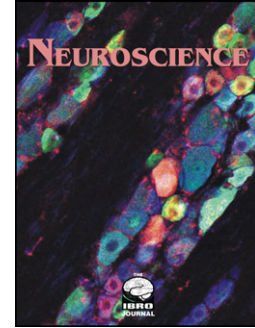


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## Combined Effect of Brain-Derived Neurotrophic Factor and LINGO-1 Fusion Protein on Long-term Survival of Retinal Ganglion Cells in Chronic Glaucoma

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**Abstract**

Glaucoma is a progressive neuropathy characterized by loss of vision as a result of retinal ganglion cell (RGC) death. There are no effective neuroprotectants to treat this disorder. Brain-derived neurotrophic factor (BDNF) is well known to transiently delay RGC death in the ocular hypertensive eyes. The central nervous system-specific leucine-rich repeat protein LINGO-1 contributes to the negative regulation to some trophic pathways. We thereby examined whether BDNF combined with LINGO-1 antagonists can promote long-term RGC survival after ocular hypertension. In this study, intraocular pressure was elevated in adult rats using an argon laser to photocoagulate the episcleral and limbal veins. BDNF alone shows slight neuroprotection to RGCs after a long-term progress of 4 weeks following the induction of ocular hypertension. However, combination of BDNF and LINGO-1-Fc prevents RGC death in the same condition. We further identified that 1) LINGO-1 was co-expressed with BDNF receptor, TrkB in the RGCs, and 2) BDNF combined with LINGO-1-Fc activated more TrkB in the injured retina compared to BDNF alone. These results indicate that the combination of BDNF with LINGO-1 antagonist can provide long-term protection for RGCs in a chronic ocular hypertension model. TrkB may be the predominant mediator of this neuroprotection.

Keywords: Ocular hypertension; Neural cells; Neuronal survival; Neurotrophic factors; TrkB

Glaucoma represents a group of neurodegenerative diseases characterized by structural damage to the optic nerve and slow, progressive death of retinal ganglion

cells (RGCs) (Quigley et al., 1995). Although the most important trigger for progression of glaucomatous damage is an elevation of intraocular pressure (IOP), the exact mechanism remains unknown. Current standard therapy for glaucoma is to lower the IOP by medication or surgery that may delay disease progression but does not alter RGC loss and axon degeneration. Therefore, treatment of glaucomatous neuropathy requires the preservation, protection, and rescue of RGCs. Although several approaches for neuroprotection have been described including the use of neurotrophins (Cui et al., 1998), no effective clinical neuroprotectants are available.

Neurotrophic agents have been implicated in survival- and growth-promoting activity in the central nerve system (CNS). Brain-derived neurotrophic factor (BDNF) is an important survival factor for RGCs. BDNF rescues RGCs from death after optic nerve axotomy (Yan et al., 1999; Zhang et al., 2005; Mansour-Robaey et al., 1994; Mey and Thanos, 1993) and in cell culture (Johnson et al., 1986). Exogenously applied BDNF can delay the death of RGCs in animal glaucoma models (Ko et al., 2001; Quigley et al., 2000). However, a series of studies showed that BDNF did not rescue all RGCs after optic nerve axotomy and only delayed RGC death (Mansour-Robaey et al., 1994; Mey and Thanos, 1993; Peinado-Ramon et al., 1996; Klocker et al., 1998). Even repeated intravitreal injections of BDNF, or persistent availability of bioactive BDNF did not promote long-term survival of RGCs after axotomy (Mansour-Robaey et al., 1994; Di et al., 1998; Isenmann et al., 1998). The protective effect of exogenous BDNF or overexpression of BDNF gene on RGCs was also limited in a chronic glaucoma model (Martin et al., 2003; Ko et al., 2001). A clearer mechanistic understanding of the limited neuroprotection of BDNF can be crucial for the development of treatments for glaucoma.

LINGO-1 is a leucine-rich repeat Ig-containing protein first identified as a critical component of Nogo receptor /p75 or TROY signaling complexes that prevent

axonal regeneration in the presence of myelin inhibitors in the CNS (Shao et al., 2005; Mi et al., 2004). LINGO-1 is specific to the CNS and functions as a negative regulator of axonal regeneration and neuronal survival. We previously found that blocking the function of LINGO-1 protected a major proportion of injured RGCs in rats with ocular hypertension (Fu et al., 2008a). LINGO-1 binds to the epidermal growth factor receptor to negatively regulates its activation (Inoue et al., 2007). We just identified that LINGO-1 binds with BDNF receptor, TrkB and inhibits TrkB activation *in vitro* and in the rat retinas (Fu QL et al., unpublished data). These data suggest that the negative regulatory functions of LINGO-1 may be involved in the limited neuroprotective effect of BDNF and it could be reversed after blocking the LINGO-1 function.

In this study, we investigated the combined neuroprotective effect of BDNF and a LINGO-1 antagonist, soluble LINGO-1 protein in a rat ocular hypertension model. We also investigated the involvement of TrkB activation with neuroprotection in this model.

