# Hydrogen Sulfide Protects Retinal Ganglion Cells Against Glaucomatous Injury In Vitro and In Vivo

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Submitted: May 30, 2017
Accepted: September 6, 2017

Citation: Liu H, Anders F, Thanos S, et al. Hydrogen sulfide protects retinal ganglion cells against glaucomatous injury in vitro and in vivo. *Invest Ophthalmol Vis Sci*. 2017;58:5129–5141. DOI:10.1167/iovs.17-22200

**Purpose.** Hydrogen sulfide ($H_2S$) is recognized as a novel third signaling molecule and gaseous neurotransmitter. Recently, cell protective properties within the central nervous and cardiovascular system have been proposed. Our purpose was to analyze the expression and neuroprotective effects of $H_2S$ in experimental models of glaucoma.

**Methods.** Elevated IOP was induced in Sprague-Dawley rats by means of episcleral vein cauterization. After 7 weeks, animals were killed and the retina was analyzed with label-free mass spectrometry. In vitro, retinal explants were exposed to elevated hydrostatic pressure or oxidative stress ($H_2O_2$), with and without addition of a slow-releasing $H_2S$ donor Morpholin-4-ium-methoxyphenyl-morpholino-phosphinodithioate (GYY4137). In vivo, GYY4137 was injected intravitreally in animals with acute ischemic injury or optic nerve crush. Brn3a+ retinal ganglion cells (RGCs) were counted in retinal flat mounts and compared. Optical coherence tomography (OCT) was performed to examine the vessels. Comparisons were made by *t*-test and ANOVA ($P < 0.05$).

**Results.** IOP elevation caused significant RGC loss ($P < 0.001$); 3-mercaptosulfurtransferase, an $H_2S$ producing enzyme, showed a 3-fold upregulation within the retina after IOP elevation. GYY4137 protected RGCs against elevated pressure and oxidative stress in vitro depending on the concentration used ($P < 0.005$). In vivo, intravitreal administration of GYY4137 preserved RGCs from acute ischemic injury and optic nerve crush ($P < 0.0001$). Retinal vessel diameters enlarged after intravitreal GYY4137 injection ($P < 0.0001$).

**Conclusions.** $H_2S$ is specifically regulated in experimental glaucoma. By scavenging reactive oxygen species and dilating retinal vessels, $H_2S$ may protect RGCs from pressure and oxidative stress–induced RGC loss in vitro and in vivo. Therefore, $H_2S$ might be a novel neuroprotectant in glaucoma.

Keywords: hydrogen sulfide, retinal ganglion cell, elevated pressure, glaucoma, neuroprotection

Glaucoma is a group of diseases characterized by the accelerated death of retinal ganglion cells (RGCs) and their axons. The death of RGCs ultimately leads to progressive visual field loss and irreversible blindness.[1] Elevated IOP is a main risk factor for glaucoma and the mainstay of treatment. Despite lowering of IOP, RGC loss may proceed.[2] Thus, the demand for additional therapies is high. Various other treatment options have been examined to delay or halt RGC loss to preserve visual function in patients with glaucoma without combating results. In recent years, the role of other factors, like glutamate excitotoxicity, disorganized metabolism of nitric oxide (NO), overproduction of reactive oxygen species (ROS), increased endothelin-2 levels, and reduction of ocular blood flow, also has been discussed to play a key role.[3]

An interesting molecule in this context is hydrogen sulfide ($H_2S$). $H_2S$ is the third identified gaseous transmitter after NO and carbon monoxide and it is endogenously generated in mammalian endothelial cells.[4] Recently cell protective properties within the cardiovascular system have been found, and thus its action in the cardiovascular system excessively studied. It has been shown that endogenously generated and exogenously administered $H_2S$ exerts a wide range of actions in vascular and myocardial cells, including vasodilator/vasoconstrictor effects via modification of the smooth muscle tone.[5]

Besides these vascular effects, neuroprotective properties have been assumed. In the central nervous system, $H_2S$ has been found to facilitate long-term potentiation and regulate intracellular calcium concentration and pH level in brain cells. Antioxidant, antiapoptotic, and anti-inflammatory effects of $H_2S$ have been found in, for example, Alzheimer's disease, Parkinson's disease, and vascular dementia. Furthermore, an abnormal generation and metabolism of $H_2S$ has been seen involved in most of these neurodegenerative disorders.[6] Moreover, $H_2S$ protects neurons against glutamate-mediated oxidative stress, or oxytosis, through the pleiotropic effects of maintaining the activities of cystathionine-γ-lyase and cystine transport, leading to an increase in glutathione levels.[7]

However, the role of $H_2S$ in glaucoma remains obscure. There are different donors releasing $H_2S$. One of them is Morpholin-4-ium-methoxyphenyl-morpholino-phosphinodithioate (GYY4137), which slowly releases low but consistent concentrations of $H_2S$.

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The purpose of our study was to analyze first the expression changes of $H_2S$ in an experimental animal model of glaucoma and second its potential neuroprotective effect on RGCs toward elevated pressure in glaucoma models in vitro and in vivo by addition of the slow-releasing $H_2S$ donor GYY4137.

## Materials and Methods

### Animals

Female Sprague-Dawley rats ($n = 39$; 250–300 g) were matched for age and body weight. All experimental procedures were done in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the guidelines of the Institutional Animal Care and Use Committee. The use of animals for research purposes was approved by the Landesuntersuchungsamt Rheinland-Pfalz (permission number 14-1-085). All animals were housed at the Translational Animal Research Center of the Johannes Gutenberg University Mainz. Food and water were provided ad libitum with a day- and night-cycle of 12 hours, respectively. Experimental interventions were conducted by using 0.05 mL medetomidine hydrochloride (Pfizer, New York, NY, USA) for anesthesia. Medetomidine was administered intramuscularly into the hamstrings. All animals were observed directly after each intervention and following daily in terms of their health condition and general behavior.

