Siponimod exerts neuroprotective effects on the retina and higher visual pathway through neuronal S1PR1 in experimental glaucoma

Devaraj Basavarajappa, Vivek Gupta, Nitin Chitranshi, Roshana Vander Wall, Rashi Rajput, Kanishka Pushpitha, Samridhi Sharma, Mehdi Mirzaei, Alexander Klistorner, Stuart L. Graham

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Abstract

Sphingosine-1-phosphate receptor (S1PR) signaling regulates diverse pathophysiological processes in the central nervous system. The role of S1PR signaling in neurodegenerative conditions is still largely unidentified. Siponimod is a specific modulator of S1P1 and S1P5 receptors, an immunosuppressant drug for managing secondary progressive multiple sclerosis. We investigated its neuroprotective properties in vivo on the retina and the brain in an optic nerve injury model induced by a chronic increase in intraocular pressure or acute N-methyl-D-aspartate excitotoxicity. Neuronal-specific deletion of sphingosine-1-phosphate receptor (S1PR1) was carried out by expressing AAV-PHP.B-cre recombinase under Syn1 promoter in S1PR1^lox/lox mice to define the role of S1PR1 in neurons. Inner retinal electrophysiological responses, along with histological and immunofluorescence analysis of the retina and optic nerve tissues, indicated significant neuroprotective effects of siponimod when administered orally via diet in chronic and acute optic nerve injury models. Further, siponimod treatment showed significant protection against trans-neuronal degenerative changes in the higher visual center of the brain induced by optic nerve injury. Siponimod treatment also reduced microglial activation and reactive gliosis along the visual pathway. Our results showed that siponimod markedly upregulated neuroprotective Akt and Erk1/2 activation in the retina and the brain. Neuronal-specific deletion of S1PR1 enhanced retinal and dorsolateral geniculate nucleus degenerative changes in a chronic optic nerve injury condition and attenuated protective effects of siponimod. In summary, our data demonstrated that S1PR1 signaling plays a vital role in the retinal ganglion cell and dorsolateral geniculate nucleus neuronal survival in experimental glaucoma, and siponimod exerts direct neuroprotective effects through S1PR1 in neurons in the central nervous system independent of its peripheral immuno-modulatory effects. Our findings suggest that neuronal S1PR1 is a neuroprotective therapeutic target and its modulation by siponimod has positive implications in glaucoma conditions.

Key Words: glaucoma; intraocular pressure; neurodegeneration; neuroprotection; optic nerve injury; retinal ganglion cells; siponimod; sphingosine-1-phosphate

Introduction

Sphingosine-1-phosphate (SIP) is a crucial lipid signaling molecule generated from ceramide metabolism and binds to five G-protein-coupled receptors, termed S1P1–5 receptors (S1PR1–5) (O’Sullivan and Dev, 2017). S1PRs are expressed ubiquitously and play major roles in cell survival, growth, migration, and differentiation. Consequently, S1PR modulation has emerged as a potential therapeutic strategy in various diseases, including inflammatory, cardiovascular, and brain disorders (Cartier and Hla, 2019). Siponimod (BAF312) and fingolimod (FTY720) are SIP structural analogues that modulate S1P receptor signaling and are approved immunosuppressive drugs for the treatment of multiple sclerosis (Kappos et al., 2006, 2018; Gajofatto, 2017). The active form, fingolimod-phosphate (fingolimod-P), formed following in vivo phosphorylation, binds potently to four SIP receptors, S1P1, S1P3, S1P4, and S1P5. Siponimod, in contrast, is a selective modulator of S1P1 and S1P5 receptors. Immunomodulation has been primarily regarded as the mechanism of action of both drugs in MS. Modulation of S1PR signaling is implicated in the pathophysiology of various CNS disorders of the CNS. S1PRs in neuronal precursor cells and neurons play roles in cell migration, neurogenesis, neural development, and survival (Gupta et al., 2012; Karunakaran and van Echten-Deckert, 2017). S1PR signaling pathways in microglia and astrocytes regulate their activation, proliferation, gliosis, and neuroinflammation (Lucaciu et al., 2020). S1PRs in oligodendroglia and oligodendrocyte precursor cells participate in differentiation, survival, and myelination (Martin and Sospedra, 2014; Roggeri et al., 2020). Siponimod, in addition to immunomodulatory effects on the peripheral immune cells, can cross the blood-brain barrier (Hunter et al., 2016; Kipp, 2020; Bigaud et al., 2021). Recent data on the protective effects of siponimod in clinical trials...
involving secondary progressive MS patients suggests that siponimod may directly interact with S1PR1 in the CNS (Behrangi et al., 2019; Dumitruc et al., 2019) which is important as secondary progressive MS is largely driven by local CNS degenerative mechanisms (Bartocci et al., 2018; Simkins et al., 2021). There is limited evidence to demonstrate the in vivo role of S1PR1 in the CNS cell types in degenerative conditions.