## EXPERIMENTAL PROCEDURES

### Generation of recombinant LINGO-1-Fc

LINGO-1-Fc protein (soluble LINGO-1) was prepared as described previously (Mi et al., 2004). Residues 1-532 of human LINGO-1 was fused to the hinge and Fc region of human IgG1, expressed in CHO cells and purified on Protein A Sepharose (Pharmacia). The purified protein (> 95% pure) ran on SDS-PAGE with  $M_r = 90$  kDa under reducing conditions and  $M_r = 180$  kDa under non-reducing conditions. Recombinant human BDNF was purchased from Regeneron Pharmaceutical (Tarrytown, NY).

## **Animals**

Adult female Sprague-Dawley rats weighing approximately 250-280 g were reared in a temperature-controlled room on a 12-hour light–dark cycle in the Laboratory Animal Unit of The University of Hong Kong. A total of 32 rats was used for the study of RGC survival ( $n = 8$  for each group). The animal number is 4-5 for each group for the experiments on histochemistry and western blotting. All the experimental and animal handling procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were also reviewed and approved by the Faculty Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong. The animals were anesthetized with intraperitoneal injection of ketamine (80 mg/kg, 10% alfasan, Woerden, Netherlands) and xylazine (8 mg/kg, 2% alfasan, Woerden, Netherlands) during the experiments and were euthanized with an overdose of pentobarbital sodium (150 mg/kg, Alcon-Couvreur, Rijksweg, Puurs, Belgium). Alcaine 0.5% (Alcon-Couvreur, Rijksweg, Puurs, Belgium) was applied to the eyes before all operations and antiseptic eye drops (Tobres [Tobramycin 0.3%], Alcon-Couvreur, Rijksweg, Puurs, Belgium) were used to prevent infection after the treatment. Rimadyl (0.025mg/ml, Pfizer, NY, USA) in drinking water was used to relieve the pain for 7 days after the surgeries.

## **Ocular hypertension model and treatments**

To induce experimental ocular hypertension, the rats received argon laser (Ultima 2000SE Argon Laser, Coherent, Palo Alto, CA) photocoagulation of the episcleral and limbal veins two times with 7 days apart in the right eye. This technique was adopted from the method by WoldeMussie et al (WoldeMussie et al., 2001) and has been used in our laboratory with a track record of publications (Fu et al., 2008b; Fu et al., 2008a; Ji et al., 2004; Li et al., 2006b; Li et al., 2006a). About 90 spots were

applied on the three episcleral veins and 70 spots on the limbal vein (270° around the limbus, except on the nasal side) with the following settings: power of 1000 mW; spot size of 50 µm in diameter; and duration of 0.1s. The contralateral (left) eyes were used as controls. Animals were allowed to survive for 4 weeks post first laser exposure before they were sacrificed. The IOP of the eyes were measured using a Tonopen XL Tonometer (Mentor, Norwell, MA, USA) at different time points.

The eyes with elevated IOP received intravitreal injection of proteins once a week in the 4-week glaucoma animals on day 0, 7, 14 and 21. The animals were divided into 4 groups, 0.01M phosphate buffered saline (PBS) group (2 µl), combination of BDNF (1 µg in 1 µl) and PBS (2 µl) group, LINGO-1-Fc group (2 µg in 2 µl), combination of BDNF (1 µg in 1 µl) and LINGO-1-Fc group (2 µg in 2 µl). The protein solution was injected intravitreally into the right eye using a 10 µl Hamilton microsyringe fixed with a 26-s gauge needle (#80300, Hamilton, Reno, Nevada, USA). The microsyringe was held inside the eyeball for more than 1 minute before being pulled out. The site of injection was just below of the limbus of the cornea, which provides minimal possibility of injury to the retina. The injections for combined proteins were performed at 30 min intervals.

### **Retrograde labeling of RGCs and RGC counting**

In order to retrogradely label RGCs, both superior colliculi were exposed and a piece of Gelfoam (Pharmacia & Upjohn, NJ, USA) soaked with Fluoro-Gold (FG, 6% in distilled H<sub>2</sub>O, Fluorochrome, Denver, CO) was placed on the surface of superior colliculi seven days before sacrifice. At 4 weeks after laser coagulation, the rats were transcardially perfused with 0.9% saline for 30 min. Both eyes of each animal were enucleated and fixed in 4% paraformaldehyde for 60 min. Retinas were prepared as flat-mounts and the FG labeled RGCs were counted under fluorescence microscope

using an ultra-violet filter (excitation wave length = 330-380 nm). The RGCs were quantified under an eyepiece grid of  $200 \times 200 \mu\text{m}^2$  along the mid line of each quadrant, from the optic disc to the border at 500  $\mu\text{m}$  intervals (Ji *et al.*, 2004). Eight microscopic fields for each quadrant and a total of 32 per retina for four quadrants were counted. The data were expressed as the density of cells (number of cells/ $\text{mm}^2$ ) and also analyzed in terms of relative percent RGC loss in the injured right eye to the contralateral left intact eye from the same animal.

