Sigma 1 Receptor Contributes to Astrocyte-Mediated Retinal Ganglion Cell Protection

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PURPOSE. Sigma 1 receptor (S1R) is expressed in retinal ganglion cells (RGCs) and astrocytes, and its activation is neuroprotective. We evaluated the contribution of S1R within optic nerve head astrocytes (ONHAs) to growth and survival of RGCs in vitro.

METHODS. Wild-type (WT) RGCs and WT or S1R knockout (S1R KO) ONHAs were cocultured for 2, 4, or 7 days. Total and maximal neurite length, neurite root, and extremity counts were measured. Cell death was measured using a TUNEL assay. Signal transducer and activator of transcription 3 phosphorylation levels were evaluated in ONHA-derived lysates.

RESULTS. The coculture of WT RGCs with WT or S1R KO ONHAs increased the total and maximal neurite length. Neurite root and extremity counts increased at 4 and 7 days when WT RGCs were cocultured with WT or S1R KO ONHAs. At all timepoints, the total and maximal neurite length decreased for WT RGCs in coculture with S1R KO ONHAs compared with WT ONHAs. Root and extremity counts decreased for WT RGCs in coculture with S1R KO ONHAs compared with WT ONHAs at 2 and 7, but not 4 days. RGC apoptosis increased in S1R KO ONHA coculture and S1R KO-conditioned medium, compared with WT ONHA coculture or WT-conditioned medium. S1R KO ONHA-derived lysates showed decreased phosphorylated signal transducer and activator of transcription 3 levels compared with WT ONHA-derived lysates.

CONCLUSIONS. The absence of S1R within ONHAs has a deleterious effect on RGC neurite growth and RGC survival, reflected in analysis of WT RGC + S1R KO ONHA indirect cocultures. The data suggest that S1R may enhance ganglion cell survival via glia-mediated mechanisms.

Keywords: optic nerve head astrocyte, retinal ganglion cells, neuroprotection

 Sigma 1 receptor (S1R) is a small (25.3 kDa), unique, transmembrane protein that is enriched within the endoplasmic reticulum.1,2 S1R functions to modulate multiple intracellular signaling and cell survival pathways through a variety of mechanisms that are not fully understood.3–7 Importantly, S1R is expressed in both neuronal and glial cell types throughout the central and peripheral nervous systems, including the optic nerve head.8–11 Within the eye, studies indicate that targeting S1R is beneficial in optic nerve and retinal disorders that affect ganglion cells and photoreceptors.12–17 For example, retinal ganglion cell (RGC) loss was attenuated and retinal structure preserved by administration of the S1R agonist (+)-pentazocine ((+)-PTZ) in rodent models of diabetic retinopathy and NMDA-induced inner retinal toxicity.18,19 Furthermore, investigations of S1R knockout (S1R KO) animals indicate that loss of S1R leads to accelerated neurodegenerative pathology in the brain, spinal cord, and optic nerve.20–25

In addition to in vivo investigations, in vitro studies have shown that S1R activation can protect isolated primary RGC cultures from apoptosis under conditions of excitotoxic and oxidative stress.13,14,26 Furthermore, our recent studies using primary cultures of optic nerve head-derived astrocytes (ONHAs) show that stimulation of S1R using (+)-PTZ mitigates the generation of intracellular reactive oxygen species and protects against astrocyte cell death.27 Whether S1R within ONHAs can function to protect RGCs independent of S1R activation within the RGCs themselves is an unknown but critically important question.

Astrocytes throughout the central nervous system (CNS) have both neuroprotective and neurotoxic properties, and these characteristics depend on contextual and environmental factors.28,29 For example, reactivity responses associated with ischemia (stroke), traumatic injury, or neurodegenerative disease show variation at different stages of the same disorder and among astrocyte cohorts derived from different CNS regions.28,30–32 In vivo studies performed specifically in the ON and ONH under glaucomatous conditions are consistent with the CNS paradigm of disorder and context-specific astrocytic stress

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responses. In addition, in vitro studies of astrocyte-neuronal cocultures indicate that astrocytes derived from the brain frontal cortex region can robustly protect RGCs from apoptosis, but that astrocytes derived from the optic nerve region may only weakly promote RGC survival. However, few studies have examined RGC–ONHA interactions in controlled, ex vivo environments.