The retina and optic nerve (ON) are considered unique extensions of the CNS and show specific changes in response to various neurological disorders (London et al., 2013). Glaucoma is a age-related multifactorial neurodegenerative disease, clinically characterized by cupping of the optic nerve head with irreversible visual field loss due to ON damage and retinal ganglion cell (RGC) death, with anterograde trans-neuronal degeneration in higher visual centers (Ionas et al., 2017; Melik Parsadanians et al., 2020). The retinal involvement in the progression of glaucoma is supported by the elevated intraocular pressure (IOP) (Pang and Clark, 2020). Elevated IOP causes axonal transport disruption, microvascular abnormalities, extracellular matrix remodeling, glial activation, and loss of RGCs (Imerze et al., 2013; Arteaga-Callejo et al., 2020; Rolle et al., 2020). Based on the above discussion, we hypothesized that targeting neuronal S1PR1 by siponimod could produce neuroprotective effects in glaucoma conditions, independent of the drug’s peripheral immunomodulatory effects. The present study investigated the neuroprotective effect of siponimod on the visual pathway in glaucomatous conditions using combined physiological, structural, and biochemical measures. Further, we established the role of S1PR1 in RGCs in situ as a baseline and the drug-mediated effects using cell-specific S1PR1 ablated mice.

Methods

Experimental animals

All animal experiments in this study were conducted in compliance with the Animal Ethics Committee for the Care and Use of Animals (AEC) Purposes and the guidelines of the ARVO (the Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research and approved by Macquarie University Animal Ethics Committee (AEC No. 2018/75). Tochigi Laboratory, Tochigi, Japan). All procedures were performed using an operating microscope (OPMI Vario S88, Carl Zeiss, Oberkochen, Germany), and care during the procedure. After pupils were dilated with 1% tropicamide, a single drop of Gentamicin 0.3% was applied to the cornea under dim red light. The ground and reference electrodes were inserted into the tail and subcutaneously into the forehead of the animal, respectively. The body temperature of the animals was maintained at around 37°C with an electric heating pad during recordings. The recording electrode (gold-plated objective) was placed on the eye’s corneal surface after applying hypromellose to maintain the corneal contact with the corneal surface. After stabilizing the baseline in darkness, the ERG responses were recorded from each eye individually. ERGs were recorded using a flash intensity of 6.1 log cd·s/m². Dim stimulation (~4.3 log cd·s/m²) was delivered 20 times at a frequency of 0.5 Hz. The amplitude of positive scotopic threshold response (pSTr) was measured from baseline to the positive peak observed around 120 ms.

Intravitreal NMDA injection

Retinal neurotoxicity was induced by a single intravitreal injection of NMDA (30 nmol per eye) (Dheer et al., 2019). Mice were anesthetized in an induction chamber with 2–5% isoflurane in oxygen, and then animals were maintained on 3% isoflurane in oxygen using a nose cone (min flow of 2 L/min of oxygen) on a warming pad during the procedure. After pupils were dilated with 1% tropicamide, a single drop of Gentamicin 0.3% was applied to the cornea. Micropipettes were positioned using a micromanipulator and loaded with 33G glass micropipettes with a tip diameter of ~0.8 μm. The micropipettes were positioned using a micromanipulator and loaded with 33G glass micropipettes with a tip diameter of ~0.8 μm. The micropipettes were positioned using a micromanipulator and loaded with 33G glass micropipettes with a tip diameter of ~0.8 μm.
Results

Siponimod preserves retinal structure and function in optic nerve injury models.

The potential protective effects of siponimod on inner retinal function and structure were measured in a high IOP mouse model (Figure 1A). For functional assessment of the inner retina, we measured the pSTR amplitudes (You et al., 2013). Siponimod-treated mice with intracameral injections of microbeads showed elevated IOPs comparable to the untreated control animals (Figure 1B). After 8 weeks, the high IOP subjected eyes exhibited a decline in pSTR amplitudes, while mice treated with siponimod (10 mg/kg in the diet and fed ad libitum) showed significant protection against the pSTR amplitude loss (P < 0.01, Figure 1C and D).

Assessment of cellular changes in the ganglion cell layer (GCL) and ON damage were then performed. Histological analysis of the retinal sections stained with H and E revealed that the untreated mice showed neurodegenerative changes induced by chronic high IOP. The animals subjected to four experimental groups were utilized to analyze the untreated mice (P < 0.001). GCL loss was significantly attenuated in the siponimod-treated mice group compared with the untreated mice group in high IOP conditions, where GCL cell density loss decreased from 60.64 ± 8.73% to 25.29 ± 8.21% (P < 0.001; Figure 1E and F). Neurofilament heavy chain in RGC axes has been reported to undergo dephosphorylation in eyes subjected to elevated IOP (Kashiwagi et al., 2003; Chidlow et al., 2011). Immunofluorescence staining of ON cross-sections with phosphorylated neurofilament heavy chain (pNFH) antibody and pNFH area measurements revealed a significant decrease in pNFH immunoreactivity in high IOP ONs. This ON axonal damage in elevated IOP conditions was decreased from 64.88 ± 8.25% to 38.21 ± 9.15% with siponimod treatment compared with the untreated control mice (P < 0.001). These results indicated that siponimod exerts neuroprotective effects on the retina and ON against neurodegenerative changes induced by chronic high IOP.
The neuroprotective effect of siponimod on the retina was further tested in an acute NMDA excitotoxicity model. A significant retinal functional deterioration in response to NMDA toxicity was observed after 7 days post-injection. The pSTR amplitudes in NMDA-treated mice were reduced by 65.33 ± 3.78%, and this reduction was significantly protected with siponimod treatment (P < 0.05; Figure 2A and B). H and E staining of retinal sections and examination of ONs stained with pNFH further revealed the protective effects of siponimod. NMDA-induced cell loss in GCL decreased from 71.36 ± 9.61% to 47.58 ± 11.50% in the siponimod treated group compared with the untreated mice group (P < 0.05; Figure 2C and D). Axonal damage assessed by pNFH Immunohistochemistry showed pNFH immunoreactivity loss was decreased from 51.39 ± 10.99% to 22.02 ± 10.74% in siponimod treated mice in NMDA excitotoxicity condition compared with the untreated mice group (P < 0.05; Figure 2E and F). These results emphasize the protective effects of siponimod treatment on the retina in the NMDA injury model.