### **Immunohistochemistry for TrkB and LINGO-1**

RGCs were retrogradely labeled with FG 7 days before sacrifice. The eyes were enucleated at 4 weeks after laser treatment following transcardial perfusion with 0.9% saline and were fixed in 4% paraformaldehyde for 1 h. After removing the cornea and lens, the eye cups were fixed further in paraformaldehyde for 4-6 h and then transferred to 30% sucrose solution at 4°C for 16 h. The eye cups were embedded in OCT compound and cryosections (10  $\mu\text{m}$  thick) were prepared at -20°C. The retinal sections were washed with 0.01M PBS (PH 7.4), incubated in 0.5% Triton/PBS for 10 min, and blocked with 10% normal goat serum and 0.1% Triton/PBS for 1 h. Incubation with mouse antibody for LINGO-1 (1:100, Biogen Idec, Inc. MA, USA) and chicken IgY TrkB antibody (1:100, Promega, Mannheim, Germany) was performed at 4°C for 16 h. After washing with PBS, the sections were treated with Qdot@655 goat IgG anti-chicken IgY (1:400, Molecular probes, Oregon, USA) or Alexa-labeled goat anti-mouse 488 antibodies (1:400, Molecular probes, Oregon, USA) at room temperature for 2 h. After washing, the sections were mounted with fluorescent mounting medium (DakoCytomation, Denmark) and analyzed under Carl Zeiss LSM 510 META confocal microscope. We used the multi-photon laser (720 nm)



to excite the FG because this confocal microscope has no UV excitation laser. We regarded blue as the color of FG. The animal number was 4-5 for each group.

### **Western blotting**

To measure the effects of BDNF and LINGO-1-Fc on TrkB phosphorylation, we injected recombinant human BDNF intravitreally (1  $\mu$ g/injection) together with PBS (2 $\mu$ l) or BDNF together with LINGO-1-Fc (2  $\mu$ g/injection) just after laser coagulation and then euthanized the animals 5 days later.

Retinas were dissected and homogenized in lysis buffer (10 mM Tris pH7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA) supplemented with 10% protease inhibitor cocktail and 1% phosphatase inhibitor cocktails from Sigma. Following centrifugation at 13,000 rpm for 30 min to remove cell debris, the protein concentration of the supernatant was measured using a Bio-Rad DC protein Assay Kit (Bio-Rad Laboratories, CA, USA). A 80  $\mu$ g aliquot of proteins from each sample was subjected to 6-10 % SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membrane (Bio-Rad Laboratories, CA, USA). The membranes were blocked with 5% non fat dry milk and 2% bovine serum albumin (Sigma, MO, USA) in Tris-buffered saline containing 0.1% Tween 20 for 1 h in room temperature. Incubation with primary antibodies for phosphor-TrkB (p-TrkB, Tyr785, 1:100, a gift from Dr. B. Sun, Shanghai Institutes of Biological Sciences, Shanghai, China) (Ji et al., 2005) or chicken IgY total TrkB antibody (1:100, Promega, Mannheim, Germany) After washing, the membranes were incubated with Horseradish Peroxidas-conjugated goat anti-rabbit (1:2000, DakoCytomation, Denmark) or anti-IgY antibody (1:1000, Promega, Mannheim, Germany) in 5% non fat dry milk and 2% bovine serum albumin for 2 h at room temperature and immunoreactive proteins were detected

using the enhanced chemiluminescence method (Amersham, Piscataway, NJ, USA). The intensity of each band was quantified with densitometric scanning using Labworks gel documentation (UVP, Inc, Upland, CA). All experiments for western blotting were performed with 4-5 animals in each group and the samples from each animal were run on separate lanes. The levels of p-TrkB were finally expressed as relative values compared to total TrkB.

### **Statistics**

The data were expressed as Mean  $\pm$  SEM. Statistical analysis was performed by one-way analysis of variance followed by post-hoc tests (Turkey HSD) for comparisons of more than two groups or using paired Student's *t* test for comparisons between the right and contralateral left eyes in the same group. Data were analyzed statistically with the software SPSS 12.0. The mean difference is significant at 0.05 level.

## **RESULTS**

### **Long-term RGC survival with the combined treatment of BDNF with LINGO-1 antagonist after ocular hypertension**

Our previous findings that LINGO-1 negatively regulates TrkB function suggest that BDNF may activate more TrkB after blocking the function of LINGO-1. To determine the long-term effect of BDNF on RGC survival, we induced chronic ocular hypertension in rats and examined RGC survival 4 weeks after the induction of elevated IOP. The right eyes received a total of 4 intravitreal injections of proteins. As reported in our previous study (Fu *et al.*, 2008a), there was a significant loss of  $20.9 \pm 1.2\%$  RGCs at 4 weeks after laser coagulation in animals receiving PBS injection [ $1638 \pm 32$  cells per  $\text{mm}^2$  in experimental right eyes vs  $2070 \pm 59$  cells per  $\text{mm}^2$  in left