### Thermic Episcleral Vein Occlusion to Induce Elevated IOP

IOP was induced by thermic occlusion of three episcleral veins, which reduces 50% of the venous outflow as previously described by Shareef et al. in 1995.[8] Surgery was carried out in each animal on the left eye ($n = 17$), while the right eye served as contralateral control. IOP was measured before surgery to obtain a baseline, and followed weekly by a TonoLab rodent rebound tonometer (iCare, Vantaa, Finland) between 9:00 AM and 11:00 AM; IOP was followed up for 7 weeks after elevation. Animals were fully conscious during the measurement and just fixated through handholding. Ten consecutive readings were taken from the same area of cornea and a mean value was calculated. Animals with fluctuating IOP or without noticeable IOP elevation were excluded from further experiments.

### Preparation of Retinal Explants

Sprague-Dawley rats were euthanized under $CO_2$ atmosphere. Eyes were enucleated immediately postmortem and transferred to a Petri dish containing ice-cold sterile Hank's Balanced Salt Solution (Gibco BRL, Eggenstein, Germany). The anterior segment of the eye was removed and the retina exposed. Intact retina was dissected from the optic cup and the vitreous body removed. Explants were placed with the ganglion cell side up on Millipore filters (Millipore; Millicell, Cork, Ireland), and cut equally into four pieces.

### Quantification of RGCs

For the in vivo and ex vivo in vitro experiments, rats were euthanized and the eyes enucleated. Retinal wholemounts were isolated. RGCs were quantified by anti-Brn3a (Santa Cruz Biotechnology, Dallas, TX, USA) immunostaining; identification of RGCs by Brn3a immunodetection is a powerful tool to assess RGC survival in several mouse and rat injury models, such as ocular, traumatic optic nerve, excitotoxicity, and optic neuritis,

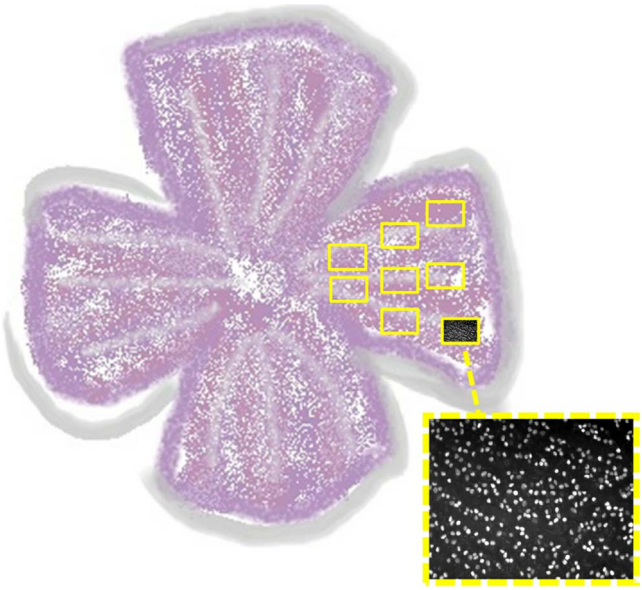

Figure 1. Images of retinal whole mounts were photographed from eight different regions of each quadrant of the retina.

and to quantify the efficacy of neuroprotective therapies.[9,10] In brief, the isolated retina was fixed in formalin-solution (4% para-formaldehyde in PBS, pH 7.4), transferred in 30% sucrose solution overnight, and finally frozen in methylbutane for 10 seconds (Merck, Darmstadt, Germany). The primary antibody was diluted in 10% fetal calf serum and incubated overnight at 4°C. Immunofluorescent RGCs were further visualized with a fluorescent microscope (Axiophot Carl Zeiss, Gottingen, Germany) using a 20-fold magnification.

### Microscopy and Analysis

Images of retinal whole mounts were photographed from eight different regions of each quadrant of the retina (Fig. 1) using a Zeiss fluorescent microscope (Carl Zeiss, Ltd., Hertfordshire, UK). Images were captured at a 20-fold magnification using a fluorescent camera (Carl Zeiss, Ltd.). Total numbers of Brn3a-positive cells were counted (total = 8 counts/quadrant, and eight retinal quadrants from different animals per treatment group). The mean number of RGCs per quadrant was calculated from a mean count at each of the eight different regions.

### Quantitative Proteomic Measurements

Proteins were extracted from the retinal tissue for the proteomic measurements ($n = 14$). 0.5% n-Dodecyl β-D-maltoside (Sigma-Aldrich Corp., St. Louis, MO, USA) in Tris-buffered saline, which was used to ensure chemical breakdown. Protein concentration was determined by Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA): 80 µg of total protein amount per retinal sample was loaded on NuPAGE Novex 12% Bis-Tris Protein Gel (ThermoFisher Scientific) and separated subsequently using standard PAGE. Gel lanes were singularized into 15 pieces; in-gel digestion was achieved by using sequence-grade modified trypsin (Promega, Mannheim, Germany). The peptide digestion products were extracted from the gel pieces using acetonitrile (AppliChem, Darmstadt, Germany), water, and formic acid (AppliChem). Mass spectrometric measurements were performed using an LTQ Orbitrap XL system (ThermoFisher Scientific) with an upstream-connected liquid-chroma-

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Table 1. Experiments In Vitro

| Glaucoma Models In Vitro | *n* | Stressing Agent | Duration, h | Concentrations of GYY4137 | Outcome |
|---|---|---|---|---|---|
| Oxidative stress on retinal explants | 4 | With or without 100 nM hydrogen peroxide. | 24 | 1 nM, 100 nM, and 10 μM | RGC quantification |
| Elevated hydrostatic pressure on retinal explants | 6 | With or without 60 mm Hg elevated hydrostatic pressure. | 48 | 1 nM, 5 nM, 10 nM, 100 nM, 1 μM, and 10 μM; in addition, 1 nM and 100 nM GYY4137, which released $H_2S$ in advance | RGC quantification |
| Elevated hydrostatic pressure rBMECs | N/A | With or without 60 mm Hg elevated hydrostatic pressure. | 72 | 1 nM, 5 nM, 10 nM, 100 nM, 1 μM, and 10 μM | Morphologic change |

tography system to ensure maximum fractioning of the peptides. The full-scan mass spectrum had a range from 300 to 2000 m/z and was acquired with a resolution of 30,000. Obtained raw files were analyzed with MaxQuant v.1.5.3.30 (Max-Planck-Gesellschaft, Halle, Germany).[11] Parameters were set to a false-discovery rate of 0.01 with a minimum peptide length of six. Only unique peptides accounted for the follow-up label-free quantification (LFQ) process. No retinal samples were pooled, but measured individually to counterbalance possible measuring inaccuracy. The obtained LFQ data were averaged within the experimental groups (elevated IOP; untreated control) and subsequent fold-change calculations were performed to analyze potential alterations with respect to protein levels.