Figure 2 | Neuroprotective effects of siponimod on function and structure of the retina and optic nerve (ON) against acute retinal N-methyl-D-aspartate (NMDA) excitotoxicity.

(A) Positive scotopic threshold responses (pSTR) of the eyes after 7 days of NMDA excitotoxicity and (B) quantification of the pSTR amplitudes in acute retinal NMDA excitotoxicity (P < 0.05, n = 10 per group). (C) Histological analysis of sagittal cross-sections of the eyes stained with hematoxylin and eosin (representative images, arrows indicate the changes in cell densities, scale bar: 50 μm; GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer) and (D) cell counts in the GCL (P < 0.05, n = 5 per group). (E) Immunofluorescence images of ON cross-sections stained with pNFH (green) and DAPI (blue) (scale bar: 50 μm) and immunoreactivity measurements from magnified areas (boxes, scale bars: 10 μm) (F) and their quantitative analysis (P < 0.05, n = 5 per group). Statistical significance was determined using one-way analysis of variance with Tukey’s multiple comparisons test (mean ± SD).

Protective effects of siponimod on trans-neuronal degenerative changes in dLGN

Neuronal degenerative changes in the dLGN have been documented in ON injury conditions (Gupta et al., 2007; Yucel and Gupta, 2008; Sreem et al., 2012). The effect of siponimod on trans-neuronal degeneration under chronic elevated IOP in the dLGN region of the brain was evaluated by staining coronal brain sections with Nissl blue and NeuN. Quantitative neuronal cell density analysis revealed a significant protective effect of siponimod on neuronal cell loss in the dLGN. A decline of 38.95 ± 7.26% in neuronal cell density was observed in high IOP mice, and this degeneration was reduced to 12.3 ± 7.85% (P < 0.03) in the siponimod treated group compared with the untreated mice group (Figure 3A and B). Further, in high IOP eyes, the frequency of NeuN+ cells was decreased by 39.34 ± 6.77%, whereas this loss was reduced to only 13.72 ± 7.36% in the siponimod treated group (Figure 3C and D). The higher visual cortex (V1) analysis in this high IOP condition for 2 months period showed no significant trans-neuronal degenerative changes. These results together establish a significant protective effect of siponimod on the neuronal cell population in the dLGN region of the brain.

Siponimod regulates pro-survival signaling pathways

Phosphatidylinositol-3 kinase (PI3K)/Akt and the mitogen-activated protein kinase (MAPK)/Erk1/2 signaling play a critical role in cell survival (Kimmel, 2019; Xu et al., 2020). FTY720 treatment has been shown to promote Akt/Erk activation in cultured neuronal cells and in vivo conditions (Zhang and Wang, 2020). Akt and Erk1/2 phosphorylation levels were evaluated by western blotting. A decrease in Akt and Erk phosphorylation levels was observed under high IOP conditions in the retina and dLGN region of the brain. Mice treated with siponimod showed significantly enhanced Akt (P < 0.01) and Erk1/2 phosphorylation in the retina (P < 0.01) and the dLGN (P < 0.01 for Erk1; P < 0.001 for Erk2; Figure 4A and B) compared with the untreated mice group in high IOP conditions.

Figure 3 | Protective effects of siponimod on the dorsolateral geniculate nucleus (dLGN) of the brain in chronic glaucoma condition (8 weeks).

(A) Nissl staining of coronal sections (contralateral) of mouse brains (contralateral, representative) stained with anti-NeuN (green) a neuronal marker and DAPI (blue) (scale bar: 200 μm), the dLGN region is marked. (B) Percentage of NeuN+ cells out of DAPI-positive cells in the dLGN from magnified areas (scale bar: 20 μm). **P < 0.01, one-way analysis of variance with Tukey’s multiple comparisons test, n = 5 per group.

Figure 4 | Siponimod upregulates Akt and Erk1/2 phosphorylation in the retina and the brain.

(A) Western blot analysis of Akt (phospho-S473 antibody) and Erk1/2 (phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) phosphorylation levels in retinas and (B) in the dorsolateral geniculate nucleus (dLGN) of the brain tissues lysates (8 weeks of chronic elevated IOP) and their densitometric quantitative analysis (P < 0.05, **P < 0.01, ***P < 0.001, one-way analysis of variance with Tukey’s multiple comparisons test, n = 4 per group).

Siponimod reduces microglial activation and reactive gliosis in the glaucoma model

Chronic neuroinflammation in glaucoma is implicated as an important component in disease pathogenesis. The early reactivity of glial cells (microglial activation and reactive gliosis) has been observed in the retina (Melik Parsadaniantz et al., 2020; Rolle et al., 2020). Glial activation along the visual pathway was evaluated by investigating Iba1 and GFAP expression alterations. Immunofluorescence staining of the retina, ON, and the brain tissue sections with Iba1 and GFAP revealed activation of glial cells along the visual pathway in chronic high IOP condition (Figure 5 and Additional Figure 1). Activated microglia in the retina, ON, and dLGN were reduced with siponimod treatment in chronic high IOP conditions (Figure 5A, C and D).
Additional Figure 1A. Microglial activation in the ON evaluated by measuring changes in Iba1+ areas indicated a significant increase (3.02 ± 0.29-fold) of Iba1 immunoreactivity in the high IOP model. This activation was reduced in the siponimod-treated group to 1.84 ± 0.41 fold (Additional Figure 1A).