control eyes; Paired Student *t*-test: paired differences,  $431.8 \pm 76.4$ ,  $t(7) = 5.65$ ,  $P = 0.001$ ] (Fig. 1B). The treatment by BDNF and LINGO-1-Fc resulted in a significant reduction of the RGC loss in experimental eyes (one-way analysis of variance,  $F_{3,28} = 31.48$ ,  $P < 0.0001$ ). Consistent with previous report (Ko et al., 2001), four injections of BDNF only showed a partial neuroprotective effect on RGCs compared with PBS treatment group ( $P = 0.018$ , RGC loss,  $15.3 \pm 1.7\%$ ) (Fig. 1A). Combined treatments with BDNF and LINGO-1-Fc rescued almost all of the RGCs compared with the BDNF+PBS group ( $P < 0.0001$ ) and LINGO-1-Fc alone group (RGC loss,  $8.8 \pm 1.5\%$ ,  $P = 0.004$ ) (Fig. 1A). Blocking the function of LINGO-1 with LINGO-1-Fc significantly reduced RGC loss compared with PBS group ( $P < 0.0001$ , Fig. 1A). However, there was still a significant RGC loss with the LINGO-1-Fc treatment alone [paired Student *t*-test between right and left eyes,  $t(7) = 2.431$ ,  $P = 0.034$ ] (Fig. 1B). In this study we also analyzed the data for RGC density between the treated eyes with normal eyes (Fig. 1C). Similar conclusions were drawn after using the normal eyes as the control. The administration of PBS and BDNF resulted in a significant loss for RGCs compared to normal group ( $P < 0.0001$ ). After the treatment of LINGO-1-Fc alone, more RGCs survived compared to PBS and BDNF treated eyes ( $P < 0.0001$ ). However, there was still RGC loss in LINGO-1-Fc group compared to normal group ( $P < 0.05$ ). We have previously investigated the death of RGCs in our rat glaucoma model 2, 4, 8 and 12 weeks after laser coagulation. The results showed that the loss of RGCs reaches a maximal level after 4 weeks (Li et al., 2006a). So the findings of the significant neuroprotection of BDNF and LINGO-1-Fc in 4 weeks after laser coagulation provide sufficient evidence for the positive efficiency of LINGO-1-Fc and anti-LINGO-1 antibody in this glaucoma model. Representative photomicrographs of retinal flat-mount 4 weeks after ocular hypertension are shown in Fig. 1D.

## **IOP profile**

IOP in the contralateral left eye of treated animals remained at about the level of 13 mmHg throughout the experiments. The IOP of the laser treated right eye in all groups increased after the first laser surgery and remained at the level of 23 mm Hg until sacrifice (Fig. 2). The treatments did not change the level of the IOP in treated rats compared with control animals. It suggests that RGC survival induced by combined BDNF/LINGO-1 treatments occurs in the face of maintained elevated IOP.

## **TrkB Activation with the combined treatment of BDNF and LINGO-1-Fc**

To reveal whether BDNF could activate TrkB more efficiently in the presence of LINGO-1-Fc, we injected exogenous BDNF into the eye and studied the TrkB activation 5 days after the induction of ocular hypertension (Fig. 3A, B). Normal rat retinas expressed total TrkB (lane 1-3). While *p*-TrkB level was very low in normal control retinas (lane 1-3). BDNF treatment alone increased *p*-TrkB levels but the change was not statistically significant (lane 4-6). However, BDNF combined with LINGO-1-Fc significantly increased *p*-TrkB levels under the same conditions ( $P = 0.004$  for LINGO-1-Fc compared to PBS group) (lane 7-9). This suggests that BDNF activates TrkB level after blocking the function of LINGO-1. These findings indicate that exogenous BDNF stimulation produced only limited activation of TrkB receptors but blocking of LINGO-1 function reversed this limitation.

## **Co-localization of TrkB and LINGO-1 in the retinal ganglion cells**

To further support the claim that LINGO-1-Fc supports BDNF to promote the activation of TrkB, we examined the co-localization of LINGO-1 and TrkB in the retina. The results presented in this study confirm our previous findings (Fu et al., 2008a) that LINGO-1 was expressed in RGCs (Fig. 4A) and up-regulated after the induction of ocular hypertension (Fig. 4B). We found that there was moderate immunostaining for TrkB in the inner plexiform layer and in the ganglion cell layer in the normal retina (Fig. 4C). TrkB expression increased at 4 weeks after the induction of ocular hypertension (Fig. 4D). LINGO-1 and TrkB were co-expressed in the RGCs retrogradely labeled with FG (Fig. 4E-H).

## DISCUSSION

Current neuroprotectants used to treat glaucoma only delay and do not prevent RGC death. This study showed that a combined treatment of BDNF and LINGO-1 antagonist provides long term RGC neuroprotection after the induction of elevated IOP. We further identified that a combination of BDNF and LINGOO-1-Fc accelerates TrkB activation in the eyes with elevated IOP. Our study suggests an effective strategy for the treatment of glaucomatous neuropathy.

### **Neuroprotective effects of combined application of BDNF and LINGO-1-Fc after ocular hypertension**

The loss of RGCs in patients suffering from glaucoma appears progressively over many years. An ideal neuroprotectant to treat glaucoma should enhance the survival of RGCs and preserve the function of RGCs without adverse effects on normal retinal signaling. Although numerous clinical trials in stroke, Parkinsonism and Alzheimer's disease are underway, there are currently no clinical neuroprotectants that prevent

RGC death in human glaucoma. The major type of RGC death in glaucoma is probably apoptosis (Quigley et al., 1995). Neurotrophin can hinder the induction of apoptotic cell death and promote RGC survival. However, several neuroprotectants such as BDNF, neurotrophin-3 and 4, insulin-like growth factor, glial cell-derived neurotrophic factor show only partial protective effects on RGCs after retinal injury (Kermer et al., 2000; Klocker et al., 1997; Peinado-Ramon et al., 1996). Here we found that BDNF combined with LINGO-1-Fc rescued almost all of RGCs up to 4 weeks after ocular hypertension. Unlike the slow course of human chronic glaucoma, the loss of RGCs in this rat ocular hypertension model becomes stable after 4 weeks (Li et al., 2006a). We have previously investigated the death of RGCs in our rat glaucoma model 2, 4, 8 and 12 weeks after laser coagulation. The results showed that the loss of RGCs reaches a maximal level after 4 weeks (Li et al., 2006a). The findings of the significant neuroprotection of combined treatment of BDNF and LINGO-1-Fc in 4-week course provide sufficient evidence for the positive efficiency of the combined treatment in this ocular hypertension model.

