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Relationship between Sigma-1 receptor and BDNF in the visual system

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Abstract

Glaucoma is an incurable optic neuropathy characterized by dysfunction and death of retinal ganglion cells (RGCs). Brain derived neurotrophic factor (BDNF) is an essential neurotrophin that supports RGC function and survival. Despite BDNF's importance, our knowledge of molecular mechanisms that modulate BDNF processing and secretion is incomplete. Sigma-1 receptor (S1R) is associated with increased BDNF in hippocampus and with BDNF secretion by brain-derived astrocytes and neuronal cell lines. Much less is known about the relationship between S1R and BDNF in the visual system. Here, we examine how S1R activation and deletion alter expression of mature BDNF (mBDNF) and proBDNF in retina and cultured optic nerve head (ONH) astrocytes. For S1R activation, the S1R agonist (+)-pentazocine (PTZ, 0.5 mg/kg) was administered by intraperitoneal injection to C57BL/6J mice, 3 times per week, for 5 weeks. Expression of proBDNF and mBDNF was also examined in S1R knockout and age-matched C57BL/6J mice. *In vitro*, cultured ONH astrocytes were treated with 3 μ M PTZ for 24 hours followed by collection of media and ONH astrocyte lysates. Results showed that treatment with (+)-PTZ increased mBDNF protein in both retina and hippocampus. In contrast, S1R deletion was associated with retinal mBDNF deficits. In ONH astrocytes S1R agonist (+)-PTZ significantly increased levels of secreted BDNF and proBDNF in cell lysates. These findings support a role for S1R in the modulation of BDNF levels within the retina and optic nerve head. Treatment with S1R agonists might provide benefit in diseases such as glaucoma by increasing BDNF levels from endogenous sources.

Keywords

BDNF; retina; optic nerve head astrocytes; glaucoma

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1.0 Introduction

Glaucoma is an incurable optic neuropathy and second leading cause of blindness worldwide (Quigley and Broman, 2006). Glaucoma is characterized by the death and dysfunction of retinal ganglion cells (RGCs), the final output neurons of the retina. RGC axons leave the retina at the optic nerve head (ONH) where they form the optic nerve and project to the brain. The most common risk factors for glaucoma are aging and elevated intraocular pressure (IOP) (Friedman et al., 2004; Worley and Grimmer-Somers, 2011). Because lowering IOP is currently the only treatment strategy for slowing glaucoma-induced degeneration (Gordon et al., 2002; Heijl et al., 2002; Kass et al., 2002), a great need exists to identify novel targets that will protect RGCs. Development of these new therapies depends, in part, upon expanding our understanding of molecular mechanisms that regulate expression and secretion of brain derived neurotrophic factor (BDNF), a critical factor for RGC function and survival (reviewed by (Johnson et al., 2009; Mysona BA, 2017)).

The study of BDNF within the visual system is complicated by the presence of multiple forms and sources of BDNF. BDNF is synthesized as a precursor protein, proBDNF (30–35 kD), that is proteolytically cleaved and processed to form mature BDNF (mBDNF, 14 kD) (Kolbeck et al., 1994; Suter et al., 1991; Teng et al., 2010). This cleavage occurs either intracellularly in the endoplasmic reticulum (ER) and golgi (Seidah et al., 1996), or extracellularly (Lee et al., 2001; Pang et al., 2004). Whether a cell secretes proBDNF, mBDNF, or both varies with tissue, cell type, and culture conditions (Koshimizu et al., 2009; Matsumoto et al., 2008; Yang et al., 2009). In healthy retina, BDNF is produced endogenously by RGCs and glia (Avwenagha et al., 2006; Perez and Caminos, 1995; Vecino et al., 1998) or can enter the retina via retrograde transport from the brain via RGC axons (Pease et al., 2000; Quigley et al., 2000).