Here, we report use of an in vitro, indirect coculture system of primary RGCs and primary ONHAs. We observe a clear increase in viability when RGCs are cocultured with adult, murine-derived ONHAs. Using the ONHA–RGC coculture system, we evaluate the extent to which lack of S1R in ONHAs alters RGC neurite growth and survival compared with WT ONHAs. We find that, compared with WT-derived ONHAs, S1R knockout (KO)-derived ONHAs are significantly less protective of WT RGCs in coculture. These results suggest that, in vitro, S1R may provide neuroprotection through glia-mediated mechanisms.

**METHODS AND MATERIALS**

**Animals**

Experiments requiring animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Our animal protocol is approved by Augusta University Institutional Animal Care and Use Committee (IACUC, 2011-0338). C57BL/6j (WT) mice (The Jackson Laboratory, Bar Harbor, ME) and S1R KO mice (see Wang et al.40) were kept under controlled lighting conditions (12-h light: 12-h dark).

**Primary Mouse Optic Nerve Head Astrocytes (ONHAs) Culture**

Primary mouse ONHAs were isolated from optic nerve head of WT mice and S1R KO mice according to our previously published protocol.41 Briefly, 6-week-old mice were euthanized. Six mice were used for each ONHA isolation and cell culture experiment. Optic nerve head tissue was dissected proximal to the sclera and was digested for 15 minutes using 0.05% trypsin (Invitrogen, Carlsbad, CA) at 37 °C. Cells were washed once with ONHA growth media (Dulbecco’s modified Eagle’s medium/F12; Invitrogen) containing 10% of fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 0.2 mg/mL L-cysteine (Sigma). Tissue was triturated in DPBS containing 0.15% trypsin inhibitor and 1% BSA. Resulting cells were pelleted by centrifugation (5 minutes, 2000 rpm) and the pellet was resuspended in panning buffer (DPBS, 0.02% BSA, 5 μg/mL insulin [Sigma], 60 μg/mL N-acetyl-L-cysteine [Sigma]). The cell suspension was incubated in a 75 cm² flask precoated with AffiniPure donkey anti-rabbit IgG (H+L) antibody (Jackson ImmunoResearch, West Grove, PA) at room temperature for 1 hour to remove macrophages and microglial cells. Nonadherent cells were incubated on a 100-mm Petri dish precoated with AffiniPure donkey anti-rat IgG (H+L) antibody (Jackson ImmunoResearch) conjugated to rat anti-mouse Thy-1.2 antibody (BD Biosciences, Franklin Lakes, NJ) at room temperature for 1 hour. Adherent RGCs were released by incubation with 0.125% trypsin (Sigma) in EBSS at 37°C for 5 minutes, followed by the addition of 30% fetal bovine serum in Neurobasal medium (Thermo Fisher Scientific, Waltham, MA). The suspension of RGCs was collected by centrifugation (5 minutes, 2000 rpm). The pellet was resuspended in Neurobasal medium containing 5 μg/mL insulin (Sigma), 1 mM sodium pyruvate (Thermo Fisher Scientific), 0.1 mg/mL transferserin (Sigma), 60 ng/mL progesterone (Sigma), 16 μg/mL putrescine (Sigma), 40 ng/mL sodium selenite (Sigma), 40 ng/mL tri-iodo-thyronine (Sigma), 1 mM L-glutamine (Thermo Fisher Scientific), 60 μg/mL N-acetyl cysteine (Sigma), 2% B27 (Thermo Fisher Scientific), 50 ng/mL brain-derived neurotrophic factor (Sigma), 10 ng/mL CNTF (Sigma), 10 ng/mL forskolin (Sigma), 10 ng/mL basic fibroblast growth factor (Sigma), 0.1 mg/mL BSA. Cells were plated on coverslips coated with poly-D-lysine (Sigma) and laminin (ThermoFisher). One-half of the culture medium was changed every other day.