The limited ability of BDNF to confer neuroprotection remains unresolved until now. The finding of long-term neuroprotection of combination of BDNF and LINGO-1-Fc provides a clearer understanding of the activity of BDNF. Free radicals were suggested to be responsible for limiting the rescue effect of BDNF after retinal injury (Klocker et al., 1998; Ko et al., 2000). However, a combined treatment of BDNF and antioxidants only showed partial survival of RGCs in the eyes of hypertensive rats (Ko et al., 2000). It is possible that the RGCs lose the responsiveness of TrkB to BDNF after retinal injury, as has been observed in other neurons (Carter et al., 1995; Frank et al., 1996). However, axotomized RGCs can express sufficient levels of TrkB (Di et al., 1998) and transferring the TrkB gene into RGCs combined with exogenous BDNF administration provided a transient increased neuronal survival after optic

nerve transection (Cheng et al., 2002). Here we found that the limited protection of BDNF on RGCs could be reversed after blocking of LINGO-1 function. We further identified a possible mechanism underlying this protective effect, as described in the following discussion.

We previously found that blocking LINGO-1 function promoted RGC survival in a rat ocular hypertension model. However, there was still some RGC loss at 4 weeks after the induction of ocular hypertension. In this study we found that the effect of a combined treatment of BDNF and LINGO-1-Fc on RGC survival in this animal model of ocular hypertension is better than LINGO-1-Fc alone. It suggests LINGO-1-Fc has limited neuroprotection.

### **The effect of combined application of BDNF and LINGO-1-Fc on TrkB activation after ocular hypertension**

Here we found that a combined treatment of BDNF and LINGO-1-Fc could activate more TrkB compared to BDNF alone. Neurotrophins can activate two types of receptors, the Trk family and p75 receptor (Huang and Reichardt, 2003). Trk receptors consist of a family of three receptor tyrosine kinases, TrkA, TrkB and TrkC, each of which can be activated by respective neurotrophins. TrkB is the dominant Trk receptors in the retina. BDNF exerts its neuroprotective activity by activating TrkB. The p-TrkB subsequently activates the downstream signaling pathway such as PI-3K/Akt (Huang and Reichardt, 2003). These data suggest that LINGO-1 may negatively regulate the activity of BDNF and TrkB. We just identified that LINGO-1 binds with TrkB and negatively regulates its activation in a culture system of RGCs and in the retina of rats (Fu QL et al., unpublished data). These data suggest a possible molecular mechanism that the partial neuroprotection of BDNF may largely

attributable to negative regulation of LINGO-1 to the BDNF-TrkB signaling receptor. It may be the novel molecular mechanism that a combination of BDNF with LINGO-1 antagonists promotes long-term survival on RGCs in this model of ocular hypertension. We found that LINGO-1-Fc as a soluble protein of LINGO-1 directly binds on the surface of LINGO-1 to block its function (data now shown). The involved signal pathways is described in Figure 5.

## CONCLUSIONS

The marked neuroprotection of a combined treatment of BDNF and LINGO-1-Fc in 4-week course has important significance for the long-term prevention of RGC damage in this glaucoma model. The application of BDNF combined with LINGO-1 antagonists leads to a long term protection for RGCs in this chronic hypertension model. It provides a new avenue to combat RGC death after chronic retinal injury. Neurotrophins are being tested currently in clinical trials for the treatment of various neurological disorders. If our observations obtained in RGCs can be extended to other neuronal populations and injury paradigms, the use of LINGO-1 antagonists may provide a promising way to optimize the neuroprotective effects of neurotrophins. This could help in designing new strategies in the treatment of human neurodegenerative diseases.

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## Figure Legends

### **Figure 1. Blocking of LINGO-1 function reinforces the neuroprotective activity of BDNF after ocular hypertension**

(A) Percentage of RGC loss in the hypertensive eyes at 4 weeks post laser coagulation (Mean  $\pm$  SEM). BDNF exhibits a slight neuroprotection to injured RGCs (\*  $P = 0.018$  compared to PBS group). Blocking the function of LINGO-1 with LINGO-1-Fc significantly reduced RGC loss (\*\*\*  $P < 0.0001$  compared to PBS group). A combined administration of BDNF and LINGO-1-Fc rescued almost all of the RGCs compared to BDNF (\*\*\*  $P < 0.0001$ ) and LINGO-1-Fc (\*\*  $P = 0.004$ ) alone in this ocular hypertension model.  $n = 8$  for each group. (B) Analyses for the density of RGCs between the right eyes and left eyes in each group (the number of RGCs/mm<sup>2</sup>,

