Neuroprotective effects of exogenous brain-derived neurotrophic factor on amyloid-beta 1–40-induced retinal degeneration

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Abstract
Amyloid-beta (Aβ)-related alterations, similar to those found in the brains of patients with Alzheimer’s disease, have been observed in the retina of patients with glaucoma. Decreased levels of brain-derived neurotrophic factor (BDNF) are believed to be associated with the neurotoxic effects of Aβ peptide. To investigate the mechanism underlying the neuroprotective effects of BDNF on Aβ<sub>1–40</sub>-induced retinal injury in Sprague-Dawley rats, we treated rats by intravitreal administration of phosphate-buffered saline (control), Aβ<sub>1–40</sub> (5 nM), or Aβ<sub>1–40</sub> (5 nM) combined with BDNF (1 µg/mL). We found that intravitreal administration of Aβ<sub>1–40</sub> induced retinal ganglion cell apoptosis. Fluoro-Gold staining showed a significantly lower number of retinal ganglion cells in the Aβ<sub>1–40</sub> group than in the control and BDNF groups. In the Aβ<sub>1–40</sub> group, low number of RGCs was associated with increased caspase-3 expression and reduced TrkB and ERK1/2 expression. BDNF abolished Aβ<sub>1–40</sub>-induced increase in the expression of caspase-3 at the gene and protein levels in the retina and upregulated TrkB and ERK1/2 expression. These findings suggest that treatment with BDNF prevents RGC apoptosis induced by Aβ<sub>1–40</sub> by activating the BDNF-TrkB signaling pathway in rats.

Key Words: amyloid-beta 1–40; brain-derived neurotrophic factor; Fluoro-Gold; neuroprotection; retinal ganglion cells (RGC); retinal toxicity; tropomyosin receptor kinase B (TrkB)

Introduction
Amyloid-beta (Aβ) deposition is a major neuropathological abnormality in Alzheimer’s disease (AD) (Murphy and LeVine, 2010; Sadigh-Eteghad et al., 2015; DeTeure and Dickson, 2019). Aβ deposition also occurs in other neurodegenerative diseases, such as glaucoma, where its deposition in the retina is associated with a loss of retinal ganglion cells (RGCs) (Mancino et al., 2018; Wang and Mao, 2021). Multiple studies have shown a relationship between AD and glaucoma (Ratnayaka et al., 2015; Musuzzo et al., 2016). Aβ is one of the most widely investigated neurodegenerative diseases. It is characterized by the deposition of Aβ fibrils and formation of senile plaques in the brain. These pathological changes are associated with the formation of aggregates around cerebral blood vessels, oxidative stress, and glutamate excitotoxicity, leading to neuronal apoptosis (Dong et al., 2009; Uitara et al., 2009; Nita and Grzybowski, 2016). Interestingly, in patients with AD, similar neuropathological changes have been observed in association with RGC apoptosis and axonal loss, reduced thickness of the retinal nerve fibre layer, and increased cupping of the optic disc (Goldstein et al., 2003; Liu et al., 2009; Moncaster et al., 2010; Kesler et al., 2011; Frost et al., 2013; Javadi et al., 2016). In transgenic AD mouse models, RGC apoptosis and impaired visual functions have been found to be associated with increased Aβ deposition (Chiu et al., 2012; Parnell et al., 2012). These findings suggest that retinal neurodegeneration is associated with AD-like pathology (Normando et al., 2009). More recently, mechanisms underlying RGC loss in glioma have been shown to resemble those involved in AD-associated changes in the brain, suggesting that a common pathway could be involved in cell death in the two diseases (Vrabec and Levin, 2007; Caprioli, 2013; Ghiso et al., 2013; Pescosolido et al., 2014; Davis et al., 2016). Several studies have shown that glaucomatous RGC apoptosis is associated with increased retinal Aβ deposition (Guo et al., 2005, 2007; Guo and Cordeiro, 2008; Davis et al., 2016; Yan et al., 2017). For example, RGC apoptosis was significantly reduced in an experimental glaucoma model by targeting the endogenous Aβ synthesis pathway (Guo et al., 2007). Accumulating evidence has shown increased vitreous levels of Aβ in patients with glaucoma (Guo et al., 2007; Tsolaki et al., 2011; Mancino et al., 2018). Hence, Aβ-associated molecular pathways are potential targets for the prevention of glaucomatous neurodegeneration. We previously showed that intravitreal administration of Aβ<sub>1–40</sub>-induced apoptosis of retinal cells in rats, leading to time- and dose-dependent changes in retinal and optic nerve morphology (Mohd Lazaldin et al., 2018). Aβ<sub>1–40</sub>-induced changes, marked by increased retinal oxidative stress and significantly reduced levels of retinal brain-derived neurotrophic factor (BDNF), were more severe following 14 days of intravitreal administration (Mohd Lazaldin et al., 2018).