**Coculture of Primary RGCs With Primary ONHAs**

The RGC/ONHA indirect coculture system was established based on the protocol by Navneet et al.44 with minor modifications. Briefly, 1 day before RGC isolation, WT or S1R KO ONHAs were seeded on a 0.4-μm pore size cell culture insert (ThermoFisher) at a density of 25,000 cells per insert. RGCs were isolated by a two-step immunopanning procedure from WT mice as described elsewhere in this article. After 1 hour of seeding RGCs on coverslips, ONHAs inserts were placed within the wells containing RGCs (Fig. 1A). In this coculture system, RGCs and ONHAs were cultured in the RGCs culture medium as described elsewhere in this article.

**Immunocytochemistry**

After 2, 4, and 7 days, RGCs on coverslips were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) at room temperature for 15 minutes, followed by washing three times with PBS. Cells were then permeabilized with 0.3% triton X-100 in PBS at room temperature for 10 minutes. Next, cells were blocked with 0.1% triton X-100 in PBS with 10% goat serum (Sigma) at room temperature for 1 hour, then incubated in βIII-tubulin antibody (1:500, Abcam, Cambridge, MA) at 4 °C overnight. Next, cells were incubated in secondary antibody (Alexa Fluor...
Figure 1. Coculture and neurite outgrowth measurements. (A) Coculture model of RGCs and ONHAs. Porous membrane transwell inserts (0.4 μm pore size) separated the culture system into two chambers. The RGCs were placed on the bottom of the lower chamber, and the ONHAs were placed on the membrane of the upper chamber. (B) Illustration of neurite outgrowth evaluation for total length, maximal length, root and extremity counts.

Western Blot Analysis

Mouse ONHAs were lysed in RIPA buffer (Sigma, St. Louis, MO) containing 2 mM sodium orthovanadate and 1% protease inhibitor cocktail (Thermo Fisher Scientific). Cell lysates were centrifuged at 14,000 g for 30 minutes. Protein concentration was measured by Bradford assay (Bio-Rad, Hercules, CA). Proteins were separated by electrophoresis on a 4% to 15% SDS-polyacrylamide gel (BioRad) and then transferred to a nitrocellulose membrane (Thermo Fisher Scientific). The membrane was blocked with 5% nonfat milk in Tris-buffered saline-0.05% Tween 20 for 1 hour at room temperature, then incubated overnight at 4°C with primary antibodies (pSTAT3 1:500 [Cell Signaling, Danvers, MA]; STAT3 1:500 [Cell Signaling]; S1R 1:500). After three washes in Tris-buffered saline-0.05% Tween 2, the membrane was incubated for 1 h with an appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX) at room temperature. Proteins were visualized by incubating with a SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) and quantified by densitometry with ImageJ software (http://imagej.nih.gov/ij/) provided in the public domain by the National Institutes of Health (Bethesda, MD). The S1R rabbit polyclonal antibody was raised from peptide sequence SEVYPGETVVGPEATDVEWG (corresponds with residues 143–165 of rat S1R) and generated within the laboratory of Dr Sylvia Smith. This antibody has been used in numerous publications since 2002 as a tool for study of S1R expression and function.

TUNEL Assay

After mouse RGCs were cultured on coverslips for 7 days, TUNEL analysis (ApopTag Fluorescein In Situ Apoptosis Detection Kit) was performed per the manufacturer’s instructions. The coverslips were mounted with Fluoroshield with DAPI (Sigma). The number of cells emitting the green fluorescence indicating apoptosis were counted. Data are expressed as apoptotic cells per total number of cells in the field (nuclei visualized by DAPI).