Mean  $\pm$  SEM). Paired Student's *t* test was used to analyze the data. There was loss of RGCs in right eyes compared to left eyes in PBS [ $t$  (7) = 5.65, \*\*  $P$  = 0.001] and BDNF [ $t$  (7) = 9.611, \*\*\*  $P$  < 0.0001] groups. The application of LINGO-1-Fc showed slight RGC loss [ $t$  (7) = 2.431, \*  $P$  = 0.045] after laser coagulation. However, the combined treatment of BDNF and LINGO-1-Fc rescued almost all the RGCs after a long-term progress of 4 weeks following the induction of ocular hypertension [ $t$  (7) = 1.161,  $P$  = 0.284]. (C) The data were analyzed with RGC density between experimental eyes and normal eyes. (D) Flat-mounted retinas showing surviving Fluoro-Gold labeled RGCs 4 weeks after laser coagulation with different treatments.

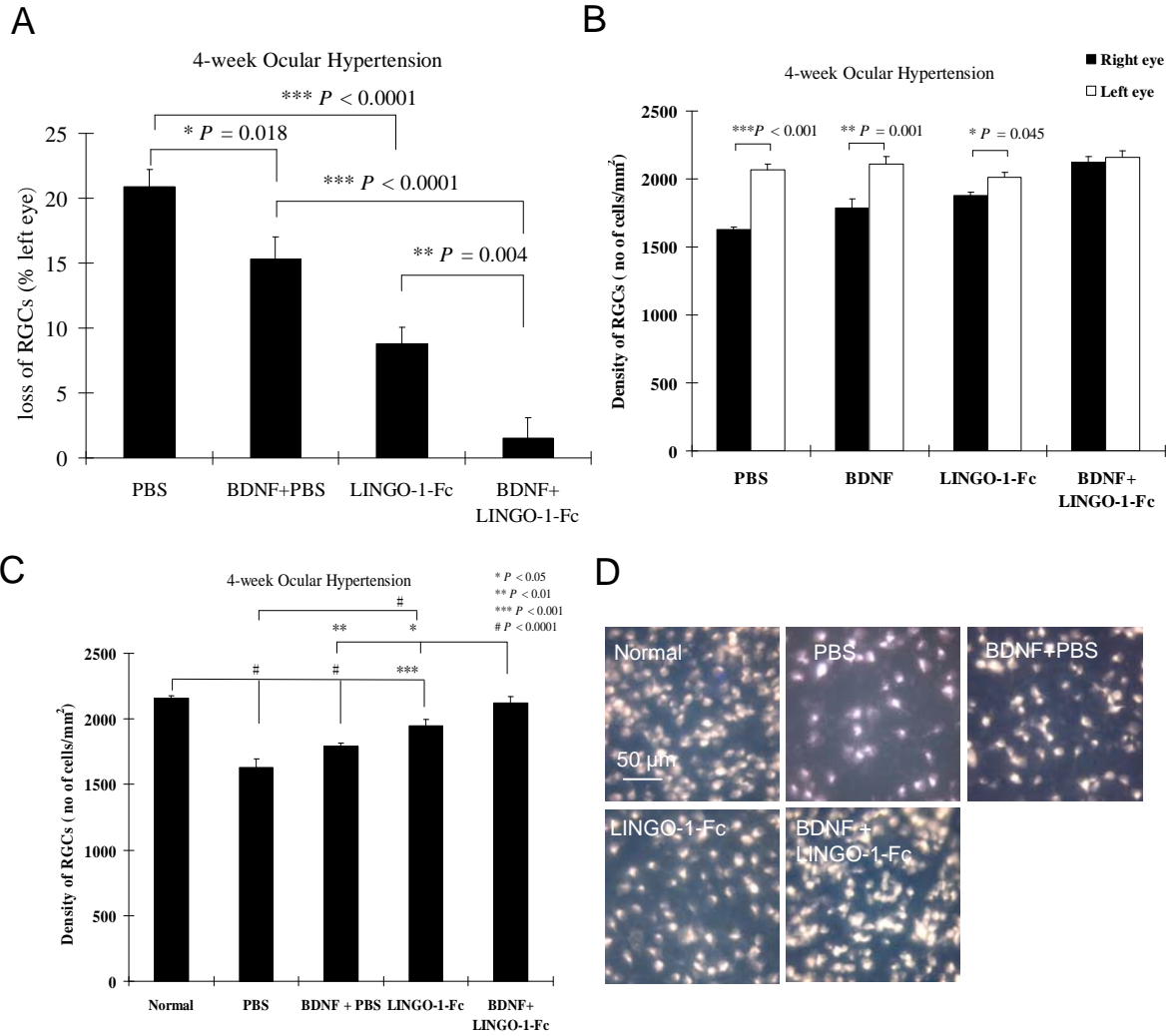
**Figure 2. IOP profile after induction of ocular hypertension.** The IOP of experimental right eyes was significantly elevated after the first laser treatment compared with those of control left eyes for each group. The elevated IOP remained at a steady level throughout the experimental period. The treatments did not reduce IOP. IOP values are expressed as the Mean  $\pm$  SEM.