Western blot analysis of retinal and dLGN tissues for Iba1 expression revealed a 3.63 ± 0.25-fold increase in the retina and 2.43 ± 0.32-fold increase in dLGN in high IOP conditions, and this upregulation was significantly diminished with siponimod treatment (2.27 ± 0.31-fold in retina and 1.63 ± 0.22-fold in dLGN) (Figure 5B and D).

In control retinas, GFAP immunoreactivity was primarily confined to the GCL. High IOP retinas showed GFAP labeling as a dense network of Müller cell processes in the GCL along with the emergence of a radial pattern of GFAP immunopositivity (Figure 5E). Similar hypertrophic reactive astrogliosis with upregulated GFAP was observed in ON (Additional Figure 1B) and dLGN of high IOP subjected mice (Figure 5G). This reactive gliosis was diminished with siponimod treatment. Western blot quantitative analysis of GFAP expression in the retina and dLGN tissues showed 4.33 ± 0.35-fold and 2.96 ± 0.27-fold upregulation, respectively in high IOP conditions compared with the control normal IOP (sham) mice group. Siponimod treatment resulted in its suppression to 2.23 ± 0.31-fold in the retina and 1.65 ± 0.23-fold in dLGN compared with the untreated mice (Figure 5F and H). GFAP immunoreactivity in the ON was increased to 3.47 ± 0.5-fold in high IOP condition compared with the control normal IOP (sham) mice but was reduced significantly to 1.96 ± 0.30 fold in siponimod treated mice (P < 0.001; Additional Figure 1B). These results indicated that the siponimod treatment effectively reduced microglial activation and reactive gliosis in chronic high IOP experimental glaucoma.

S1PR1 loss in RGCs and neurons exacerbates glaucoma injury attenuating siponimod protective effects

To define the role of S1PR1 intrinsic signaling in neuronal survival and its involvement in mediating the protective effects of the drug in glaucoma conditions, neuronal-specific ablation of S1PR1 was carried out in S1PR1fox/fox mice. The Cre-recombinase under neuron-specific promoter, Syn1 was integrated into AAV-PHP.eB vector and viral vectors were administered intravenously to S1PR1fox/fox mice (Additional Figure 2A). Efficient delivery of AAV construct crossing the blood-brain barrier was evident by GFP expression in the neuronal cells of mice receiving AAV injections. The transduction efficiency measured by the ratio of GFP+ cells to the total NeuN+ cells achieved 83.20 ± 7.63% for GFP control and 82.36 ± 6.87% for Cre recombinase AAVs in the dLGN regions of the brain. Similarly, the transduction efficiency of 76.93 ± 4.73% for GFP control and 75.31 ± 5.53% for Cre AAVs was observed for RGCs in the retina (Additional Figure 2B–D).

Western blot analysis of dLGN and retinal tissues further validated the loss of S1PR1 expression in AAV-Cre recombinase-expressing mice. Densitometric quantification demonstrated that the S1PR1 expression in AAV-GFP-Cre injected mice was decreased by 67.95 ± 7.15% in the dLGN and about 61.43 ± 7.79% in the retina compared with the AAV-GFP injected control mice (P < 0.0001, Additional Figure 2E and F).

The mice injected with AAV-GFP referred to as S1PR1+ control mice (AAV-GFP) and the neuron-specific S1PR1 deleted mice (termed as Neu-S1PR1−/− mice (AAV-GFP-Cre)) were administered siponimod diet (Additional Figure 3). No significant functional or structural changes were observed in the retina upon neuronal S1PR1 ablation under normal conditions. However, upon microbead-induced ON injury, neuronal S1PR1 deleted mice showed a significant decrease in the pSTR amplitudes and GCL thinning. Interestingly, the protective effects of siponimod were significantly attenuated in the group subjected to neuronal S1PR1 impairment (Neu-S1PR1−/−, AAV-GFP-Cre) compared with control S1PR1+ animals (AAV-GFP). High IOP-induced pSTR amplitude loss of 57.69 ± 5.03% was reduced to 32.58 ± 4.57% in siponimod-treated S1PR1+ control animals. However, Neu-S1PR1−/− animals exhibited 77.62 ± 2.74% loss in high IOP eyes compared with 66.43 ± 2.45% loss in the siponimod treated group (P < 0.01; Figure 6A–C).

Further, quantitative analysis of the GCL from H and E stained retinal sections revealed a significant decrease (P < 0.05) in GCL density in Neu-S1PR1−/− ablated (AAV-GFP-Cre) mice compared with S1PR1+ control (AAV-GFP) mice in high IOP conditions. The siponimod-mediated protective effect was significantly diminished in Neu-S1PR1−/− ablated mice compared with control S1PR1+ animals (P < 0.01; Figure 6D–F). ON damage was assessed by performing pHF immunoreactivity measurements that showed similar changes between Neu-S1PR1−/− ablated and S1PR1+ control mice. However, the siponimod-treated group showed significantly greater immunoreactivity of pHF only in S1PR1+ control mice compared with Neu-S1PR1−/− mice (P < 0.01; Additional Figure 4).