Statistical Analysis

Data were analyzed by one-way ANOVA followed by Tukey-Kramer post hoc test for multiple comparisons. Significance was set at a P value of less than 0.05 (Prism; GraphPad Software, Inc. La Jolla, CA).

RESULTS

Our previous in vitro studies have shown that activation of S1R enhances ONHA reactivity characteristics. To evaluate the effects of ONHAs, which express S1R versus those that do not, on RGCs in vitro, we adapted an indirect coculture method as previously described for combined culture of Müller cells with RGCs (Fig. 1A). After 2, 4, and 7 days, we measured neurite outgrowth parameters as described in the Methods.

Two days after seeding, comparison between groups revealed a significant increase in both total and maximal neurite length when WT RGCs were cocultured with either WT ONHAs or S1R KO ONHAs compared with WT RGC culture alone (Figs. 2A–D). A further analysis of the measurements showed a significant decrease in both total and maximal neurite length for WT RGCs cocultured with S1R KO ONHAs compared with WT RGCs cocultured with WT ONHAs or S1R KO ONHAs compared with WT RGC culture alone (Figs. 2A–D).
**Figure 2.** RGC neurite outgrowth after 2 days. WT RGCs were isolated from neonatal mouse pups and cultured for 2 days. Scale bar, 50 μm. (A) WT RGCs were plated alone on coverslips in the lower coculture chamber. (B) WT RGCs were plated on coverslips in the lower coculture chamber, and WT ONHAs were seeded on the membrane of the upper coculture chamber. (C) WT RGCs were plated on coverslips in the lower coculture chamber, and S1R KO ONHAs were seeded on the membrane of the upper coculture chamber. (D) RGC neurites were traced using Image J, Simple Neurite Tracer function, with green-colored overlay reflecting traced neurites. The total and maximal neurite length were measured and root and extremity numbers were counted. Significantly different from control at **P < 0.01, ****P < 0.0001. Data were analyzed using one-way ANOVA followed by the Tukey-Kramer post hoc test for multiple comparisons. Two coverslips, and eight microscopic fields per coverslip were quantified from each group of each isolation. The total number of cells analyzed per group was N = 200–350. The experiments were repeated in triplicate with cells isolated from different dates, different animals.

**Figure 3.** RGC neurite outgrowth after 4 days. WT RGCs were isolated from neonatal mouse pups and cultured for 4 days. Scale bar, 50 μm. (A) WT RGCs plated alone on coverslips. (B) WT RGCs were plated on coverslips in the lower chamber and WT ONHAs were seeded on the membrane of the upper coculture chamber. (C) WT RGCs were plated on coverslips in the lower coculture chamber, and S1R KO ONHAs were seeded on the membrane of the upper chamber. (D) RGC neurites were traced using Image J, Simple Neurite Tracer function, with green-colored overlay reflecting traced neurites. The total and maximal neurite length were measured and root and extremity numbers were counted. Significantly different from control at *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Data were analyzed using one-way ANOVA followed by the Tukey-Kramer post hoc test for multiple comparisons. Two coverslips, and eight microscopic fields per coverslip were quantified from each group of each isolation. The total number of cells analyzed per group was N = 150–400. The experiments were repeated in triplicate with cells isolated from different dates, different animals.
KO ONHAs compared with WT RGCs cocultured with WT ONHAs. (Figs. 2B–D). Quantification of neurite root and extremity counts was then performed (Fig. 2D). This analysis revealed significantly increased root and extremity counts when WT RGCs were cocultured with WT ONHAs, compared with WT RGC culture alone. In addition, there was a trend toward increased root and extremity counts when WT RGCs were cocultured with S1R KO ONHAs, compared with WT RGC culture alone (Fig. 2D). However, this trend was not significant. A further analysis of measurements showed a significant decrease in root and extremity counts when WT RGCs were cocultured with WT ONHAs compared with S1R KO ONHAs (Fig. 2D).