BDNF depletion is associated with both AD and glaucoma (Chitranshi et al., 2018; Mancino et al., 2018). BDNF, which exists as a precursor (proBDNF) and in a mature form, plays a critical role in neuronal functions and survival (Bathina and Das, 2015; Xue et al., 2022).

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The sera and cerebrospinal fluids of patients with AD show low levels of BDNF (Diniz et al., 2014). Similarly, post-mortem brain tissues from patients with AD show decreased BDNF mRNA levels (Connor et al., 1997). Connor et al. (1997) also proposed that BDNF acts as a neuroprotective agent and delays the progression of neurodegenerative disorders, including AD (Connor et al., 1997). The differentiation and survival of neurons in the hippocampus and basal forebrain is promoted by BDNF (Tapia-Aracibia et al., 2008). In vivo studies, interruption of the retrograde transport of BDNF and the accumulation of its TrkB receptor at the optic nerve head in rats suggest that BDNF deprivation plays a role in the pathogenesis of RGC death (Pease et al., 2009). In contrast, overexpression of BDNF delays the progressive loss of RGCs and axons in the eyes of rats with elevated intracocular pressure (IOP) (Feng et al., 2016, 2017).

The neuroprotective effects of BDNF against retinal damage caused by Aβ1–40 have been reported previously (Mohd Lazaldin et al., 2020). Pre-treatment with BDNF significantly reduces Aβ1–40-induced increase in the number of TUNEL-positive RGCs. It also ameliorates Aβ1–40-induced changes in optic nerve morphology, axonal swelling, and the expression of retinal glutathione, superoxide dismutase, and catalase. Furthermore, pre-treatment with BDNF restores Aβ1–40-induced deterioration of the ability of a rat to recognize visual cues (Mohd Lazaldin et al., 2020). However, the mechanisms underlying the anti-apoptotic effects of BDNF against RGC loss remain unknown. Hence, in this study, we investigated whether the BDNF-TrkB signaling pathway acts by altering ERK1/2 activation and suppressing caspase-3 activation, thereby protecting RGCs against Aβ-induced apoptosis.

Methods

Animals

Forty-two male Sprague-Dawley rats (9 weeks old, 200–250 g body weight) free from any contamination under strict quarantine observation were housed on a 12-hour light/dark cycle before use. Normal pelleted diet and water were provided ad libitum. The experimental protocols complied with the Association for Research in Vision and Ophthalmology’s Resolution on the Use of Animals in Research (ARVO, 2021). Ethical approval for this study was obtained from Committee on Animal Research & Ethics of Universiti Teknologi MARA, Puncak Alam, Selangor, Malaysia (UiTM Care: 117/2015, approval date: October 9, 2015).

Preparation and validation of aggregated Aβ1–40

Fresh Aβ1–40 solution was prepared as previously described (Watts et al., 2010; Li et al., 2011). Aβ1–40 peptides (AnaSpec, Fremont, CA, USA) were diluted with sterile PBS to a final concentration of 1 µg/µL. The Aβ1–40 stock solution was sonicated for 1 minute and incubated at 37°C for 1 week to aggregate the peptides. This stock solution of Aβ1–40 was stored at –20°C (Resende et al., 2008).

Scanning electron microscopy (SEM) images of amyloid fibrils were obtained using an ESEM system (Thermo Fisher Scientific, Waltham, MA, USA) with a backscatter electron detector. Images were collected at 5600×, 10,000×, and 20,000×. Fibril’s diameter data were collected from software build-up in the peptides. This stock solution of Aβ1–40 was stored at –20°C (Resende et al., 2008).

The average number of Fluoro-Gold-labeled RGCs in the 12 images representing 12 areas (120 × 160 µm²) was calculated to determine the cell density of each retina (LaFuentet et al., 2002; Schlamp et al., 2013, Nadal-Nicolás et al., 2015, Nor Arfuzi et al., 2020; Lambuk et al., 2021).

BDNF preparation

BDNF solution was prepared in accordance with manufacturer’s instructions (BioVision revision 11/14, Mountain View, CA, USA). Briefly, the BDNF-containing signal transduction (6088°C, 20 seconds at 4°C, Ependorf 5430R, Germany) and then diluted in ddH₂O to obtain a final 1.0 µg/mL concentration. BDNF aliquots were kept at –20°C for future use.

Study design

The rats were randomly divided into three groups using the random number table method, each independent selection containing 14 animals. Animals in group 1 received bilateral intravitreal injection of 3 µL PBS; those in group 2 received 3 µL of 5 nmol/µL Aβ1–40 in PBS, while those in group 3 received 2 µL of 5 nmol/µL Aβ1–40 in PBS, followed by 1 µL of 1 µg/mL BDNF in PBS. In all cases, the PBS solution was sonicated and incubated in a manner similar to that of the Aβ1–40 solution (Figure 1).