The link between S1R and BDNF first became evident when several selective serotonin uptake inhibitors used as anti-depressants were shown to bind S1R and to enhance BDNF expression and signaling (Larsen et al., 2008; Narita et al., 1996; Yagasaki et al., 2006). In addition, treatment with S1R specific agonists SA4503 (Matsuno et al., 1996) and PRE-084 (Su et al., 1991) enhances BDNF expression and signaling in hippocampal regions of the brain (Kikuchi-Utsumi and Nakaki, 2008; Xu et al., 2015). In neuroblastoma cell lines, S1R has been shown to act as a chaperone associated with enhanced secretion of mature BDNF (Fujimoto et al., 2012). Whether the relationship between S1R agonists and BDNF extends to other regions within the central nervous system (CNS) is uncertain. For example, the antidepressants imipramine and fluvoxamine both enhance BDNF signaling through a S1R dependent mechanism in cortex-derived neuronal cultures. However, chronic treatment with the S1R agonist, SA4503, did not increase BDNF protein in rodent cortex (Kikuchi-Utsumi and Nakaki, 2008; Larsen et al., 2008; Matsuno et al., 1996; Narita et al., 1996; Xu et al., 2015; Yagasaki et al., 2006). Given the importance of BDNF in supporting RGC health and function in the retina and the need for neuroprotective therapies in neurodegenerative diseases such as glaucoma, we were interested in investigating the relationship between S1R and BDNF in the visual system.

In this paper, we hypothesized that S1R modulates BDNF levels within the visual system. Effects of S1R activation and deletion were examined *in vivo* in retina and *in vitro* in ONH astrocyte cultures. Results of this study showed that *in vivo*, S1R activation by (+)-PTZ increased mBDNF protein in retina, whereas S1R deletion caused a mBDNF deficit. *In vitro*, (+)-PTZ treatment of ONH astrocyte cultures increased proBDNF levels within cell lysates and stimulated cellular secretion of BDNF. Together these findings suggest that an important relationship exists between S1R and BDNF within the retina and ONH. Treatment with S1R agonists might provide benefit in diseases such as glaucoma by increasing BDNF levels from endogenous sources.

2.0 Methods

2.1 Animals and injections

Experiments requiring animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the National Institutes of Health guide for the care and use of laboratory animals. C57BL/6J (The Jackson Laboratory, Bar Harbor, Maine) and S1R-KO mice (Wang et al., 2015), ages 4–18 months, were used in experiments. Mice were kept under controlled lighting conditions (12-h light: 12-h dark). A subset of C57BL/6J mice (4 months old) received intraperitoneal injection of (+)-PTZ (Sigma-Aldrich, St. Louis, MO) 0.5 mg/kg, every other day, 3 days per week, for 5 weeks. (+)-PTZ was dissolved initially in DMSO and diluted 1:10 with 0.01M phosphate buffered saline (PBS) and administered at the final dosage of 0.5mg/kg. In addition to mice, Sprague-Dawley rat breeder pairs (ENVIGO Laboratories, Indianapolis, IN) were maintained for the generation of 3–5 day old rat pups used in the isolation and culture of ONH astrocytes.

2.2 Collection of tissue

Following euthanasia by CO₂, retina and hippocampus were removed and immediately frozen over dry ice and stored at –80°C until needed.

2.3 Western blot

Tissue and cells were lysed and sonicated in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA) and 2 mM sodium orthovanadate (Sigma-Aldrich) phosphatase inhibitor. Lysates were centrifuged at 14,000g for 15 minutes. Protein concentration in the supernatant was measured using the Bio-Rad DC Protein assay (Bio-Rad, Hercules, CA). Proteins were separated by electrophoresis using SDS-polyacrylamide gel (PAGE). For mBDNF (14 kD) 15% SDS-PAGE or gradient (4–20%, Bio-Rad) gels were used. Gels were transferred to a nitrocellulose membrane (Thermo Fisher Scientific). Membranes were blocked with 5% nonfat milk in Tris-buffered saline-0.1% Tween 20 (TBST) for 15–30 min at room temperature and then incubated overnight at 4°C with primary antibody. After three washes in TBST, membranes were incubated for 1.5 hour with appropriate HRP-conjugated secondary antibody in 5% milk TBST solution at room temperature. Proteins were visualized by incubating with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) or Immobilon Western Chemiluminescent HRP substrate (EMD Millipore, Billerica, MA) and quantified by densitometry with ImageJ software (Schneider et al.,

2012). Blots were stripped and re-probed for loading controls, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -actin, or γ -tubulin. Different loading controls were required as blots were sometimes cut to facilitate probing for proteins with different molecular weights from the same blot.

2.4 BDNF ELISA

Total BDNF levels in mouse tissue were quantified using Quantikine ELISA kit for total BDNF (R&D Systems, Minneapolis, MN) per the manufacturer's instructions. For retina 50 μ l of retinal lysate containing 30 μ g of total protein was loaded for each condition. BDNF in rat ONH astrocyte cell culture media was quantified using RayBio Rat BDNF ELISA Kit (RayBiotech, Norcross, GA) per the manufacturer's instructions.