Four days after seeding, neurite outgrowth was evaluated in RGCs alone versus in coculture with WT or S1R KO ONHAs (Figs. 3A–D). An analysis of RGC morphology parameters revealed a significant increase in both total and maximal neurite length when WT RGCs were cocultured with either WT or S1R KO ONHAs compared with WT RGC culture alone (Figs. 3A–D). Further analysis showed a significant decrease in both total and maximal neurite length for WT RGCs cocultured with S1R KO ONHAs compared with WT RGCs cocultured with WT ONHAs. (Fig. 3D). Quantification of neurite root and extremity counts showed a significant increase in both parameters when WT RGCs were cocultured with either WT or S1R KO ONHAs compared with WT RGC culture alone. Finally, a comparison after 4 days showed no significant difference in root or extremity counts between WT RGCs cocultured with S1R KO ONHAs versus WT RGCs cocultured with WT ONHAs. (Fig. 3D).

Next, we evaluated the WT RGC neurite outgrowth after 7 days in culture. An assessment of RGC morphology parameters revealed a significant increase in both total and maximal neurite length when WT RGCs were cocultured with either WT ONHAs or S1R KO ONHAs compared with WT RGC culture alone (Figs. 4A–D). Additional analysis showed a significant decrease in both total and maximal neurite lengths when WT RGCs were cocultured with S1R KO ONHAs compared with WT RGCs cocultured with WT ONHAs. Next, quantification of neurite root and extremity count showed a significant increase in both parameters when WT RGCs were cocultured with either WT ONHAs or S1R KO ONHAs compared with WT RGC culture alone (Fig. 4D). Furthermore, an analysis of measurements showed a significant decrease in both root and extremity counts in WT RGCs cocultured with KO ONHAs compared with WT RGCs cocultured with WT ONHAs. (Fig. 4D).

To compare RGC neurite outgrowth over the time period studied, the complete data shown in Figures 2 through 4, were then analyzed. The values for RGC neurite total and maximal length, root and extremity counts were all compared over 2, 4, and 7 days in culture (Fig. 5). This analysis indicated that, across multiple timepoints, indirect coculture of RGCs with either WT or S1R KO ONHAs led to an improvement in measures of RGC neurite extension and complexity compared with RGC culture alone (Fig. 5). In addition, Figures 5A and B illustrate a steady increase in the total and maximal RGC neurite lengths for WT RGCs in coculture with WT ONHAs compared with S1R KO ONHAs or WT RGCs alone.
Furthermore, in Figures 5C and D, the significant increase in RGC neurite root and extremity counts in coculture with either WT or S1R KO ONHAs is obvious after 4 days in culture. The further increase in root and extremity counts in WT RGCs cocultured with WT ONHAs becomes even larger after 7 days in culture compared with either coculture with S1R KO ONHAs or WT RGCs alone (Figs. 5C and D).

To evaluate whether indirect coculture of ONHAs that expressed S1R or not altered RGC survival, we performed further ONHA/RGC coculture experiments. TUNEL assay was used to evaluate apoptosis of RGCs at the 7-day coculture time-point (Fig. 6A). Quantification of TUNEL-positive cells showed a significant decrease in cellular apoptosis when WT RGCs were cocultured with either WT or S1R KO ONHAs, compared with WT RGCs alone (Fig. 6B). Further comparison between groups showed a significant increase in TUNEL positivity when RGCs were cultured with S1R KO ONHAs compared with WT ONHAs (Fig. 6B). This finding indicates that a lack of S1R within ONHAs leads to decreased protection against WT RGC apoptosis in ONHA/RGC coculture.