For intravitreal injections, ketamine (80 mg/kg, intraperitoneal) combined with xylazine (12 mg/kg, intraperitoneal) (Ilum Troy Laboratories, Glenndening, NSW, Australia) were administered to anesthetize the animals. A 30-gauge needle mounted on 10 µL Hamilton syringe was used. Using a microscope, the tip of the needle was used to puncture the sclera at the dorso-temporal limbus of the eye, following which the Hamilton needle was inserted through the puncture site. The solution was injected slowly over at least 2 minutes to avoid retinal damage resulting from pressure caused by fluid injection. Neomycin-polymyxin ointment (Alcon, Geneva, Switzerland) was administered after the procedure.

For retrograde labeling, Fluoro-Gold (Fluorochrome, Denver, CO, USA) was injected very slowly into the superior colliculus of each hemisphere, and a stereotaxic instrument (Stoelting Co., Wood Dale, IL, USA) was used to locate the specified area. The fur was shaved, povidone-iodine was applied to the overlying skin, and a 2-cm-long incision was made in the midline of the scalp over the cranium to expose the skull. A micro dental drill was used to drill a hole 7.5 mm behind the bregma and 2.0 mm on each side from the midline at a depth of 4.0 to 4.5 mm to reach the superior colliculus area (Paxinos and Watson, 2007; Schlamp et al., 2013). Four microliters of 3% Fluoro-Gold (Fluorochrome) in sterile PBS was injected using a Hamilton syringe at a rate of 1 µL/min for 2 minutes on either side as previously described (Schlamp et al., 2013). The exposed skull was fully covered with dental cement, and the scalp was closed using a nylon surgical suture. The rats were allowed to fully recover from anesthesia before being transferred back to the animal house.

Intraperitoneal ketamine (> 80 mg/kg) combined with xylazine (> 12 mg/kg) was used to sacrifice the animals. The eyes were enucleated at 24 hours, 3 days, and 7 days for western blotting (WB) and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis, and at 14 days for Congo red staining and counting of Fluoro-Gold-stained RGCs. For both WB and qRT-PCR, samples were derived from three rats at each time point in each group. Furthermore, for WB, each biological sample was subjected to assessment in two technical replicates whereas for qRT-PCR there were three technical replicates. Fluoro-Gold and Congo red staining was performed in four rats in each group on days 7 and 14, respectively. For histopathological examination, a 10% formaldehyde solution was used to fix the eyeballs for 24 hours at room temperature. Fluoro-Gold-stained RGCs were counted in freshly isolated retinas.

Congo red staining

To visualize Fluorib A plaques in the retina, Congo red (MilliporeSigma, Burlington, MA, USA) staining was performed (Wang et al., 2010). Briefly, deparaffinized retinal sections were dehydrated using decreasing ethanol concentrations and stained with Congo red solution for 1 hour. Slides were rinsed, counterstained with hematoxylin, and examined under a light microscope (20× magnification, Nikon, Tokyo, Japan).

Retrograde labeling

To count Fluoro-Gold-stained RGCs, enucleated eyes were fixed with 4% formaldehyde solution for 2 hours at 4°C. The eyeballs were then washed in 5 mL of 1 M PBS at room temperature to remove excess formaldehyde. The anterior segment was removed, and the vitreous was carefully detached from the retina. The retinal cup was then isolated and four incisions were made at the retinal periphery to flatten it. Finally, the retinal flat mount was examined under a fluorescence microscope.

A fluorescence microscope (Olympus, Tokyo, Japan) was used to observe Fluoro-Gold-labeled RGCs using ultraviolet filters with maximal absorption at 560 nm (40× magnification). Fluoro-Gold-labeled RGCs were counted by three blinded investigators using the image analysis program, ImageJ (version 1.52, National Institutes of Health, Bethesda, MD, USA), based on ≥12 images representing 12 areas (120 × 160 μm²), three, each in two quadrants of each retina (Figure 2). The number of Fluoro-Gold-labeled RGCs in each image was then converted into RGC density per square millimetre (RGCs/mm²) of the retina. The average number of Fluoro-Gold-labeled RGCs in the 12 images was calculated to determine the cell density of each retina (LaFuentet et al., 2002; Schlamp et al., 2013; Nadal-Nicolás et al., 2015; Nor Arfuzi et al., 2020; Lambuk et al., 2021).

Figure 1 | Study design.

Ab: Amyloid-beta; BDNF: brain-derived neurotrophic factor; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; i.v.: intravital administration; PCR: polymerase chain reaction; RGC: retinal ganglion cell.
Extracted retinal tissues were rinsed with ice cold PBS (0.1 M) and stabilized in RNeater Stabilization Solution (Ambion, Austin, TX, USA). AgNeET RNA purification kit (Thermo Fisher Scientific) was used for RNA extraction and purification of the macrophages. cDNA was then used to synthesize cDNA. Up to 1 µg of DNA- and DNase-free RNA was added to 5x reaction mix (containing reaction buffer, dNTPs, oligo (dT)18, and random hexamer primers), Maxima Ex Taq DNA Synthesis Kit (Thermo Fisher Scientific) was used to synthesize cDNA. For all experiments, anti-β-actin mouse antibody (1:10,000, Santa Cruz Biotechnology, Cat# sc-16102, RRID: AB_2687626) was used as the secondary antibody.