## Effects of GYY4137 In Vitro

To test the effect of GYY4137 on the survival of RGCs in vitro, two different models imitating glaucoma were used: elevated hydrostatic pressure and oxidative stress. Besides this, rat brain microvessel endothelial cells (rBMECs) were cultured under elevated pressure with or without GYY4137. An overview of the protocol is listed in Table 1.

Retinal explants were prepared as described above then transferred into lumox dish 35 (Sarstedt, Nümbrecht, Germany). The retinal tissue was cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12; Gibco BRL, Eggenstein, Germany), supplemented with 10 μg/mL porcine insulin, 100 U/mL penicillin, 100 μg/mL streptomycin, and aerated with humidified 5% $CO_2$, balance air, at 37°C. Allocation of the tissue to treatment or control was randomized to minimize variability.

**Culture Under Increased Oxidative Stress.** Retinal explants from four rats were cultured with and without addition of different concentrations (1 nM–10 μM) of GYY4137(Sigma-Aldrich, Darmstadt, Germany) in the presence or absence of hydrogen peroxide (100 mM) for 24 hours.

**Culture Under Elevated Hydrostatic Pressure.** In addition, retinal explants from six rats were cultured with and without addition of different concentrations (1 nM–10 μM) of GYY4137 for 48 hours without elevated pressure or under elevated hydrostatic pressure (60 mm Hg) within a pressure incubation chamber. The metallic incubation chamber was self-fabricated from steel. The metallic high-pressure incubator was fabricated for this purpose with screwable cover and an undirected valve to allow for entrance of incubator air. The intracameral air pressure is adjusted with a nanometer with readings in mm Hg. Constant air pressure can be obtained over several days up to 200 mm Hg (266.64 hpascal) keeping this pressure stable or changing the pressure on demand. A valve allowed for entrance of 5% $CO_2$ containing atmosphere from the main incubator (Heraeus, Hanau, Germany). A manometer was used to continually monitor the air pressure within the high-pressure incubator.

We also incubated GYY4137 solution in a water bath for 4 hours to release $H_2S$ in advance, then applied it to the retinal explants to see if GYY4137 itself had any effect on RGCs. After culturing of the retinal explants, Brn3a staining and cell quantification was performed as mentioned above to measure RGC survival. Mean values were built for each experimental group and compared.

## Preparation and Culture of Primary rBMECs Under Elevated Hydrostatic Pressure

The rBMECs were isolated according to the method described by Ji et al.[12] All procedures were carried out under aseptic conditions. The brains were removed after decapitation from male and female pups from the Sprague-Dawley strain at postnatal days P5 to P8. The brains were immediately placed into ice-cold PBS. Connective tissue and meninges were discarded. Cortex gray matter was minced into small, homogeneous fragments made by crosscutting the tissue with scalpels. The tissue fragments were suspended and incubated in 10 mL PBS containing 0.05% trypsin for 25 minutes at 37°C. After incubation, cells were pelleted by centrifugation at 800*g* for 5 minutes. The pellet was resuspended in 5 mL PBS containing 20% BSA. After centrifugation at 2000*g* for 5 minutes, fat, cell debris, and myelin were floating on the aqueous BSA-phase. These and the aqueous phase were removed and discarded. The pellet containing the microvessels was resuspended in 2 mL PBS containing 0.1% collagenase A and incubated at 37°C for 30 minutes. The microvessel endothelial cells were finally collected by centrifugation at 800*g* for 5 minutes, washed two times in PBS, resuspended in DMEM/F12 supplemented with 20% fetal calf serum, 15 mM HEPES and 1% penicillin/streptomycin and cultured at 37°C in 5% $CO_2$ humidified atmosphere. The medium was changed every 3 days. Before use, cell culture flasks and dishes were gelatinated with 0.5% gelatin for 30 minutes at 37°C and subsequently allowed to dry for 10 minutes at room temperature. For incubation in the pressure chambers, only confluent primary cultures were used. rBMECs were cultured with and without addition of different concentrations (1 nM–10 μM) of GYY4137 within the pressure incubation chamber at 60 mm Hg for 3 days. Pictures were taken to detect any morphologic changes.

## Effect of GYY4137 In Vivo

IOP was measured at baseline before any intervention and then monitored according to the intervention (Table 2). Measurement was carried out as described above.

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**Table 2.** Experiments In Vivo

| Types of Intervention | *n* | Protocol of IOP Measurement | Animal Status During IOP Measurement | Intravitreal Injection of GYY4137 | Follow-up |
|---|---|---|---|---|---|
| Episcleral vein cauterization | 17 | Weekly in bilateral eyes between 9 AM and 11 AM for 7 wk. | Fully conscious and fixated through handholding during the measurement. | N/A | OCT scanning was performed in all animals before the cauterization and after 7 weeks of IOP elevation. Afterward, retinae were harvested for quantitative proteomic measurement. |
| | | | | 3 of the animals received intravitreal injection after 6 wk of IOP elevation. | 3 of which received intravitreal injection, had OCT scanning at multiple time points after injection, and also 1 wk later. |
| 2% GYY4137 applied as eye drop | 5 | Immediately after application of the eye drops and 3, 6, and 9 h after the application, and repeated 3 d in a row. | Fully conscious and fixated through handholding during the measurement. | N/A | N/A |
| Ischemic-reperfusion injury | 6 | IOP was raised to 55 mm Hg and monitored every 10 min for 60 min. | Properly anesthetized. | Before the injury was induced. | Retinae of the animals were harvested and stained against Brn3a. |
| ONC | 6 | N/A | N/A | Immediately after the operation. | Retinae of the animals were harvested and stained against Brn3a. |

## Topical Application of GYY4137

To analyze any effect of GYY4137 on IOP in normotensive, untreated female Sprague-Dawley rats, 2% GYY4137 dissolved in saline was applied topically ($n = 5$). To test this, 50 µL of 2% (53 nM) GYY4137 was applied at 9:00 AM, while the contralateral eye received the same quantity of vehicle (saline) eye drops. IOP was measured at baseline, immediately after application of the eye drops, and 3, 6, and 9 hours after the application. In addition, the same procedure was repeated every day over 3 days. Baseline IOP was measured at the same time 1 week before the experiment. The eyes of the animals were monitored for potential side effects (i.e., tearing, hyperemia).