2.5 Antibodies

Antibodies were purchased from manufacturers as follows: anti-ProBDNF (AGP-031) with blocking peptide (Alomone, Jerusalem, Israel); anti-actin and anti- γ -tubulin (Sigma-Aldrich); anti-BDNF(SC-546), anti-GAPDH (sc-365062), HRP-conjugated anti-rabbit, anti-goat, and anti-mouse IgG all from Santa Cruz Biotechnology, Dallas, TX; HRP-conjugated donkey anti-guinea pig (Jackson ImmunoResearch Laboratories, West Grove, PA), goat anti-rabbit Alexa-Fluor 546 (Invitrogen, Carlsbad, CA). S1R rabbit polyclonal antibody was raised from a peptide sequence generated by the laboratory of Dr. Sylvia Smith (Ola et al., 2002).

2.6 Rat ONH astrocyte culture

Primary ONH astrocytes were isolated and cultured from 3–5 day old Sprague Dawley rat pups as published previously by our group (Zhao J, under revision) per the protocol modified from Mandal (Mandal et al., 2010) and Beckel (Beckel et al., 2014). Briefly, after sacrifice, eyes were enucleated and ONH tissue dissected proximal to the sclera and digested for 15 minutes using 0.25% trypsin (Invitrogen) at 37°C. After washing, cells were spun and resuspended in growth media of Dulbecco's modified Eagle's medium DMEM/F12 (Invitrogen) containing 10% heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA), 1% penicillin/streptomycin and 1% Glutamax (Invitrogen) and 25ng/ml epidermal growth factor (EGF) (Sigma-Aldrich), and plated on 0.2% gelatin (Sigma-Aldrich) coated T75 cell-culture flasks. Cells were maintained in a humidified incubator containing 5% CO₂ at 37°C. Cells were passaged after 7–10 days and were used between passages 2–5. Immunocytochemistry for GFAP was performed to confirm purity of ONH astrocyte cultures as published previously by our group (Zhao J, under revision).

2.7 ONH astrocyte cell treatment

For cell culture experiments, ONH astrocytes were seeded 250,000 cells per 25 cm² filter flask and maintained for 3 days after which cells were washed 1x with serum free DMEM F-12 with 0.1% Penn-Strep and 1% glutamax and maintained in 4 ml of serum free media with or without treatment with (+)-PTZ (3 μ M) for 24 hours.

2.8 ONH astrocyte collection of media and cell lysates

After 24 hours, 3.5 ml media was collected and concentrated using Amicon Ultra-4 Centrifugal filters, Ultracel – 3K (EMD Millipore). For secreted BDNF, the concentrated media was analyzed by ELISA (RayBiotech) and normalized to protein content of the corresponding cell lysate. To collect lysates, ONH astrocytes were washed 1X with PBS then lysed in 100 μ l of RIPA buffer prepared as described above for Western blot and stored at -80°C for further analysis. For ONH astrocyte studies, 6 experiments using ONH astrocytes from independent isolations were used.

2.9 Statistical analysis

Statistical analysis was performed with the aid of GraphPad Prism (Prism; GraphPad Software Inc. La Jolla, CA). For comparison of two groups the unpaired t test was used with Welch's correction for unequal variances. For multiple comparisons of groups with a normal distribution and equal variances one-way ANOVA was used followed by Tukey-Kramer post hoc test. Significance was set at $P < 0.05$.

3.0 Results

In this study, we hypothesized that S1R activation and deletion would have the opposite effects of increasing versus decreasing BDNF levels in the visual system. Administration of the S1R agonist (+)-PTZ was used to activate S1R. C57BL/6J mice (4 weeks old) were treated for five weeks with (+)-PTZ and compared to age-matched C57BL/6J (WT) controls (Figure 1A). At end of treatment, retina and hippocampus tissues were collected and lysates analyzed by Western blot to determine whether S1R activation caused changes in expression levels of mBDNF and proBDNF proteins. We began by examining the relative level of mBDNF in hippocampus of (+)-PTZ treated mice (Figure 1B) to provide a comparison with the published finding that treatment with the S1R agonist SA4503 (Kikuchi-Utsumi and Nakaki, 2008) increases levels of mBDNF in hippocampus. Our results also showed that treatment with the S1R agonist (+)-PTZ corresponded with a significant elevation of mBDNF in hippocampus (2.04 ± 0.44 fold increase) relative to WT controls.

