 50 μm. *P<0.05.
antibodies against synaptophysin (Cell Signaling Technology), BDNF (Santa Cruz Biotecnology, Inc.), p-Akt, Akt (Cell Signaling Technology), p-CaMKII (Santa Cruz Biotecnology), p-CREB (Cell Signaling Technology), F-actin (Cell Signaling Technology), NMDAR1 (Cell Signaling Technology), NMDAR2B (Cell Signaling Technology) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Sigma-Aldrich). The blots were then probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies. Bound antibodies were detected using an enhanced chemiluminescence system (Amersham, Piscataway, NJ, USA) and X-ray film. The relative intensity was measured using an ImageMaster® VDS (Pharmacia Biotech, Piscataway, NJ, USA); the fold changes in these protein levels are indicated below the blots. The results are representative of 5 independent experiments and all data are expressed as mean±s.d.

**Statistical analysis**

All data are expressed as means±s.d. Comparisons between time points or with the controls were performed using the Student’s t-test and multiple comparisons using the Scheffe’s post hoc method. Differences with P<0.05 were considered statistically significant.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

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