## Glaucoma Animal Models

To test the effect of GYY4137 in vivo, three different models imitating glaucoma were used: elevated IOP, optic nerve crush (ONC), and ischemia- reperfusion injury (Table 2).

## Intravitreal Injection of GYY4137

Sprague-Dawley rats (250–300 g) were anesthetized as described above. Corneal analgesia was achieved by using topical drops of 0.4% oxybuprocaine (Novesine; Novartis, Basel, Switzerland). Intravitreal injections with GYY4137 were performed just posterior to the pars plana with a 5-µL syringe (Hamilton, Reno, NV, USA) and a 33-gauge needle; 3 µL of 2 µM GYY4137 in saline salt water was injected slowly. Assuming the vitreous volume of an adult rat eye to be approximately 56 µL,[13] the final intraocular concentration of GYY4137 was approximately 100 nM.

## Ischemia-Reperfusion Injury Model

The retinal ischemia-reperfusion injury model simulates clinical situations such as retinal vascular occlusion diseases and acute glaucoma, and has been a well-known animal model for studying retinal neuronal cell damage after ischemic insult. Female, Sprague-Dawley rats ($n = 6$; 250–300 g) were anesthetized as described above. Intraocular injection was performed as described above. Immediately after the injection, the anterior chamber was carefully cannulated with a 30-gauge infusion needle from the superotemporal cornea of Sprague-Dawley rats, without injuring the lens. An air bubble served as an "air embolus" to keep the anterior chamber tight. The needle was connected to a plastic container of 200 mL sterile saline solution. By elevating the saline container, the IOP was raised to 55 mm Hg (measured with the TonoLab) for 60 minutes. Retinal ischemia was confirmed by observing whitening of the iris and loss of the red reflex of the retina. A group without previous injection of GYY4137 served as the corresponding control. Animals were kept alive for 24 hours after the reperfusion injury. After euthanizing the animals, the retina was harvested and stained against Brn3a, as described earlier.

## ONC Surgery

ONC was performed as previously described.[14] Briefly, female, Sprague-Dawley rats ($n = 6$; 250–300 g) were anesthetized as described above. Corneal analgesia was achieved by using topical drops of 0.4% oxybuprocaine (Novesine; Novartis). A lateral canthotomy was performed to the upper eyelid parallel to the superior orbital edge, and the optic nerve was exposed and clamped for 3 seconds using fine forceps. After surgery, an intravitreal injection was performed as previously described, and the eye was covered with antibiotic ointment. The right eye was left as an untreated control. Twenty-four hours after the operation, animals were killed, and the retina was harvested and stained against Brn3a, as described above.

## Measurement of Retinal Vessel Calibers by Optical Coherence Tomography

Optical coherence tomography (OCT) was carried out to survey the vessel diameter. Analyses were conducted using a spectral-domain OCT (SD-OCT) device from Heidelberg Engineering (Heidelberg, Germany). To ensure high-quality measurements for rodents, the corneal radius was fixed to 7.7 mm, while the focus and reference arm were adjusted

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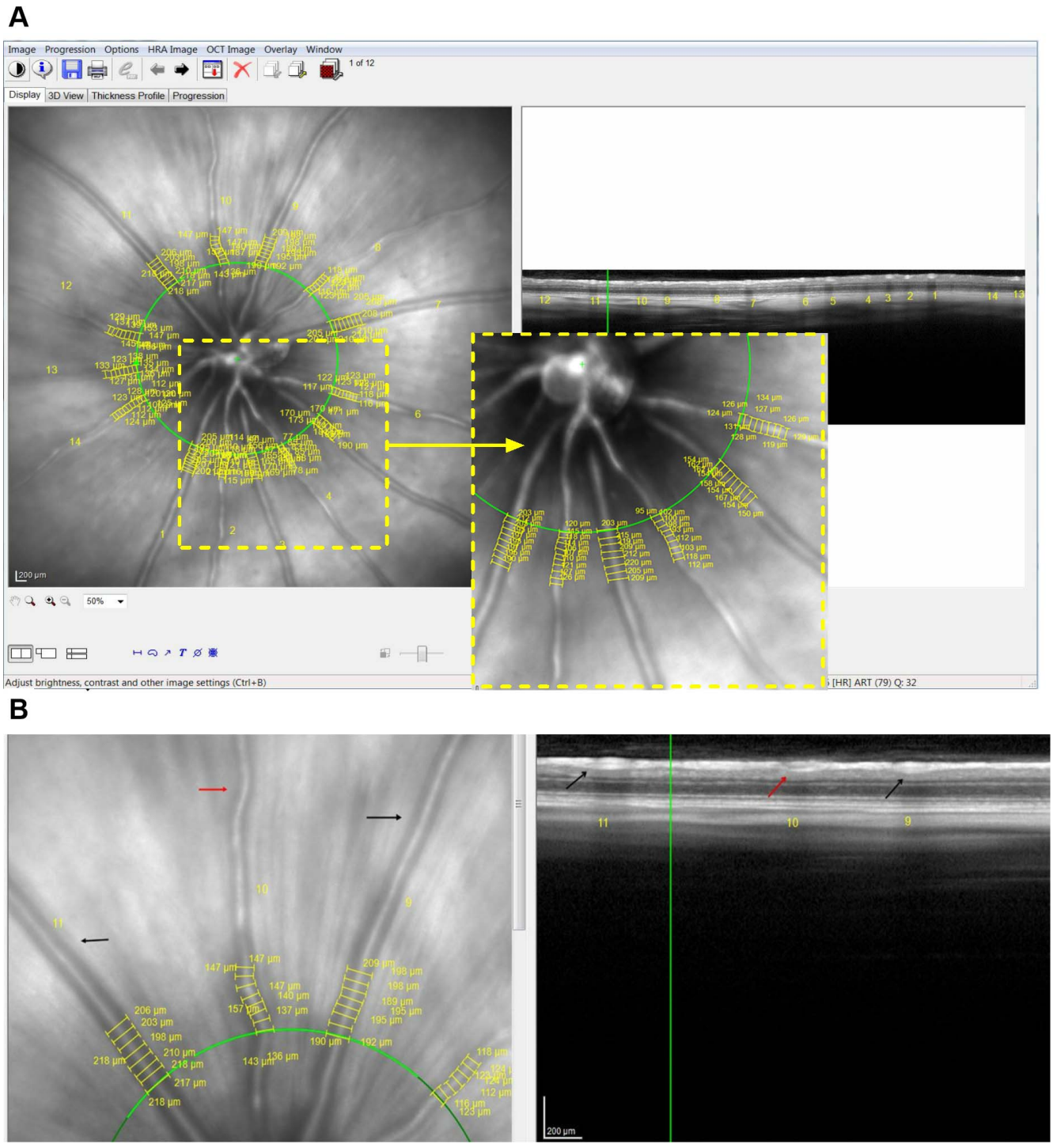