To test whether conditioned medium alone derived from ONHAs, with or without S1R, was protective against RGC apoptosis, we cultured isolated WT RGCs for 7 days with regular whole growth medium or with conditioned medium derived from WT ONHAs or S1R KO ONHAs. The day before WT RGCs isolation, WT and S1R KO ONHAs were placed in RGC whole growth medium, and the media was

Figure 5. Comparison of neurite outgrowth over time among groups of WT RGCs, alone or in coculture with WT or S1R KO ONHAs. (A) Total neurite length measurement. (B) The maximal neurite length measurement. (C) The root count. (D) The extremity count. The experiments analyzed were repeated in triplicate with cells isolated from different dates, different animals.

Figure 6. RGC apoptosis alone and in coculture with ONHAs. (A) Representative images of TUNEL assay for RGCs alone and in coculture with WT and S1R KO ONHAs. (B) Quantification of TUNEL-positive cells. Significantly different from control at *P < 0.05, ****P < 0.0001. Data were analyzed using one-way ANOVA followed by the Tukey-Kramer post hoc test for multiple comparisons. Two coverslips, and eight microscopic fields per coverslip were quantified from each group of each isolation. The total number of cells analyzed was N = 1000–3000. These experiments were repeated in triplicate with cells isolated from different dates, different animals.
conditioned by the ONHAs for 24 hours. The freshly isolated WT RGCs were then plated on coverslips in either RGC whole growth medium or with conditioned medium derived from WT or S1R KO ONHAs. One-half of the medium was changed and replaced with corresponding medium every other day for 7 days. A TUNEL assay was then used to evaluate RGC apoptosis (Fig. 7A). Results were similar to those observed for TUNEL evaluation of indirect ONHA–RGC cocultures (Fig. 6A–B). Quantification of TUNEL-positive cells showed a significant decrease in cellular apoptosis when WT RGCs were incubated with conditioned medium derived from either WT or S1R KO ONHAs (Fig. 7B). In addition, a comparison between groups showed a significant increase in TUNEL positivity when RGCs were cultured with S1R KO ONHA-derived conditioned medium compared with WT ONHA-derived conditioned medium (Fig. 7B). This finding indicates that factors present in the medium derived from ONHAs can protect cultured RGCs from apoptosis, independent of any communication between RGCs and ONHAs in indirect coculture. Furthermore, the lack of S1R within ONHAs causes medium derived from these cells to provide less protection against WT RGC apoptosis than medium derived from WT ONHAs.

Our previous work showed that S1R mediates aspects of ONHA reactivity, and studies indicate that modulation of astrocyte reactivity responses can contribute to neuroprotection, in vitro and in vivo.28,34,38,41 We previously found that treatment of ONHAs with an S1R agonist enhanced phosphorylation of the signaling molecule, signal transducer and activator of transcription 3 (STAT3), a critical astrocyte reactivity regulator.41 To evaluate whether presence or absence of S1R in ONHAs affected levels phosphorylated STAT3 (pSTAT3), we prepared cellular lysates from WT versus S1R KO ONHA cultures. Lysates were analyzed by Western blot and probed with antibodies to STAT3 and pSTAT3 (Fig. 8A). Results showed significantly higher levels of pSTAT3 in lysates derived from WT ONHAs compared with lysates derived from S1R KO ONHAs (Figs. 8A and B).
**DISCUSSION**

We report here the use of a novel ex vivo indirect coculture method to evaluate ONHA-RGC interactions in the presence and absence of S1R. Results of our investigations indicate that, in vitro, the loss of S1R within ONHAs decreases the ability of these cells to support healthy growth and prevent apoptosis of RGCs. Ligands for S1R are in clinical development for a wide range of disorders including neurodegenerative diseases, cancer, drug abuse, and viral infection owing to severe acute respiratory syndrome coronavirus 2.\textsuperscript{97–95} Furthermore, stimulation of S1R may offer a useful treatment strategy for optic neuropathies, including glaucoma.\textsuperscript{94} Within the optic nerve head, S1R is expressed in both RGCs and astrocytes.\textsuperscript{10,11} Therefore, the effects of S1R on RGC-astrocyte interaction are critically important for evaluation of this protein as a therapeutic target.