**Statistical analysis**

The sample size in this study was calculated according to the resource equation method (Arfin and Zahiruddin, 2017). In the present study, the sample size was calculated based on the “resource equation” proposed by Arfin and Zahiruddin. The sample size in each group should be a minimum of 10/k+1 and a maximum of 20/k+1 (k, the number of groups) in this equality. We calculated the sample size of each group to be a minimum of four, and a maximum of six. The sample size for western blot analysis and RT-PCR was considered adequate to test the respective biological effects and our sample sizes are similar to those reported in previous publications (Lambuk et al., 2021). In addition, the sample size was also in accordance with the recommendation of the university animal ethics research committee in this study. Investigators who collected the data were blinded to the experimental groups. Statistical analysis was performed using SPSS 26.0 (IBM Corp., Armonk, NY, USA). Fibre's diameter data are presented as the mean ± SD. Statistical significance was analyzed using independent samples t-tests. Data from retrograde labelling, western blotting and real-time PCR analyses are expressed as the mean ± SEM. Significant differences between groups were determined using one-way analysis of variance with post hoc Bonferroni tests. P-values < 0.05 were considered statistically significant.

**Results**

**Validation of aggregated AB_{1–40}**

The appearances of fresh AB_{1–40} and AB_{1–40} incubated for 7 days are shown in Figure 3A–C. Prolonged incubation led to an increase in fibrillar length (Figure 3D–F). Although generated from the same protein, fresh AB_{1–40} and AB_{1–40} incubated for 7 days had different structures. Fresh AB_{1–40} consisted of a non-branched light fibril with a diameter of 631 ± 345.74 nm. In contrast, the diameter of the AB_{1–40} fibril after 7 days of incubation was 12.12-fold larger (P = 1.4 × 10⁻³), with a mean value of 1340.8 ± 291.66 nm (Figure 4).

**Retinal AB_{1–40} deposition in rats**

Congo red staining is part of a set of histochemical tests used to confirm the presence of amyloid deposits based on a characteristic deep-red or salmon color. In this study, Congo red staining clearly demonstrated the presence of amyloid deposits in both the AB_{1–40} and BDNF groups (Figures 5A–C).

**Effects of BDNF on RGC survival in AB_{1–40}-exposed retinas**

Retrograde labeling of RGCs with Fluoro-Gold showed that RGC density was 3.98-fold lower in the AB_{1–40} group than in the control group. Rats that received BDNF showed 3.98-fold higher number of Fluoro-Gold-labeled RGCs than those that received AB_{1–40} (P = 4 × 10⁻³). The number of Fluoro-Gold-positive cells was comparable between the control and BDNF groups (Figures 6A–D).

**Effects of BDNF on TrkB expression in AB_{1–40}-exposed retina**

**TrkB gene expression**

TrkB gene expression in the retina following exposure to AB_{1–40} was significantly lower 24 hours after treatment (3.23-fold, P = 1 × 10⁻²) and 3 days (2.48-fold, P = 1 × 10⁻³) after treatment compared with the control group (Figure 7). On day 7, TrkB expression was upregulated in the BDNF group when compared with the AB_{1–40} group (2.37-fold, P = 1 × 10⁻³). However, TrkB expression in the BDNF group was significantly reduced at 24 hours (3.23-fold, P = 1 × 10⁻³) and 3 days (1.6-fold, P = 7.9 × 10⁻³) after treatment. On day 7 after treatment, it was 1.21-fold higher in the BDNF group than in the control group (P = 1.3 × 10⁻³) than that in the control group.

**TrkB protein expression**

Western blot analysis showed that at 24 hours, TrkB protein expression in the retina of rats in the AB_{1–40} group was significantly lower compared with the control group (2.48-fold, P = 1 × 10⁻³). In the BDNF group, protein expression was also significantly lower in both 24 hours (2.26-fold, P = 1 × 10⁻³) and 3 days (5.14-fold, P = 5 × 10⁻⁴) after treatment. No significant differences were observed between the BDNF and control groups at any of the time points analyzed (Figure 8).