**Figure 2.** Calibers of retinal vessels were measured by Heidelberg software. (**A**) Each vessel was measured 10 times from the same position as "progression follow-up function" indicated, the maximum value from the measurements was considered as the "diastolic diameter," and the minimum value was considered as the "systolic diameter," we take their mean value as the vessel diameter. (**B**) Retinal veins and arteries manifest with different features on SD-OCT. Retinal arteries (*red arrow*) and veins (*black arrow*) showed distinct size and thickness of vascular walls both on fundus picture and the OCT circular scan; moreover, arteries (*red arrow*) showed a stronger central reflex feature than veins (*black arrow*).

individually for each measurement. A contact lens was placed on the cornea. Centering on the optic nerve head in the fundus picture, 100 frames of a 12° diameter circular B-scan were taken. To optimize accuracy and precision, the eye-track and progression follow-up functions were activated throughout all measurements. After IOP elevation in Sprague-Dawley rats for 6 weeks, some of the rats received an intravitreal injection of 3 µL of 2 µM GYY4137 in saline. To investigate the effect of GYY4137 ($n = 3$) on retinal vessels, OCT was performed 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 1 hour 15 minutes, 1 hour 40 minutes, 2 hours 10 minutes, 2 hours 40 minutes, and 3 hours 10 minutes after the injection. OCT was performed again 1 week after the injection. The caliber of the retinal vessels was measured with the aid of the Heidelberg

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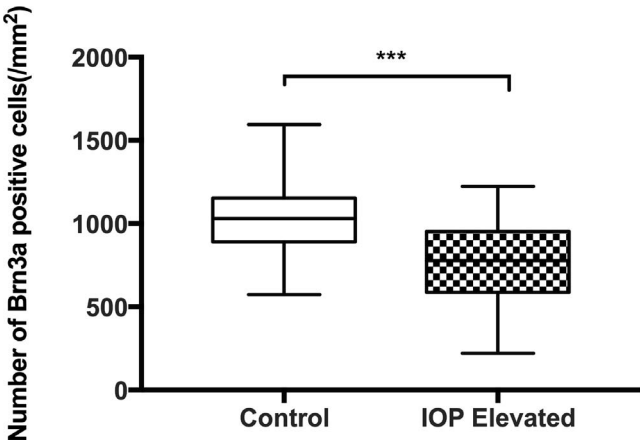


FIGURE 3. Seven weeks of IOP elevation led to significant RGC loss in vivo. After 7 weeks of IOP elevation, the number of RGCs (839.3 ± 215.4 RGC/mm$^2$) in eyes with elevated IOP decreased significantly compared with contralateral controls (1070.4 ± 191.2 RGC/mm$^2$) (*vertical bars* represent means ± SD, $^{***}P < 0.001$, $n = 14$).

software. Each vessel was measured 10 times from the same position as "progression follow-up function" indicated, the maximum value from the measurements was considered as the "diastolic diameter," and the minimum value was considered as the "systolic diameter." We have taken their mean value as the vessel diameter (Fig. 2).

Retinal veins and arteries manifest with different features on SD-OCT. They are distinguished by their size, the thickness of their wall, and their reflectivity features. Retinal veins are generally larger than arteries and have a thicker vascular wall (Fig. 1B). On the right, OCT circular scan centered at the optic disc, retinal arteries (vessel no. 10) and retinal veins (vessel nos. 9 and 11) showed a distinct size and thickness of the vascular wall. Because of their ultrastructural differences in the vascular wall, arteries and veins also have a different reflectivity. As arteries carry blood rich in oxygen, their inner part is brighter than their walls compared to veins. This makes the central reflex feature more obvious in arteries.[15]

## Statistical Analysis

All data values are expressed as the mean ± SD or mean ± SEM, either of which is indicated in the figures. Significance of difference between groups was determined by *t*-test and 1-way ANOVA. Results were considered to be statistically significant when $P < 0.05$.

# RESULTS

## Changes of 3-MST Expression Within the Retina After IOP Elevation In Vivo

IOP was elevated through thermic cauterization of episcleral veins, inducing experimental glaucoma previously shown by Anders et al.[16] In parallel with the IOP elevation, the number of RGCs (839.3 ± 215.4 RGC/mm$^2$) decreased by approximately 20% ($^{***}P < 0.001$), compared with the contralateral controls (1070.4 ± 191.2 RGC/mm$^2$) (Fig. 3). The mass spectrometric–assisted proteomics analysis of the retinal tissue 7 weeks after IOP elevation by means of cauterization of three episcleral veins led to the identification of 1573 quantifiable, retinal proteins. The predominant majority of those proteins remained unchanged with respect to their molecular level between the two observed groups. However, the 3-mercaptopyruvate sulfurtransferase (3-MST), the predominant $H_2S$-producing enzyme, was found noticeably upregulated in the experimental glaucoma samples with a fold-change of 3 (Fig. 4).