**Figure 3. Combined treatment of BDNF and LINGO-1-Fc increases TrkB activation.** (A) Western blots of *p*-TrkB and total TrkB in the retinas at 5 days after laser coagulation with the treatment of BDNF alone (lane 4-6) or combined with LINGO-1-Fc (lane 7-9). (B) Densitometric scanning of *p*-TrkB quantity in western blotting presented in the upper panel showed a significant elevation of *p*-TrkB levels with the combined treatment of BDNF and LINGO-1-Fc (lane 7-9) compared with BDNF treatment alone (lane 4-6) ( $P$  < 0.01). The relative *p*-TrkB level was calculated by the value of *p*-TrkB over the value of total TrkB. Each band stands for an individual animal.  $P$  values compared to normal and BDNF groups.  $n$  = 3 for each group (Mean  $\pm$  SEM).

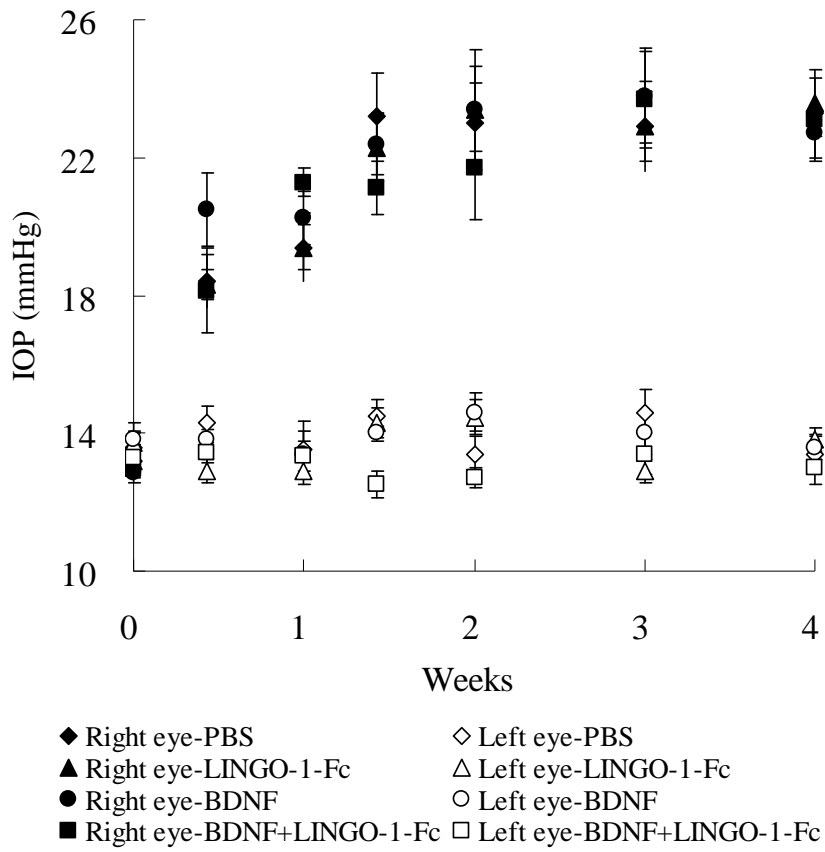
**Figure 4. LINGO-1 is coexpressed with TrkB in the retinal ganglion cells.**

Representative photomicrographs of LINGO-1 and TrkB expression in normal and injured retina following 4 weeks ocular hypertension. TrkB staining (red) (A,B), LINGO-1 staining (green) (C,D), retrograde Fluoro-Gold (FG) labeled RGCs (blue) (E,F) demonstrates the co-localization of LINGO-1 and TrkB in RGCs (magenta) (arrows) (G,H). The expression of TrkB and LINGO-1 increased after the induction of ocular hypertension. GCL: ganglion cell layer. INL: inner nuclear layer; ONL: outer nuclear layer. FG: Fluoro-Gold.

**Figure 5. The diagram of the signaling pathways involved in the combined neuroprotection of BDNF and LINGO-1-Fc on RGCs after the induction of ocular hypertension.** In the normal condition, LINGO-1 binds with TrkB and negatively modulates the activity of TrkB. Elevated IOP increased the level of LINGO-1. The activation of TrkB is not sufficient and results in transient RGC protection after the application of exogenous BDNF. LINGO-1-Fc relieves the limitation of LINGO-1 to TrkB. A combination of BDNF and LINGO-1-Fc activates more TrkB and finally promotes long-term RGC survival.