**Effects of BDNF on ERK1/2 expression in AB_{1–40}-exposed retina**

**ERK1/2 gene expression**

ERK1/2 gene expression in the retina was significantly lower at 24 hours (2.26-fold, P = 1 × 10⁻³), 3 days (2.48-fold, P = 1 × 10⁻³), and 7 days (5.14-fold, P = 5 × 10⁻⁴) after exposure compared with the control group (Figure 9). Analysis at 24 hours and 3 days showed no significant differences in ERK1/2 gene expression in the retina of rats in the BDNF group compared with the AB_{1–40} group, but ERK1/2 gene expression at these two time points was significantly lower compared with the control group (2.4-fold, P = 1 × 10⁻³, 1.6-fold, P = 8 × 10⁻⁴, respectively). On day 7, ERK1/2 gene expression was significantly upregulated (4.33-fold, P = 1 × 10⁻³) in the BDNF group compared to the AB_{1–40} group. No significant differences in ERK1/2 gene expression were observed between the BDNF and control groups on day 7.
Figure 3 | Scanning electron microscopy images of Aβ
(A–D) Fresh Aβ_{42} (A) and Aβ_{40} incubated for 7 days (D) at 2000× magnification; (B, E) fresh Aβ_{42} (B) and Aβ_{40} incubated for 7 days (E) at 4000× magnification; (C, F) Fresh Aβ_{42} (C) and Aβ_{40} incubated for 7 days (F) at 10,000× magnification. Aβ_{42}, sample solution (1 nmol/µL) was diluted 50-fold in pure water and coated with platinum. Arrows represent the amyloid fibrils. Aβ: Amyloid-beta.

Figure 4 | Fibrill diameters of fresh Aβ_{42} and the Aβ_{40} for 7 days.
Measurements of fibril diameter were repeated ten times and the photograph captured swept across the surface on the film. Data are presented as the mean ± SD. **P < 0.01 (independent samples t-test). Aβ: Amyloid-beta.

Figure 5 | Representative light photomicrograph of Congo red-stained rat retinal sections showing the effect of intravitreal administration of BDNF on Aβ_{42} deposition in the rat retina. (A) Control group; (B) Aβ_{42} group; and (C) BDNF group. Scale bars represent 100 µm at 20x magnification and arrows represent the amyloid deposition. Aβ: Amyloid-beta; BDNF: brain-derived neurotrophic factor; GCL: ganglion cell layer; IPL: inner plexiform layer; IR: inner retina.

Figure 6 | Effect of BDNF on the density of Fluoro-Gold-positive cells in Aβ_{42} exposed retina.
(A) Control group, (B) Aβ_{42} group, (C) BDNF group, and (D) quantitative expression of the effect of BDNF on the density of Fluoro-Gold-positive cells in Aβ_{42} exposed retina. The image of live retinal ganglion cells was captured from four different rats in the same group by three blinded investigators. **P < 0.01, vs. control; ***P < 0.001, vs. amyloid (one-way analysis of variance with post hoc Bonferroni test). Scale bars represent 100 µm at 100x magnification and arrows represent the live retinal ganglion cells; n = 4 (mean ± SEM). Aβ: Amyloid-beta; BDNF: brain-derived neurotrophic factor.

ERK1/2 protein expression
ERK1/2 protein expression was significantly lower in the Aβ_{42} group than that in the control group at 24 hours (2.01-fold, P = 5 × 10^{-2}) and 7 days (3.41-fold, P = 1.9 × 10^{-2}) after exposure. In contrast, on day 7, ERK1/2 expression was significantly upregulated in the BDNF group compared with the Aβ_{42} group (3.03-fold, P = 4.1 × 10^{-3}). No significant change in ERK1/2 expression in the retina was observed between BDNF and control groups at any of the time points analyzed (Figure 10).

Effects of BDNF on Aβ_{42}-induced retinal caspase-3 activation
Caspase-3 gene expression
Significant upregulation of caspase-3 gene expression in the retina was observed in the Aβ_{42} group at 24 hours (2.20-fold, P = 5 × 10^{-2}) and 3 days (1.55-fold, P = 7 × 10^{-2}) post-treatment (Figure 11). However, caspase-3 expression in this group was not significantly different from that in the control group at 7 days post-treatment. In contrast, caspase-3 gene expression in the BDNF group was significantly lower at 24 hours (1.53-fold, P = 3.9 × 10^{-2}) and at 3 days post-treatment (2.42-fold, P = 1 × 10^{-2}) compared with the Aβ_{42} group. Compared with the control group, caspase-3 gene expression in the BDNF group was significantly downregulated at 3 days post-treatment (1.56-fold, P = 5 × 10^{-2}).