## Effects of GYY4137 In Vitro

**GYY4137 Protects RGCs Against Oxidative Stress In Vitro.** We investigated the effects of GYY4137 on RGCs under elevated oxidative stress in vitro. We observed a significant cell loss caused by 100 mM $H_2O_2$ in retinal explants (Fig. 5A). Addition of 100 nM and 10 μM GYY4137 improved RGC survival significantly ($^{**}P < 0.005$), although 1 nM GYY4137 showed no protective effects toward 100 mM $H_2O_2$. At a certain concentration, GYY4137 can attenuate oxidative stress damage to RGCs (Figs. 5A, 5B).

**GYY4137 Protects RGCs Against Elevated Hydrostatic Pressure In Vitro.** Furthermore, we analyzed the effects of GYY4137 on RGCs under elevated hydrostatic pressure in vitro. Figures 6A through 6D show the density change of Brn3a-positive RGCs in retinal explants cultured under different conditions: cultured under hydrostatic pressure (60 mm Hg) for 48 hours in vitro leading to a significant loss in cell density in Figure 6B (513.0 ± 40.2 RGC/mm$^2$) compared with baseline levels (646.0 ± 32.4 RGC/mm$^2$). However, application of 1 nM GYY4137 significantly enhanced RGC density (671.0 ± 27.7 RGC/mm$^2$) to baseline levels. Meanwhile, cultured with 10 μM GYY4137, cell density (344.0 ± 41.9 RGC/mm$^2$) is significantly lower. Thus, application of GYY4137 to organotypic retinal explants showed a dose-dependent effect. Although concentration ranges of 1 to 100 nM GYY4137 could significantly improve RGC survival under elevated hydrostatic pressure in

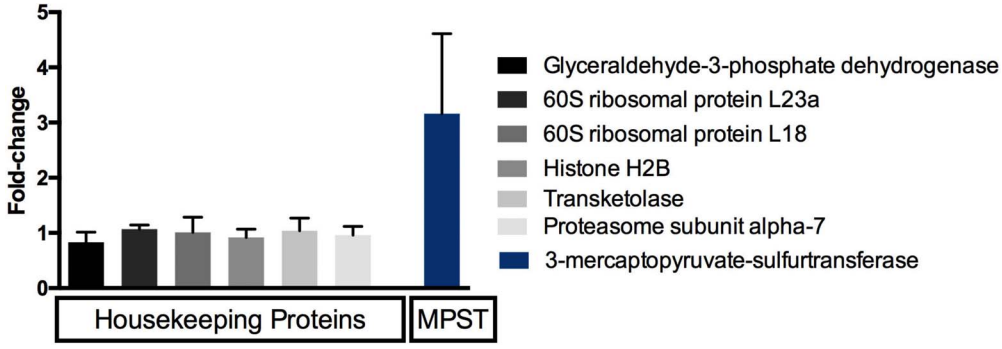


FIGURE 4. Expression of 3-MST within the retina exposed to elevated IOP in vivo. Quantitative mass spectrometric analysis of the retina revealed that within tissue that had been exposed to elevated IOP for 7 weeks, 3-MST was 3-fold upregulated compared with controls, whereas housekeeping proteins remained unchanged.

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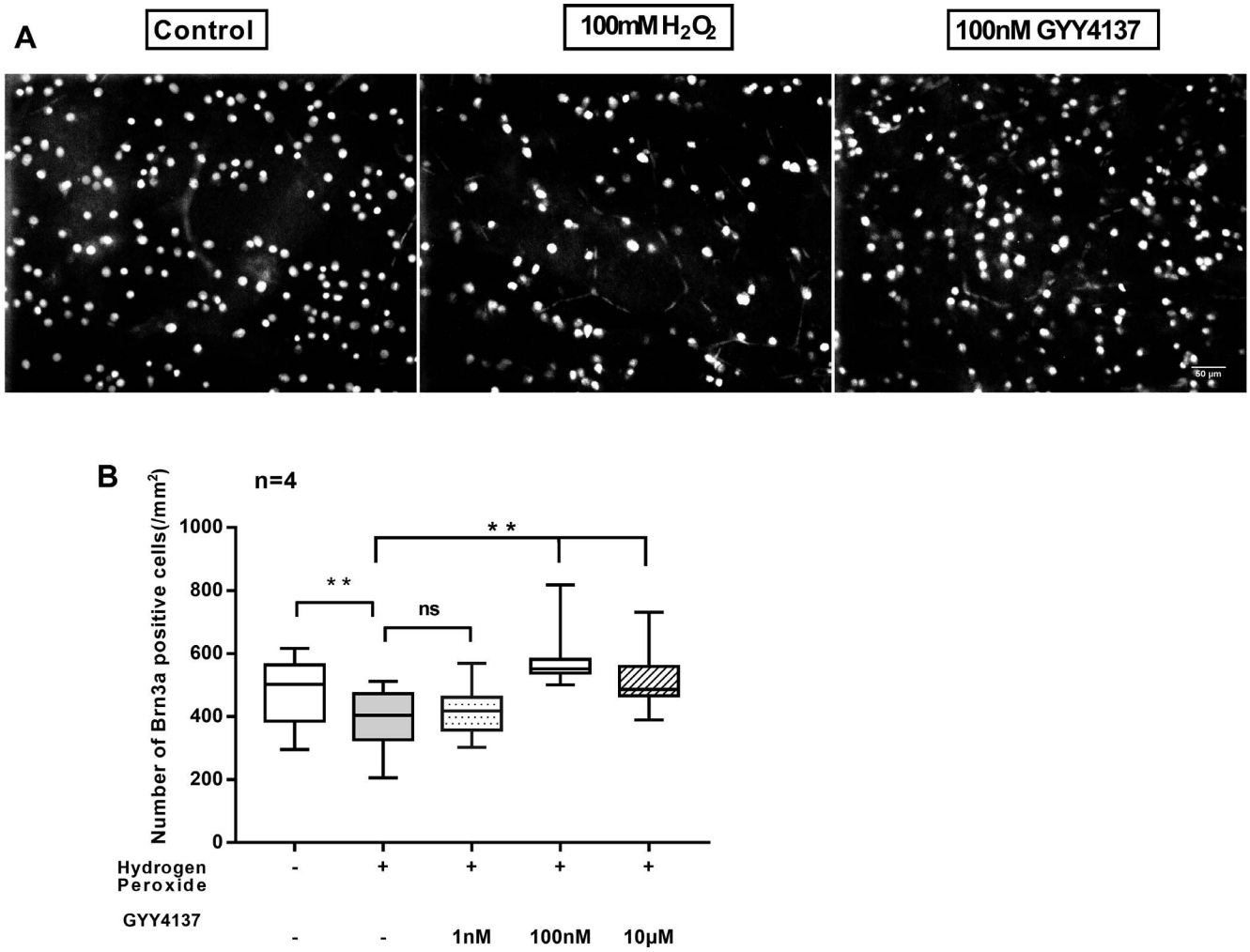