Next we examined the relative levels of mBDNF, proBDNF, and S1R in the retinas of (+)-PTZ treated mice compared to WT controls. Similar to hippocampus, retinas of (+)-PTZ treated mice showed a significant increase in mBDNF (1.57 ± 0.20 fold increase) compared to untreated controls (Figure 1C). ProBDNF protein expression in retinas of (+)-PTZ treated mice was similar to that of controls (Figure 1D). However, S1R protein levels (Figure 1E) were significantly increased in retinas of (+)-PTZ treated mice (1.75 ± 0.14 fold change).

The finding that treatment with (+)-PTZ increased mBDNF in retina prompted us to examine the effect of S1R deletion on retinal BDNF levels. Relative levels of mBDNF and proBDNF were examined in WT and KO retinas of adult (5–6mo) mice. Western blot analysis showed that KO retinas had a significant deficit in mBDNF (0.57 ± 0.06 fold decrease). In contrast, proBDNF protein was similar in both KO and WT retinas (Figure 1F and 1G, respectively). We also investigated the level of total BDNF in KO and WT retinal lysates. For this experiment, age-matched KO and WT retinal lysates from adult mice (ages

6–18 months) were examined by ELISA. Results from the pooled analyses showed that total BDNF was significantly decreased in retinal lysates of KO mice (0.51 ± 0.11 fold change) compared with their WT counterparts (Figure 1H).

Finally, we used cultured primary ONH astrocytes to investigate the effect of (+)-PTZ treatment on S1R-mediated BDNF levels at the ONH. Cultured ONH astrocytes were strongly positive for GFAP (Figure 2A). ONH astrocyte cultures were treated with (+)-PTZ (3 μ M) or vehicle alone for 24 hours, after which media was concentrated and analyzed by ELISA to determine the amount of total BDNF secreted. ONH astrocyte cell lysates were also collected and analyzed by western blot for mBDNF and proBDNF protein. Treatment with (+)-PTZ significantly increased secretion of total BDNF (1.72 ± 0.27 fold change) compared to vehicle treated control astrocytes (Figure 2C). This increase was accompanied by elevated proBDNF (1.75 ± 0.30 fold increase) in ONH astrocyte cell lysates (Figure 2D). No change was observed for mBDNF or S1R proteins in (+)-PTZ treated ONH astrocyte lysates compared to controls (Figures 2E, and 2F).

4.0 Discussion

The main finding of this study is that S1R activation and deletion exerted the opposite actions of increasing versus decreasing BDNF levels in the visual system. Treatment with the S1R agonist, (+)-PTZ, increased the level of mBDNF in mouse retina and hippocampus. These results are consistent with the findings of increased mBDNF in the hippocampi of mice treated with the S1R agonist, SA-4503 (Kikuchi-Utsumi and Nakaki, 2008). In addition, (+)-PTZ treatment of cultured primary ONH astrocytes caused these cells to increase secretion of total BDNF. We also found a corresponding increase in proBDNF protein in astrocyte lysates. Furthermore, consistent with S1R-mediated regulation of BDNF, analysis of retinas derived from S1R-KO mice showed BDNF deficits.

The discovery that S1R agonist, (+)-PTZ, increases mBDNF in retina has important implications for glaucoma treatment. BDNF supplementation delays RGC death in rodent models of ocular hypertension (Domenici et al., 2014; Ko et al., 2001; Valiente-Soriano et al., 2015). Treatment strategies that provide a long-term source of locally supplied BDNF by means of viral vectors (Martin et al., 2003) or BDNF overexpression in transgenic mice (Feng et al., 2016), may afford better protection of RGCs than simple intravitreal injection of BDNF (Ko et al., 2001). In addition, BDNF supplementation to both eye and visual cortex appear to provide better protection of RGCs than to the eye alone (Weber et al., 2010). A therapeutic strategy using an agonist for S1R has potential to systemically enhance BDNF levels. This is especially important in the visual system where sources of BDNF include both endogenous production within the retina and retrograde transport from the brain. This study provides proof of concept for the future testing of S1R agonists as a novel treatment in rodent models of glaucoma.

In contrast to treatment with the S1R agonist (+)-PTZ, S1R-KO mice had retinal BDNF deficits. BDNF deficits are associated with visual dysfunction (Gupta et al., 2014) and S1R-KO mice display an age associated optic neuropathy typified by RGC death and decreased visual function (Ha et al., 2011). Future studies should address whether the mBDNF deficit

observed in our analyses is the cause of age-related RGC death and compromised visual function previously reported in S1R-KO animals (Ha et al., 2011).