We next analyzed the impact of neuronal S1PR1 ablation on degenerative changes in dLGN of the brain. A significant decline in the frequency of NeuN+ cells was observed in neuronal S1PR1 ablated mice (AAV-GFP-Cre) compared with control S1PR1+ (AAV-GFP) mice in high IOP conditions (P < 0.05). No significant changes in dLGN neuronal cell density were evident in animals subjected to neuronal S1PR1 ablation under normal conditions. Neuronal S1PR1 ablation significantly reduced the protective effects of siponimod on the dLGN degenerative changes. Siponimod treatment showed a significantly greater frequency of NeuN+ cells in S1PR1+ control animals compared with Neu-S1PR1−/− mice (P < 0.01) in high IOP conditions (Figure 7). Quantitative histological analysis of the neuronal cell density showed a significantly reduced neuronal density (P < 0.01) in the dLGN region in Neu-S1PR1−/− ablated mice compared with the control S1PR1+ animals in high IOP conditions. The protective effect of siponimod on the dLGN neuronal density observed in the control S1PR1+ mice was significantly diminished in the Neu-S1PR1−/− ablated group under high IOP conditions (P < 0.01; Additional Figure 5). These results indicated that expression of S1PR1 in the neurons plays a pivotal role in cell survival in the retina and dLGN in high IOP-induced degeneration and that the protective effects of siponimod on the neurons under such conditions are mediated through neuronal S1PR1 expression.
The effect of S1PR1 deletion in neurons on pro-surviving Akt and Erk1/2 pathways was evaluated by Western blot analysis. Mice treated with siponimod showed enhanced Akt and Erk1/2 phosphorylation levels in the retina and dLGN in S1PR1+/+ control animals (AAV-GFP) in high IOP conditions. S1PR1 ablution in neurons diminished siponimod-induced upregulation of Akt and Erk1/2 phosphorylation. The Neu-S1PR1−/− (AAV-GFP-Cre) mice exhibited significantly decreased Akt and Erk1/2 phosphorylation levels with siponimod treatment both in the retina and dLGN (P < 0.01) in high IOP conditions (Additional Figure 6). These results demonstrated that siponimod induces phosphorylation of Akt and Erk1/2 through S1PR1 signaling in neurons.

Protective effects of siponimod on glial activation are attenuated in response to neuronal-specific S1PR1 ablution

We then determined whether the increased degeneration observed in Neu-S1PR1−/− (AAV-GFP-Cre) mice with high IOP might impact siponimod-induced effects on glial activation. Similar to the results described in Figure 5 and Additional Figure 1, hypertrophic activated microglia and reactive gliosis analyzed with Iba1 and GFAP antibodies along the visual pathway were observed in untreated control S1PR1+/+ (AAV-GFP) mice in chronic high IOP conditions and this was reduced with siponimod treatment (P < 0.001; Figure 8 and Additional Figures 7 and 8). Western blot quantification revealed a 3.16 ± 0.35-fold increase in Iba1 and 4.30 ± 0.57-fold upregulation of GFAP expression in the retina (P < 0.001) in high IOP conditions compared with the control normal IOP (sham) mice group (Figure 8C). Similarly, in the dLGN 2.57 ± 0.28-fold increase for Iba1 and 3.17 ± 0.22-fold increase for GFAP expression were observed with chronic high IOP (P < 0.001; Additional Figure 7C). This glial activation was reduced significantly to 1.52 ± 0.18 (dLGN) to 1.69 ± 0.23-fold (retina) for microglia and 2.13 ± 0.27 (dLGN) to 2.47 ± 0.37-fold (retina) for reactive gliosis with siponimod treatment in S1PR1+/+ control mice. This protective effect of siponimod on microglial activation was abolished in Neu-S1PR1−/− mice. However, siponimod treatment showed a significant suppressive effect on reactive gliosis in Neu-S1PR1−/− mice (AAV-GFP-Cre). Siponimod treatment significantly (P < 0.05) reduced GFAP expression from 4.57 ± 0.44 to 3.41 ± 0.35-fold in the retina and 3.72 ± 0.32 to 2.88 ± 0.22-fold in dLGN of the brain in Neu-S1PR1−/− mice under high IOP conditions compared with the untreated mice group. Further, similar levels of effects were observed in ONs evaluated by immunoreactivity of Iba1 and GFAP (Additional Figure 8). These results together suggest that a diminished protective effect of siponimod on glia was associated with enhanced degeneration after S1PR1 deletion in neurons.

The functional importance of S1PR1 in neurons and its deletion impairs the protective effect of siponimod on inner retinal function and structure in chronic optic nerve injury condition.

(A) Inner retinal positive scotopic threshold responses (pSTR) responses and (B) quantification of the pSTR amplitudes of control and high intraocular pressure (IOP) eyes (8 weeks) of S1PR1+/+ control (AAV-GFP) and Neu-S1PR1−/− (AAV-GFP-Cre) mice groups. (C) Comparison of pSTR amplitudes loss among different mice groups (n = 10 per group). (D) Hematoxylin and eosin staining of eye cross-sections (arrows indicate the changes in cell densities, scale bar: 50 µm). GCL: Ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer) and (E) cell counts in the GCL. (F) Comparison of cell loss in the GCL among untreated control S1PR1−/− (AAV-GFP) and Neu-S1PR1−/− (AAV-GFP-Cre) mice brains (contralateral) stained with anti-NeuN (green) and DAPI (blue) (representative, the dLG is marked with dotted circles, scale bar: 100 µm). (B) Quantification of the percentage of NeuN+ cells out of DAPI-positive cells in the dLGN from magnified different areas (scale bar: 20 µm) among different mouse groups (n = 5 per group). *P < 0.05, **P < 0.01, ***P < 0.001, one-way analysis of variance with Tukey’s multiple comparisons test. NS: Not significant; S1PR: sphingosine-1-phosphate receptor.