**Figure 5.** Effects of different concentrations (1 nM–10 µM) of GYY4137 (Sigma-Aldrich) on cell death induced by 100 mM $H_2O_2$ in retina explants. (**A**) Representative fluorescence microscopy of Brn3a staining after 24 hours of culturing with or without presence of $H_2O_2$ or GYY4137. (**B**) With the presence of 100 mM $H_2O_2$, there is a significant loss of RGCs in retina explants after 24 hours in culture. Addition of 100 nM and 10 µM GYY4137 improved RGC survival significantly, although 1 nM GYY4137 showed no protective effect against 100 mM $H_2O_2$ (**$P < 0.005$, $n = 4$). *Vertical bars* represent means ± SD of data.

vitro (***$P < 0.001$), higher doses remained toxic (1 µM–10 µM) (Figs. 6A–E).

Thus, the concentration needed toward oxidative stress is apparently higher than the one needed against elevated hydrostatic pressure. Best seen concentration for both oxidative and hydrostatic stress was 100 nM.

## GYY4137 Shows No Effect on rBMECs in Culture

However, in vitro there was no obvious effect on rBMECs under elevated hydrostatic pressure and addition of GYY4137 (data not shown).

## Effects of GYY4137 In Vivo

**Effect of GYY4137 on IOP.** As illustrated in Figure 7, between 9 AM and 5 PM there was no obvious fluctuation in IOP in normotensive conscious Sprague-Dawley rats. Furthermore, topical application of 2% GYY4137 did not decrease IOP. As treatment was carried out for 3 days in a row, no accumulation effect elicited during the observation. Treated eyes exhibited no significant side effects (e.g., hyperemia, tearing).

**GYY4137 Improves RGC Survival Against Retinal Ischemia-Reperfusion Injury In Vivo.** Sixty minutes of regional ischemia followed by 24 hours of reperfusion caused significant RGC loss in vivo (Figs. 8A, 8C). Compared with the contralateral control (1218.8 ± 253.8 RGC/mm$^2$), this procedure resulted in significant RGC loss in experimental eyes (741.3 ± 232.4 RGC/mm$^2$). Pretreatment with GYY4137 showed a significant reduction of RGC loss in vivo (1035.8 ± 241.4 RGC/mm$^2$) (****$P < 0.0001$), but RGC survival is still significantly lower than those in control eyes (**$P < 0.005$).

**GYY4137 Improves RGC Survival in ONC Injury In Vivo.** The ONC injury is an important experimental disease model for glaucoma, the crush injury to the optic nerve leads to gradual RGC apoptosis. Twenty-four hours after the ONC there was a significant RGC loss (815.2 ± 170.8 RGC/mm$^2$) compared with untreated controls (1314.8 ± 238.7 RGC/mm$^2$). Intravitreal injection of GYY4137 (final intraocular concentration = 100 nM) following ONC improved RGC survival significantly (1013.2 ± 206.2 RGC/mm$^2$), although it still remained significantly lower than in contralateral controls (****$P < 0.0001$) (Fig. 9).

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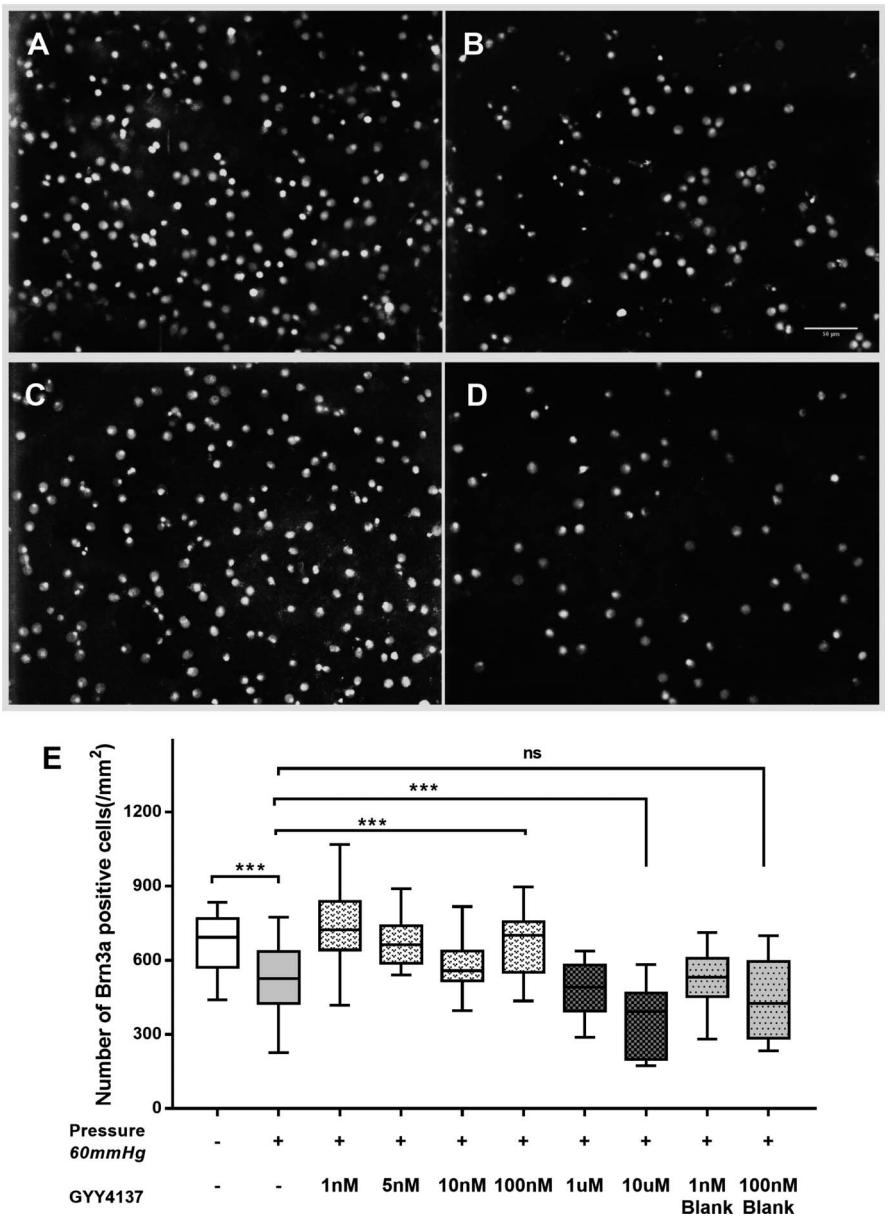