Caspase-3 protein expression
Caspase-3 protein expression was 3.25-fold higher in the retina of rats in the Aβ_{42} group than that in the control group (P = 1 × 10^{-2}) at 24 hours post-treatment. At 24 hours post-treatment, Aβ_{42}-induced retinal caspase-3 upregulation was lowered by 2.23-fold (P = 2 × 10^{-5}) in the BDNF group than that in the Aβ_{42} group. Caspase-3 protein expression in the BDNF-treated retina was comparable to that in the control group at 24 hours post-treatment. There was no significant difference in caspase-3 protein expression in the retinas across all groups at 3 and 7 days post-treatment (Figure 12).
Fluoro-Gold showed a significant increase in the RGC density in the group of animals treated with BDNF compared with the group that received Aβ<sub>40</sub>-treatment. Treatment with BDNF also abolished Aβ<sub>40</sub>-induced increase in retinal expression of caspase-3 and upregulated expression of TrkB and ERK1/2, as shown by western blot technique. These data were supported by the results of real-time PCR analysis, which demonstrated that BDNF suppressed Aβ<sub>40</sub>-induced transcriptional activity of caspase-3 and increased TrkB and ERK transcriptional activity involved in the regulation of apoptosis. Formation of the polymerized fibrous form of Aβ<sub>40</sub> which is potentially pathogenic, was confirmed using SEM. Aβ<sub>40</sub> polymerisation is involved in mediating fibril formation were observed 7 days after incubation with parental seeds (fresh Aβ<sub>42</sub>). These data are in line with the stages of Aβ polymerisation described by Brännström et al. (2011, 2018).

We used Fluoro-Gold RGC labelling to determine the effect of BDNF on Aβ<sub>40</sub>-induced changes in RGC survival. Fluoro-Gold neuronal tracer travels retrogradely through axons when injected near the superior colliculus and labels approximately 98% of RGCs (Salinas-Navarro et al., 2009). Hence, RGC labelling by Fluoro-Gold injection near the superior colliculus using a stereotaxic apparatus is one of the best approaches for studying the population of live RGCs with intact axonal transport.

The number of Fluoro-Gold-labeled RGCs in the Aβ<sub>40</sub>-treated group was significantly lower than that in the control and BDNF groups. These data concur with those from a previous study, which showed significant RGC loss in response to Aβ<sub>42</sub>-induced damage (Mohd Lazaldin et al., 2020). In that study, the BDNF group showed significantly higher RGC count than the Aβ<sub>40</sub>-treated group, with the findings being positively correlated with differences in retinal thinning between the two groups (Mohd Lazaldin et al., 2020). Furthermore, the results regarding RGC survival in both Aβ<sub>40</sub>- and BDNF groups in this study were in agreement with those from the earlier study, which showed similar changes in optic nerve morphology, retinal cell apoptosis, and retinal oxidative stress (Mohd Lazaldin et al., 2020).

Rats exposed to Aβ<sub>40</sub> showed reduced TrkB expression at the gene and protein levels in the retina compared with rats in the control and BDNF groups. The expression of TrkB mRNA in the BDNF group was significantly reduced after 24 hours and 3 days of intravitreal injection of BDNF compared with the control group. However, TrkB protein expression in the same group was similar to the control at both time points. A mismatch between gene and protein expression has often been reported. Significant post-translational modifications of proteins may impede detection of expression. Since the site of interference with ERK1/2 and PI3K/Akt signalling is downstream of the TrkB receptor, it is likely that the expression of TrkB itself is altered by Aβ<sub>40</sub> (Tong et al., 2004), and the current study provides evidence supporting this phenomenon.

The loss of TrkB signaling reportedly plays a key role in the pathogenesis of AD, Huntington’s disease, and other neurodegenerative disorders (Gupta et al., 2013, 2020). Furthermore, we previously showed that intravitreal injections of Aβ<sub>40</sub> induced retinal neurodegeneration and visual behavioural alterations (Mohd Lazaldin et al., 2020).

In the current study, intravitreal BDNF administration in rats exposed to Aβ<sub>40</sub> caused an upregulation of TrkB expression at the gene and protein levels, indicating improved TrkB signaling. TrkB receptors are activated by binding to the cognate ligand BDNF and undergo dimerization with the unliganded monomeric form, which is thought to be in equilibrium with its phosphorylated dimeric state (Massa et al., 2010). This dynamic monomer-dimer equilibrium may be of importance in the regulation of TrkB signaling in response to intracellular signaling. TrkB receptors possess an intracellular tyrosine kinase domain, which, upon phosphorylation, recruit intermediates in intracellular signaling cascades that affect gene transcription and final cellular responses to BDNF (Berra et al., 1998).