Additional Figure 1

Additional Figures 7

Additional Figures 8
The protective effects of siponimod are not primarily mediated through S1PR1 expression in oligodendrocytes

Death of oligodendrocytes in elevated IOP conditions has been reported along with axonal loss and RGC degeneration (Nakazawa et al., 2006; Son et al., 2018). To determine if S1PR1 deletion (in GFP-specific S1PR1 (GFP-S1PR1) mice) mediates these effects, siponimod-treated (GFP-S1PR1) and untreated mice were used. In the untreated group, GFP-S1PR1 mice were used, while in the treated group, GFP-S1PR1 mice were transplanted with AAVs expressing S1PR1. Immunofluorescence staining revealed that S1PR1 deletion did not significantly affect the expression of S1PR1 in oligodendrocytes, but it did change the protective effects of siponimod. Further, we observed that S1PR1 deletion increased the number of reactive astrocytes in the retina and dLGN. The protective effects of siponimod are not mediated through S1PR1 expression in oligodendrocytes.

Discussion

This study demonstrates that siponimod treatment protects the retina and higher visual pathway in the ON injury model. We further examined the impact of S1PR1 deletion in neurons and oligodendrocytes using cell-specific conditional S1PR1 ablated mice. Siponimod treatment was recently demonstrated to reduce disability progression in a clinical trial involving secondary progressive MS patients (Gajofatto, 2017; Dumitrescu et al., 2019). We observed that siponimod treatment did not significantly affect the expression of S1PR1 in oligodendrocytes, but it did change the protective effects of siponimod. Further, we observed that S1PR1 deletion increased the number of reactive astrocytes in the retina and dLGN. The protective effects of siponimod are not mediated through S1PR1 expression in oligodendrocytes.
In our findings, the mice treated with a single dose of siponimod via diet (10 mg/kg diet) showed significant neuroprotection against glaucomatous injury on the visual pathway. Further studies are required to understand the different dose effects of siponimod. We observed significant downregulation of glial activation with siponimod treatment in high IOP conditions both in the retina and the brain. However, we have not analyzed the inflammatory pathways affected by the siponimod treatment. S1PR1 is also expressed by the glial cells. Therefore, further investigations into the glial responses to siponimod treatment are required to understand the neuroproteective effects of siponimod in chronic glaucoma. Additionally, this study revealed that neuronal S1PR1 plays a crucial role in glaucomatous injury, but we have not followed the long-term effects of its deletion in normal conditions. Despite these few limitations of this study, our findings suggest that modulation of neuronal S1PR1 by siponimod treatment could be beneficial in providing neuroprotection against glaucoma injury.

In summary, our results demonstrated that siponimod exerts neuroprotective effects on the retina and the brain in an ON-injury model. S1PR1 signaling in neurons plays a crucial role in neuroprotection and induces Akt/Erk pathways activation. Furthermore, this study showed that siponimod exerts beneficial effects through S1PR1 in neurons. The drug exhibited suppressive effects on the microglial activation and reactive gliosis along with preservation of neuronal cell preservation. Therefore, this study identifies that modulation of S1P receptors within the CNS may offer promising strategies to provide neuroprotection in CNS injury conditions.

Author contributions: DB, VG, MM, AK and SLG designed the study; DB, RVW, NC, KP, and RR performed the experiments; DB, VG, and SLG analyzed the data; DB and VG wrote the manuscript; MM, SS helped to write the manuscript. All authors helped to write the manuscript preparation and approved the final version of the manuscript.

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Availability of data and materials: The authors declare that all the relevant data, associated protocols, and materials supporting the findings of this study are present in the paper and/or the Additional Materials.

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Additional files:
Additional Figure 1: Siponimod treatment reduced glial activation in optic nerve in chronic glaucoma (8 weeks) condition.
Additional Figure 2: Neuronal specific deletion of S1PR1 using AAV-PHpeB-Syn1-GFP-Cre in S1PR1 flox/flox transgenic mice.
Additional Figure 3: Chronic IOP elevation in different group mice eyes for 8 weeks following microbead injections.
Additional Figure 4: Effects of siponimod on optic nerve damage under optic nerve injury induced by high IOP (8 weeks) in control and Neu-S1PR1 flox/flox mice groups.
Additional Figure 5: Effects of neuronal-specific S1PR1 deletion on dLGN degenerative changes under chronic IOP condition (8 weeks).
Additional Figure 6: Deletion of S1PR1 in neurons reduces siponimod mediated upregulation of pro-survival signaling pathways Akt and Erk1/2 in the retina and brain.
Additional Figure 7: Siponimod suppresses microglial activation and reactive astrocytes in dLGN in control mice and its protective effect is reduced in Neu-S1PR1 flox/flox mice in chronic optic nerve injury condition.
Additional Figure 8: Effects of siponimod on glial activation in optic nerve under high IOP (8 weeks) induced optic nerve injury.
Additional Figure 9: Transduction and specific deletion of S1PR1 in oligodendrocytes using AAV-PHpeB-MBP-GFP-Cre in S1PR1 flox/flox transgenic mice.
Additional Figure 10: Chronic IOP elevation in different group mice eyes for 8 weeks following microbead injections.
Additional Figure 11: Effects of oligodendrocyte-specific S1PR1 deletion on retinal degenerational changes under chronic IOP condition.
Additional Figure 12: Effects of oligodendrocyte-specific deletion of S1PR1 on optic nerve and dLGN neurodegeneration under high IOP (8 weeks) optic nerve injury condition.