Historically, the isolation and culture of purified RGCs alone yields lower viability and neurite outgrowth than RGCs in glia-mediated coculture.\textsuperscript{55,56} Our results are consistent with this paradigm. Our cell culture model used an indirect coculture technique previously published for combined primary culture of Müller glia and RGCs that showed Müller glia-mediated RGC protection.\textsuperscript{44} In addition, studies indicate that cerebral cortex-derived astrocytes significantly extend viability and promote neurite outgrowth when in coculture with RGCs.\textsuperscript{55,56} Interestingly, additional previous studies show that the direct coculture of RGCs over a conditioning layer of optic nerve-derived astrocytes only weakly promotes RGC survival.\textsuperscript{39} In contrast with these findings, our studies show that indirect coculture with WT optic nerve head-derived astrocytes robustly protects RGCs from apoptosis and improves measures of neurite outgrowth. The apparent disparity between studies is consistent with recent work describing significant heterogeneity in neurotoxic versus neuroprotective astrocyte properties both in vitro and in vivo.\textsuperscript{57–59} These properties depend on multiple factors, including the brain or CNS region from which the astrocytes are derived.\textsuperscript{50} Further studies are needed to clarify how astrocytic subtypes are established and the conditions under which glia cells derived from specific regions of the optic nerve contribute to versus detract from RGC survival.

In addition to evaluating RGC-ONHA interactions using indirect coculture, we investigated the effects of astrocyte-secreted factors using conditioned medium derived from our cultured ONHAs. We found that conditioned medium from WT ONHA cultures alone also provided robust protection against apoptosis of WT RGCs in culture (Fig. 7). However, WT RGCs exposed to conditioned medium taken from S1R KO ONHA cultures showed significantly greater apoptosis than WT RGCs exposed to WT ONHA-conditioned medium. These results suggest that, in vitro, S1R plays a role in modulating the astrocyte secretome. Indeed, previous studies show that stimulation of S1R with its high affinity agonist, (+)-PTZ, leads to increased astrocytic release of brain-derived neurotrophic factor, a neurotrophin critical to RGC survival.\textsuperscript{61–65} In addition, as a ligand-operated protein localized at the endoplasmic reticulum membrane, S1R has been shown to regulate levels of several other proteins that are processed and transported through the cellular secretory pathway.\textsuperscript{64,65} Future studies are needed to clarify the role of S1R in modulation of the cellular secretome under conditions of varying cellular stress and tissue microenvironments.

Our previous studies of ONHAs indicate that the activation of S1R using (+)-PTZ leads to the enhancement of astrocyte reactivity characteristics under conditions of cellular stress.\textsuperscript{43} In addition, we found that S1R activation in WT ONHAs leads to an increase in phosphorylation of the astrocyte regulatory protein, STAT3.\textsuperscript{61} In the current studies, we find that cellular lysates derived from S1R KO ONHAs show significantly lower levels of phosphorylated STAT3 compared with lysates derived from WT ONHAs. The role of STAT3 in neurodegenerative diseases is incompletely understood. In some disease contexts, including models of Alzheimer’s and multiple sclerosis, inhibiting STAT3 activation in astrocytes leads to neuroprotective effects.\textsuperscript{66,67} However, interestingly, in vivo studies indicate that the conditional astrocytic KO of STAT3 within the optic nerve leads to decreased measures of astrocyte reactivity and an increased loss of RGCs under conditions of glaucomatous stress.\textsuperscript{43} Additional studies are needed to determine whether the activation or inhibition of S1R in vivo alters STAT3 levels and whether this correlates with the effects of S1R on RGC survival.

In summary, the present work illustrates the importance of S1R to ONHA-mediated support for neurite outgrowth and survival of RGCs in vitro. Future work will examine the mechanisms by which S1R in ONHAs functions to protect RGCs. This will help us to understand the complex neuroprotective role of S1R in ONHAs.

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