The current study clearly demonstrated that treatment with BDNF counteracts Aβ<sub>40</sub>-induced suppression of TrkB expression, indicating enhanced TrkB signaling. Earlier studies have shown that TrkB stimulation in the retina leads to activated PI3K/Akt and ERK1/2 signaling pathways in RGCs, and particularly, ERK1/2, appears to be of importance for promoting the survival of RGCs (Chen et al., 2000). In fact, both BDNF and TrkB are involved in RGC protection (Turner et al., 2006), and the loss of TrkB signaling, which is likely to be due to a blockade of axonal transport, leads to preferential RGCs degeneration (Pease et al., 2000; Marvanova et al., 2001; Vrabec and Levin, 2007; Itoh et al., 2005; Ly et al., 2011). Further evidence for this comes from a study that showed increased TrkB labelling behind the optic nerve head region and a decrease in RGC count under the conditions of elevated IOP (Pease et al., 2000). Other studies have also shown that the retinal toxicity induced by Aβ peptides can be prevented by upregulating TrkB signaling (Gupta et al., 2005, 2008). In the current study, higher TrkB

**Discussion**

In the present study, the effects of intravitreally administered BDNF on Aβ<sub>40</sub>-induced RGC damage via the BDNF-TrkB signaling pathway was studied in rats. The retinoprotective effect of BDNF was correlated with the number of surviving RGCs following exposure to Aβ<sub>40</sub>. Retrograde labelling with BDNF

**Figure 10** Effect of BDNF on ERK1/2 protein expression in Aβ<sub>40</sub>-exposed retina (western blotting).

Quantification was based on densitometry of three different biological samples. Total protein was extracted after 24 hours, 3 days, and 7 days of intravitreal administration. Total ERK1/2 protein expression was normalized against that of β-actin and compared with the PBS (control) group for statistical significance. Data represent three biological replicates with three technical replicates, n = 3 (mean ± SEM). *P < 0.05, **P < 0.01, vs. control group (PBS); #P < 0.05, vs. Aβ<sub>40</sub>-treated group (one-way analysis of variance with post hoc Bonferroni test). Aβ: Amyloid-beta; BDNF: brain-derived neurotrophic factor; PBS: phosphate-buffered saline; qRT-PCR: quantitative reverse transcription-polymerase chain reaction.

**Figure 11** Effects of BDNF on Aβ<sub>40</sub>-induced caspase-3 gene expression in the retina (qRTP-PCR).

Total RNA was extracted after 24 hours, 3 days, and 7 days of intravitreal administration. Caspase-3 gene expression was normalized against that of GAPDH and β-Actin and compared with the PBS (control) group for statistical significance. Data represent three biological replicates with three technical replicates, n = 3 (mean ± SEM). Statistically significant difference was calculated using one-way analysis of variance followed by a multiple comparison test using Bonferroni method: 24 hours, F<sub>5,24</sub> = 15.309, P < 0.05; 3 days, F<sub>5,24</sub> = 35.103, P < 4 × 10<sup>−3</sup>; and 7 days, F<sub>5,24</sub> = 3.207, P < 0.1. n = 3 (mean ± SEM): *P < 0.05, **P < 0.01, vs. control group (PBS); #P < 0.05, **P < 0.01, vs. Aβ<sub>40</sub>-treated group. qRTP-PCR: Quantitative reverse transcription-polymerase chain reaction.

**Figure 12** Effect of BDNF on Aβ<sub>40</sub>-induced caspase-3 protein expression in the retina (western blotting).

Quantification was based on densitometry of three different biological samples. Total protein was extracted after 24 hours, 3 days, and 7 days of intravitreal administration. Total caspase-3 protein expression was normalized against that of β-actin and compared with the PBS (control) group for statistical significance. Data represent three biological replicates with three technical replicates, n = 3 (mean ± SEM). Statistically significant difference was calculated using one-way analysis of variance followed by a multiple comparison test using Bonferroni method: 24 hours, F<sub>5,24</sub> = 33.798, P < 5 × 10<sup>−3</sup>; 3 days, F<sub>5,24</sub> = 3.867, P = 8 × 10<sup>−3</sup>; and 7 days, F<sub>5,24</sub> = 2.387, P = 0.1. n = 3 (mean ± SEM): ***P < 0.001, vs. control group (PBS); #P < 0.05, vs. Aβ<sub>40</sub>-treated group. **P < 0.05, vs. BDNF group.
expression following treatment with BDNF seemed to enhance RGC survival, as demonstrated using Fluoro-Gold-labeling. Similarly, previous studies have shown that TrkB gene therapy markedly promotes RGC survival after optic nerve injury (Cheng et al., 2002), thereby indicating that BDNF/TrkB axis activation in RGCs can be a useful therapeutic approach in the treatment of several optic neuropathies (Cheng et al., 2002).

Considering that mitogen-activated protein kinase (MAPK) cascades are fundamental downstream pathways of TrkB activation, we also evaluated the effects of BDNF on Aβ1-42-induced changes in ERK1/2 expression at the gene and protein levels in the retina of rats. Aβ1-42-induced downregulation of MAPK cascade contributes to neuronal cell degeneration. ERK1/2, a key MAPK, is commonly associated with cell survival through the activation of various transcription factors, including CREB and mTOR. In the current study, significantly reduced RGC survival was associated not only with reduced TrkB expression but also with the downregulation of ERK1/2 gene and protein expression at all three time points post-treatment in Aβ1-42–treated rats compared with the control and BDNF-treated rats. Hence, TrkB does not occur downregulation, it is logical to conclude that the sequential changes, Aβ1-42 → reduced TrkB expression → reduced ERK1/2, culminate in neurodegenerative changes in the retina. These results are also in agreement with those from a study conducted by Cruz et al. (2018), which demonstrated a significant reduction in ERK1/2 phosphorylation in primary rat hippocampal and cortical neurons with increased Aβ1-42 expression (Cruz et al., 2018).