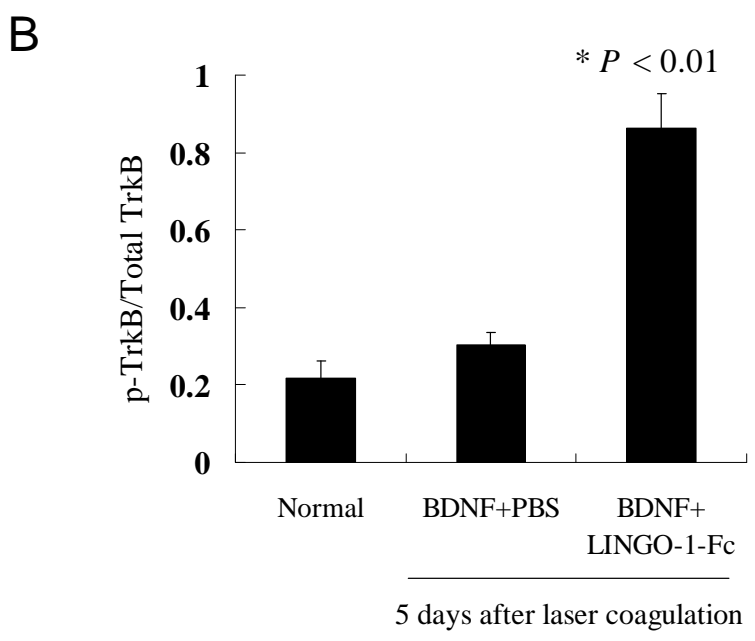
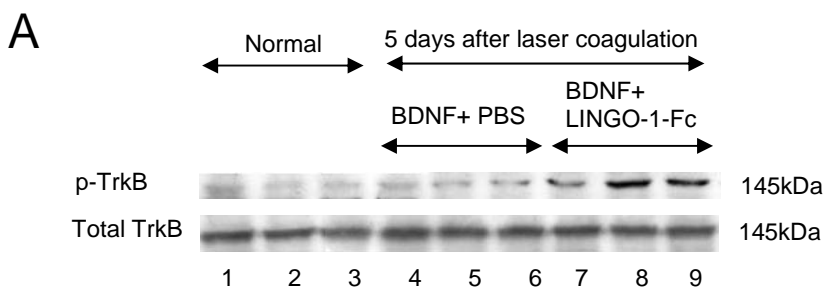
**Figure 1**



**Figure 2**

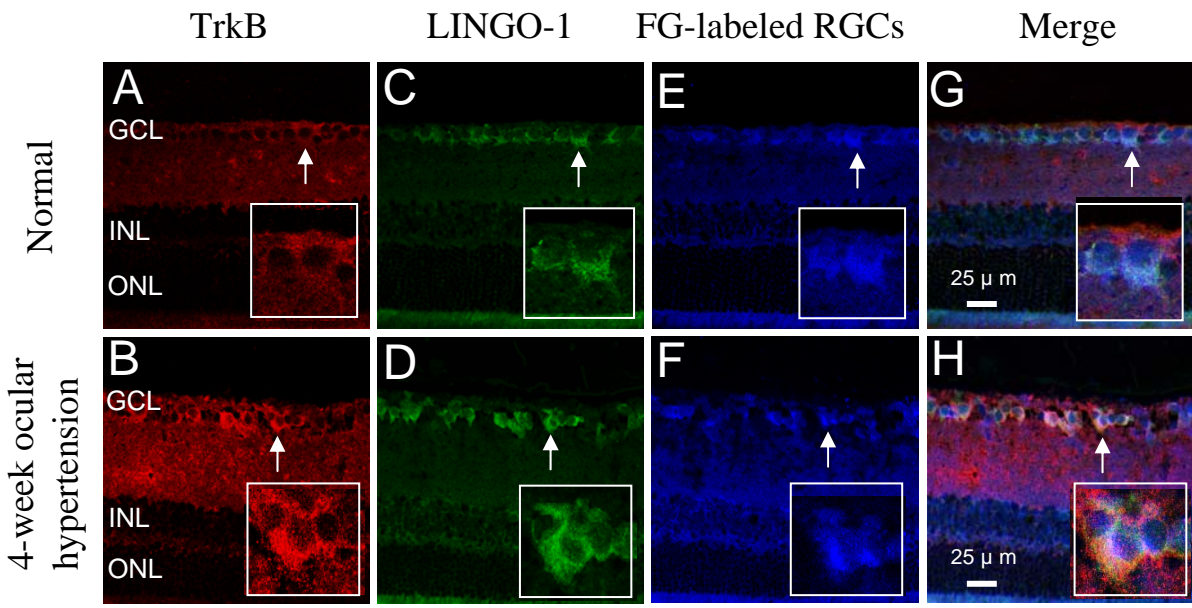


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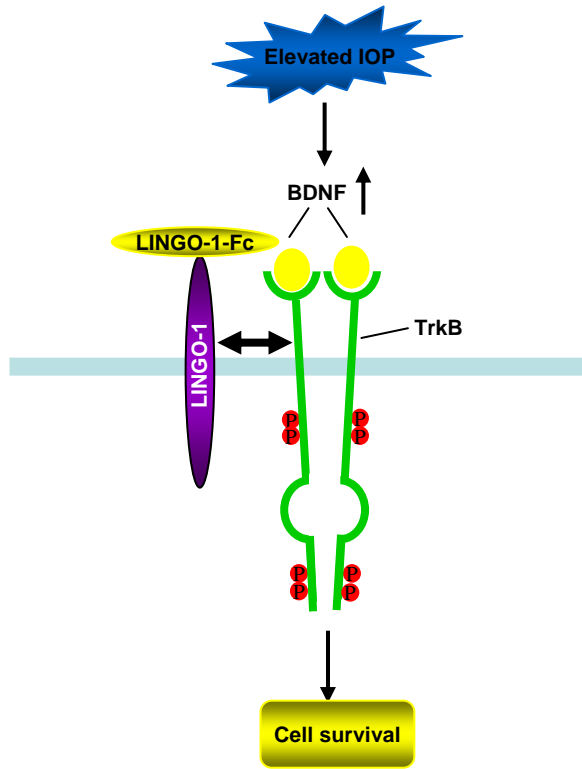


Figure 5