The relationship between S1R and BDNF is particularly important at the ONH. The ONH is susceptible to glaucoma-induced injury (Johnson et al., 2000; Johnson et al., 2007) and BDNF deficits have been detected in this tissue (Gupta et al., 2014). Both lamina cribrosa cells and ONH astrocytes derived from human ONH have been shown to secrete BDNF (Lambert et al., 2001; Lambert et al., 2004). In rodents, the ONH astrocyte is the major supporting cell type at the ONH (Morrison et al., 1995; Sun et al., 2009). Because of the large number of animals required to generate sufficient ONH tissue for analysis, we investigated the relationship between S1R and BDNF in cultured ONH astrocytes isolated from rats (Zhao J, under revision).

Our results showed that stimulation of S1R with (+)-PTZ increased ONH astrocyte secretion of total BDNF with a corresponding increase of proBDNF protein within ONH astrocyte cell lysates. This finding is in accord with a recent study showing that S1R agonists stimulate the release of BDNF from a human astrocyte cell line (Dalwadi et al., 2017). S1R has the potential to mediate BDNF secretion via multiple mechanisms. S1R is associated with upregulation of BDNF transcription (Larsen et al., 2008) as well as with S1R mediated chaperone activity (Fujimoto et al., 2012). BDNF is well-known to be the subject of both transcriptional (Zheng et al., 2012) and post translational (Lu et al., 2005) regulation. Whether S1R-mediated increases in BDNF levels result from a direct effect on BDNF transcription or translation, or are secondary to S1R's known role as a modulator of oxidative (Wang et al., 2015) and ER stress (Ha et al., 2014) is not known.

In conclusion, the results of this study support the existence of an important relationship between S1R and BDNF in the visual system. The finding that S1R activation and deletion had opposite effects of increasing versus decreasing mBDNF levels has important implications for glaucoma treatment. Treatment with S1R agonists might provide benefit by increasing mBDNF levels from endogenous sources. Further studies are required to determine whether S1R agonists are an effective neuroprotective treatment for glaucoma.

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Highlights

- Treatment with (+)-PTZ increased mBDNF protein in both retina and hippocampus.
- S1R deletion was associated with retinal mBDNF deficits.
- S1R agonist (+)-PTZ significantly increased secreted BDNF and proBDNF in cell lysates of optic nerve head astrocytes.
- These findings support a role for S1R in the modulation of BDNF levels within the retina and optic nerve head.
- Treatment with S1R agonists might provide benefit in diseases such as glaucoma by increasing BDNF levels from endogenous sources.

Figure 1. S1R activation and deletion have opposite effects on retinal BDNF levels

A) Four month old C57BL/6J mice (WT) were treated for 5 weeks with (+)-PTZ (PTZ). **B)** (+)-PTZ treatment significantly increased mBDNF protein in hippocampus ($n=6-11$, * $P < 0.05$) and **C)** retina ($n=10-17$, ** $P < 0.01$). **D)** ProBDNF protein was unchanged by (+)-PTZ treatment in retina but **E)** S1R protein was significantly increased in (+)-PTZ treated retina ($n=10-17$, *** $P < 0.001$). **F)** Significant mBDNF deficits were detected in retinas of SR1-KO (KO) mice compared to WT ($n=11-17$, * $P < 0.05$) **G)** ProBDNF protein was similar in WT and KO retinas. **H)** Decreased total BDNF was detected by ELISA in retinas of 5–18 month KO mice compared to age-matched WT ($n=9$, * $P < 0.05$). For Western blot, representative bands show mBDNF and proBDNF from three different WT and KO mice relative to internal protein controls actin, γ -tubulin or GAPDH. Graphs show quantification of mBDNF, proBDNF, and S1R proteins in tissue lysates for all n .

Figure 2. Treatment with S1R agonist (+)-PTZ modulates BDNF in ONH astrocytes
 Cultures of ONH astrocytes are positive for **A)** GFAP (red), DAPI (blue) **B)** secondary only. **C)** Treatment for 24 hours with (+)-PTZ significantly increased secretion of total BDNF into the media as determined by ELISA (n=14–17, * $P < 0.05$). Western blot analysis showed corresponding significant increase of **(E)** proBDNF in ONHA cell lysates (n=10–14, * $P < 0.05$). Although **D)** mature BDNF, and **F)** S1R proteins showed no significant differences between control and (+)-PTZ treated ONH astrocyte lysates (n=9–15).