Importantly, intraretinal BDNF administration in rats exposed to Aβ1-42 caused an upregulation of ERK1/2 gene expression at all three time points post-treatment compared with rats treated with Aβ1-42 only. The corresponding protein expression was upregulated at 3 and 7 days post-treatment, but not at 24 hours post-treatment. The relatively lower protein expression levels at the earlier time point post-treatment may be attributed to the time lag between gene and protein expression. Previously, ERK1/2 activation in RGCs was proposed to be primarily responsible for promoting the survival of RGCs (Gupta et al., 2013). Hence, it can be stated with reasonable certainty that the protective effect of BDNF against Aβ1-42-induced neurodegeneration, as observed in the current study, involves the activation of TrkB and ERK1/2. Although the current study did not evaluate whether ERK1/2 activation occurred following TrkB activation, evidence from literature indicates the likelihood of a sequential activation of BDNF → TrkB → ERK1/2. The translation of TrkB and ERK1/2 activation, in the protective effects observed in the current study was clearly demonstrated by increased RGC survival in BDNF-treated retinas. Since TrkB signaling also leads to the activation of pathways other than ERK1/2, the contribution of other activated pathways in BDNF-treated retinas. Since TrkB signaling also leads to the activation of pathways other than ERK1/2, the contribution of other activated pathways to enhanced RGC survival could not be excluded in the current study. Nevertheless, it would be logical to conclude that increased ERK1/2 activation following increased TrkB expression at least partially contributes to enhanced RGC survival in response to BDNF treatment.

Since caspases are crucial mediators of programmed cell death (apoptosis), we further corroborated the observations made in relation to the effects of BDNF on TrkB and ERK1/2 expression by studying the expression of caspase-3 at the gene and protein levels. Caspase-3 was chosen because it is a typical hallmark of apoptosis and, as a final executioner caspase, it is indispensable at the gene and protein levels. Caspase-3 was chosen because it is a typical hallmark of apoptosis and, as a final executioner caspase, it is indispensable at the gene and protein levels. Caspase-3 was chosen because it is a typical hallmark of apoptosis and, as a final executioner caspase, it is indispensable at the gene and protein levels. Caspase-3 was chosen because it is a typical hallmark of apoptosis and, as a final executioner caspase, it is indispensable (Yuan et al., 1993). Rats exposed to Aβ1-42 showed upregulated expression of retinal caspase-3 at the gene and protein levels compared with the control and BDNF groups at all three time points post-treatment, indicating significant neuronal apoptosis. Hence, it can be proposed that in the current study, Aβ1-42–induced neuronal damage in the retina is likely the outcome of reduced TrkB expression → reduced ERK1/2 expression → increased caspase-3 expression. Previous studies have also shown that the suppression of ERK1/2 is closely associated with increased caspase expression and the resulting apoptosis (Berra et al., 1998). Intraretinal BDNF administration in rats exposed to Aβ1-42 caused downregulation of the expression of caspase-3 at the gene and protein levels. Based on the results of this study, it would be logical to propose that a BDNF-induced increase in TrkB and ERK1/2 expression culminates into reduced caspase-3 activation, leading to the pro-survival effect observed on RGCs. Earlier studies have shown that the activation of the MAPK/ERK and PI3-K/AKT pathways by TrkB/BDNF can block caspase-3 activation (Yuan et al., 1993). ERK1/2 activation is associated with reduced caspase activation and improved cell survival (Holmström et al., 2000; Means et al., 2006). Taken together, these findings demonstrate the potential of BDNF/TrkB axis activation as a therapeutic approach for enhancing survival and axon growth following injury or in degenerative diseases, such as glaucoma.

Conclusion

Treatment with BDNF prevents Aβ1-42–induced retinal injury by inhibiting Aβ1-42–induced neuronal apoptosis through the downregulation of caspase-3 expression and activation of TrkB and ERK1/2 expression. Further studies are needed to fully explore the enhanced efficacy of BDNF as a neuroprotective agent to enable its application in the treatment of AD as well as its ocular manifestation glaucoma. This study used intraretinal administration, which may not be appropriate for patients with glaucoma. Hence, developing appropriate non-invasive formulations of BDNF for future clinical applications is critically important. For example, nanoparticles can not only improve the passage of BDNF through the blood-retinal barrier but may also help to slow the uptake and degradation. Based on these advantages, nanoparticles formulation may offer an enhanced therapeutic effect of BDNF in treating ocular manifestations of AD.