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Additional Figure 1 Siponimod treatment reduced glial activation in optic nerves in chronic high IOP (8 weeks) condition.

(A) Representative immunofluorescence images of optic nerve cross-sections stained for microglia activation with Iba1 areas (red) and their quantification. (B) Analysis of GFAP stained (red) for reactive astrogliosis in ONs and their immunoreactivity measurements (scale bar: 50 µm). Data are expressed as the mean ± SD. ***P < 0.001 (one-way analysis of variance with Tukey’s multiple comparisons test, n = 5 ONs per group). GFAP: Glial fibrillary acidic protein; Iba1: ionized calcium-binding adaptor molecule 1; IOP: intraocular pressure.
Additional Figure 2 Neuronal specific deletion of S1PR1 using AAV-PHP.eB-Syn1-GFP-Cre in S1PR1^flo/flox transgenic mice. 

(A) Schematic view depicting the GFP-Cre recombinase expression under neuron-specific promoter Syn1 integrated into the AAV-PHP.eB vector followed by tail vein injection of the viral particles into S1PR1^flo/flox transgenic mouse strains. (B) Efficient transduction across the brain was evident by GFP marker (green) expression (scale bar: 500 μm). (C) Representative images of immunohistochemistry stained for GFP (green) and neuron-specific staining NeuN (red) (arrows indicate the GFP expression in neurons) and bar graph showing transduction efficiency quantified by the percentage of GFP^+ cells to the total NeuN cells (colocalization of GFP in neurons) in the dorsolateral geniculate nucleus (dLGN) of the brain (n = 20 per group, scale bar: 20 μm). (D) Representative retinal sections stained for GFP (green) and NeuN (red), DAPI (blue), and their quantification for transduction efficiency (arrows indicate the GFP expression in RGCs) (n = 20 per group, scale bar: 50 μm). (E) Western blot analysis of the brain dLGN tissue and densitometry quantification showing deletion of S1PR1 (n = 12 per group). (F) Western blot analysis of the retinal tissue and densitometric quantification for S1PR1 deletion (n = 12 per group). AAV: Adeno-associated virus; DAPI: 4',6-diamidino-2-phenylindole; S1PR: sphingosine-1-phosphate receptor.
Additional Figure 3 Chronic IOP elevation in different group mice eyes for 8 weeks following microbead injections.

(A) Control S1PR1+/− mice injected with AAV-Syn1-GFP and (B) neuronal S1PR1−/− (Neu-S1PR1−/−) mice injected with AAV-Syn1-GFP-Cre recombinase (n = 10 per each group). AAV: Adeno-associated virus; IOP: intraocular pressure; S1PR: sphingosine-1-phosphate receptor.
Additional Figure 4 Effects of siponimod on optic nerve damage under optic nerve injury induced by high IOP (8 weeks) in control and Neu-SIPRI⁻/⁻ (AAV-GFP-Cre) mice groups. (A) Immunofluorescence staining of cross-section of optic nerves for phosphorylated neurofilament heavy-chain (pNFH, green) (representative, scale bar: 50 µm) and measurements of immunoreactivity (scale bar: 10 µm) from magnified areas (boxes) of ONs. (B) Quantification of pNFH⁺ areas in different mice groups (NS, not significant, *P < 0.05, **P < 0.01, n = 5 per group). Statistical significance was determined using one-way analysis of variance with Tukey’s multiple comparisons test (mean ± SD). AAV: Adeno-associated virus; IOP: intraocular pressure; S1PR: sphingosine-1-phosphate receptor.
Additional Figure 5 Effects of neuronal-specific S1PR1 deletion on dorsolateral geniculate nucleus (dLGN) degenerative changes under chronic IOP condition (8 weeks).

(A) Nissl staining of coronal sections (contralateral) of mouse brains. Marked is the dLGN region of the brain used to quantify the neuronal density (representative, Scale bars: 200 µm) in control and Neu-S1PR1−/− (AAV-GFP-Cre) mice groups. Neuronal counts were measured from magnified images of the dLGN regions (scale bars: 20 µm). (B) Quantification of neuronal density among different mice groups (*P < 0.05, **P < 0.01, n = 5 per group). Statistical significance was determined using one-way analysis of variance with Tukey’s multiple comparisons test (mean ± SD). AAV: Adeno-associated virus; IOP: intraocular pressure; S1PR: sphingosine-1-phosphate receptor.
Additional Figure 6 Deletion of S1PR1 in neurons reduces siponimod mediated upregulation of pro-survival signaling pathways Akt and Erk1/2 in the retina and brain.

(A) Western blot analysis of Akt (phospho-S473 antibody) and Erk1/2 (Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) phosphorylation levels in retinas and (B) in the dorsolateral geniculate nucleus (dLGN) region of the brain tissues and their densitometric quantitative analysis in control S1PR1+/+ and Neu-S1PR1−/− (AAV-GFP-Cre) mice under normal and high IOP (8 weeks) conditions (*P < 0.05, **P < 0.01, ***P < 0.001, n = 4 per group). Statistical significance was determined using one-way analysis of variance with Tukey’s multiple comparisons test (mean ± SD). AAV: Adeno-associated virus; IOP: intraocular pressure; S1PR: sphingosine-1-phosphate receptor.
Additional Figure 7 Siponimod suppresses microglial activation and reactive astrocytes in dLGN in control mice and its protective effect is reduced in Neu-S1PR1−/− (AAV-GFP-Cre) mice in chronic optic nerve injury condition.

(A) Representative immunofluorescence images of coronal sections of different mouse groups’ brains (contralateral, dLGN region is marked with a dotted circle, scale bar: 100 µm) stained for microglial activation with Iba1 antibody (red) and (B) reactive astrocytes with GFAP (red) in control and Neu-S1PR1−/− mice groups. (C) Western blot analysis and their quantification for Iba1 and GFAP expression in dLGN (NS: not significant, *P < 0.05, ***P < 0.001, n = 4 per group). Statistical significance was determined using one-way analysis of variance with Tukey’s multiple comparisons test (mean ± SD). AAV: Adeno-associated virus; dLGN: dorsolateral geniculate nucleus; IOP: intraocular pressure; S1PR: sphingosine-1-phosphate receptor.
Additional Figure 8 Effects of siponimod on glial activation in optic nerve under high IOP (8 weeks) induced optic nerve injury.

(A) Representative immunofluorescence staining of optic nerve-cross sections stained with Iba1 (red) for microglial and (B) their quantitative analysis of Iba1’ areas. (C) ON sections stained with GFAP (red) for reactive astrogliosis and (D) their quantitative immunoreactivity analysis (scale bar: 50 µm) in control and Neu-S1PR1−/− (AAV-GFP-Cre) mice groups. NS: not significant, *P < 0.05, **P < 0.001, n = 5 ONs per group. Statistical significance was determined using one-way analysis of variance with Tukey’s multiple comparisons test (mean ± SD). AAV: Adeno-associated virus; GFAP: glial fibrillary acidic protein; Iba1: ionized calcium-binding adaptor molecule 1; IOP: intraocular pressure; S1PR: sphingosine-1-phosphate receptor.
Additional Figure 9 Transduction and specific deletion of S1PR1 in oligodendrocytes using AAV-PHP.eB-MBP-GFP-Cre in S1PR1<sup>flox/flox</sup> transgenic mice.

(A) Schematic view depicting the GFP-Cre recombinase expression under Oligodendrocytes specific promoter MBP integrated into the AAV-PHP.eB vector followed by tail vein injection of the viral particles into S1PR1<sup>flox/flox</sup> transgenic mouse strains. (B) Transduction across the brain is evident by the GFP marker (green) expression (scale bar: 500 µm). (C) Representative images of immunohistochemistry stained for GFP (green) and Oligodendrocytes specific staining MBP (red) showing oligodendrocytes specific expression of GFP colocalization (arrows) with MBP in the brain (scale bar: 20 µm). (D) Transduction efficiency quantified by the percentage of GFP<sup></sup> area to the total MBP area in the dorsolateral geniculate nucleus (dLGN) of the brain (n=20 per group). (E) Western blot analysis of the brain dLGN tissue and (F) densitometry quantification showing deletion of S1PR1 (n = 9 per group). AAV: Adeno-associated virus; MBP: myelin basic protein; S1PR: sphingosine-1-phosphate receptor.
Additional Figure 10 Chronic IOP elevation in different group mice eyes for 8 weeks following microbead injections.

(A) Control (S1PR1⁺⁺) mice injected with AAV-MBP-GFP and (B) Oligodendrocytes S1PR1⁻⁻ mice injected with AAV-MBP-GFP-Cre recombinase (mean ± SD, n = 9 per each group). IOP: Intraocular pressure; S1PR: sphingosine-1-phosphate receptor.
Additional Figure 11 Effects of oligodendrocyte-specific S1PR1 deletion on retinal degenerative changes under chronic IOP condition.

(A) Positive scotopic threshold responses (pSTR) and (B) quantification of the pSTR amplitudes of control and high IOP eyes of control (AAV-MBP-GFP) and Oligo-S1PR1−/− (AAV-MBP-GFP-Cre) mice treated with/without siponimod (**P < 0.01, n = 9 per group). (C) H and E staining of retinal cross-sections (representative, arrows indicate changes in the cell densities, scale bars: 50 µm; GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer) and (D) cell counts in the GCL of control and Oligo-S1PR1−/− mice under normal and high IOP conditions (NS: not significant, ***P < 0.001, n = 5 per group. Statistical significance was determined using one-way analysis of variance with Tukey’s multiple comparisons test (mean ± SD). AAV: Adeno-associated virus; IOP: intraocular pressure; S1PR: sphingosine-1-phosphate receptor.)
Additional Figure 12 Effects of oligodendrocyte-specific deletion of S1PR1 on the optic nerve and dLGN neurodegeneration under high IOP (8 weeks) optic nerve injury condition.

(A) Immunofluorescence staining of cross-section of optic nerves for phosphorylated neurofilament heavy-chain (green, pNFH) (scale bar: 50 µm) and measurements of immunoreactivity (scale bar: 10 µm) from magnified areas (boxes) of ONs of control and Oligo-S1PR1−/− mice groups. (B) Quantification of pNFH+ areas in different mice groups. (C) Representative immunofluorescence images of coronal sections of mouse brains (contralateral) stained with anti-NeuN (green), a neuronal marker, and DAPI (blue) (scale bar: 100 µm) and the dLGN region is marked with dotted circles in control and Oligo-S1PR1−/− mice groups. (D) Quantification of the percentage of NeuN+ cells out of DAPI-positive cells in the dLGN from magnified areas (scale bar: 20 µm) among mice groups. NS: Not significant. **p < 0.01, n = 5 per group. Statistical significance was determined using one-way analysis of variance with Tukey’s multiple comparisons test (mean ± SD). AAV: Adeno-associated virus; dLGN: dorsolateral geniculate nucleus; IOP: intraocular pressure; S1PR: sphingosine-1-phosphate receptor.