FIGURE 6. Effects of different concentrations (1 nM–10 μM) of GYY4137 (Sigma-Aldrich) on cell death induced by 60 mm Hg hydrostatic pressure in retina explants. (**A–D**) Representative fluorescence microscopy of Brn3a stainings after 48 hours of culturing without elevated pressure or under elevated hydrostatic pressure conditions (60 mm Hg), with and without additional GYY4137. (**A**) Retinal explants cultured 48 hours without pressure or additional GYY4137. (**B**) Retinal explants cultured under pressure without additional GYY4137 showed a significant cell loss. (**C**) With the presence of 1 nM GYY4137. (**D**) With the presence of 10 μM of GYY4137. (**E**) Application of GYY4137 to organotypic retinal explants showed a dose-dependent effect. Within concentration ranges of 1 nM to 100 nM, GYY4137 could significantly improve RGC survival under pressure in vitro (***$P < 0.001$), whereas higher doses remained toxic (1 μM–10 μM), regardless the concentration GYY4137 itself 1 nM Blank and 100 nM Blank has no effect on cell survival. (*Vertical bars* represent means ± SD of data; $n = 6$.)

## Intravitreal Injected GYY4137 Showed a Dilatation Effect on Retinal Vessels In Vivo After Chronic IOP Elevation

In our study, we found that the retinal vascular diameters had significantly narrowed in glaucomatous animals after 7 weeks of IOP elevation (Fig. 10C). During the period of observation after the injection of GYY4137, retinal vessel calibers enlarged significantly (Fig. 10A). Arteries enlarged by 25.5% and veins enlarged by 14.2% (****$P < 0.0001$) due to the application of GYY4137. As illustrated in Figure 10B, shortly after the injection, vessel calibers started to enlarge. Arteries were enlarged significantly 30 minutes after the injection, and arterial calibers increased by 20.46% 1 hour after the injection, then started to narrow down gradually again. Veins showed significant enlargement 1 hour after the injection, and remained significantly enlarged up to 2 hours and 40 minutes (***$P < 0.001$). As shown in Figure 10C, vascular calibers decreased after 6 weeks of IOP elevation, especially arterial calibers, decreased by 11.13% ($P = 0.003$). However, in animals receiving intravitreal GYY4137 injections, the arterial diameter increased 10.26% compared with baseline. The arterial diameter during the diastolic period increased 11.07%; venous diameter increased 8.95%, venous diameter during the systolic

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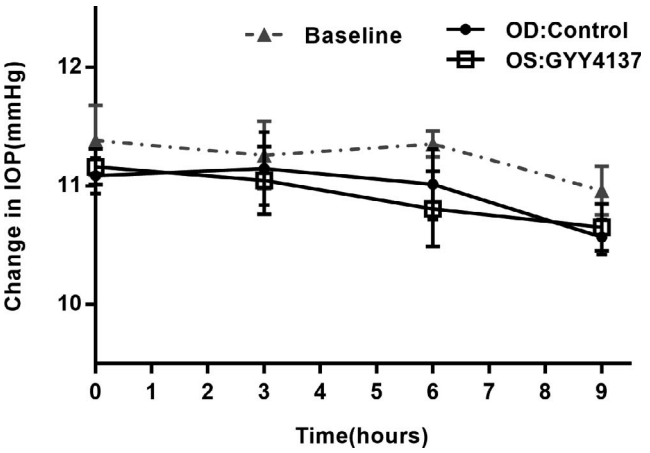


FIGURE 7. Effect of 2% GYY4137 on IOP in normotensive conscious Sprague-Dawley rats in vivo. *Vertical bars* represent means ± SEM of data obtained from five rats, repeated for 3 days in a row. In normotensive conscious Sprague-Dawley rats, there is no fluctuation in IOP between 9 AM and 5 PM. Topical application of 2% GYY4137 did not show a distinct decrease in IOP, neither in the contralateral eye. As the treatment was carried out for 3 days in a row, no accumulation effect elicited during the observation.

period increased 10.71%. Compared with those without injection, the vessel diameter was significantly enlarged (**$P < 0.005$, *$P < 0.01$).

## DISCUSSION

In this study, we investigated the expression of 3-MST, an $H_2S$-producing enzyme, and the effects of GYY4137, a slowly releasing $H_2S$ molecule, in various in vitro and in vivo animal models of glaucoma.

In brief, we had the following findings:

1. 3-MST, the $H_2S$-producing enzyme, is clearly upregulated (3-fold) due to chronically elevated IOP in vivo.
2. The slow-releasing $H_2S$ donor GYY4137 exerts neuroprotective dose-dependent effects in vitro toward glaucomatous retinal damage due to both elevated oxidative stress and hydrostatic pressure.
3. GYY4137 acts in a concentration-dependant manner, needing in general higher concentrations to preserve RGCs if the retina is exposed toward oxidative stress. Best seen concentration for both was 100 nM.
4. GYY4137 exerts neuroprotective effects against ischemia-reperfusion injury and ONC injury in vivo.
5. Presumably GYY4137 acts through dilatation of retinal vessels, therewith improving perfusion.

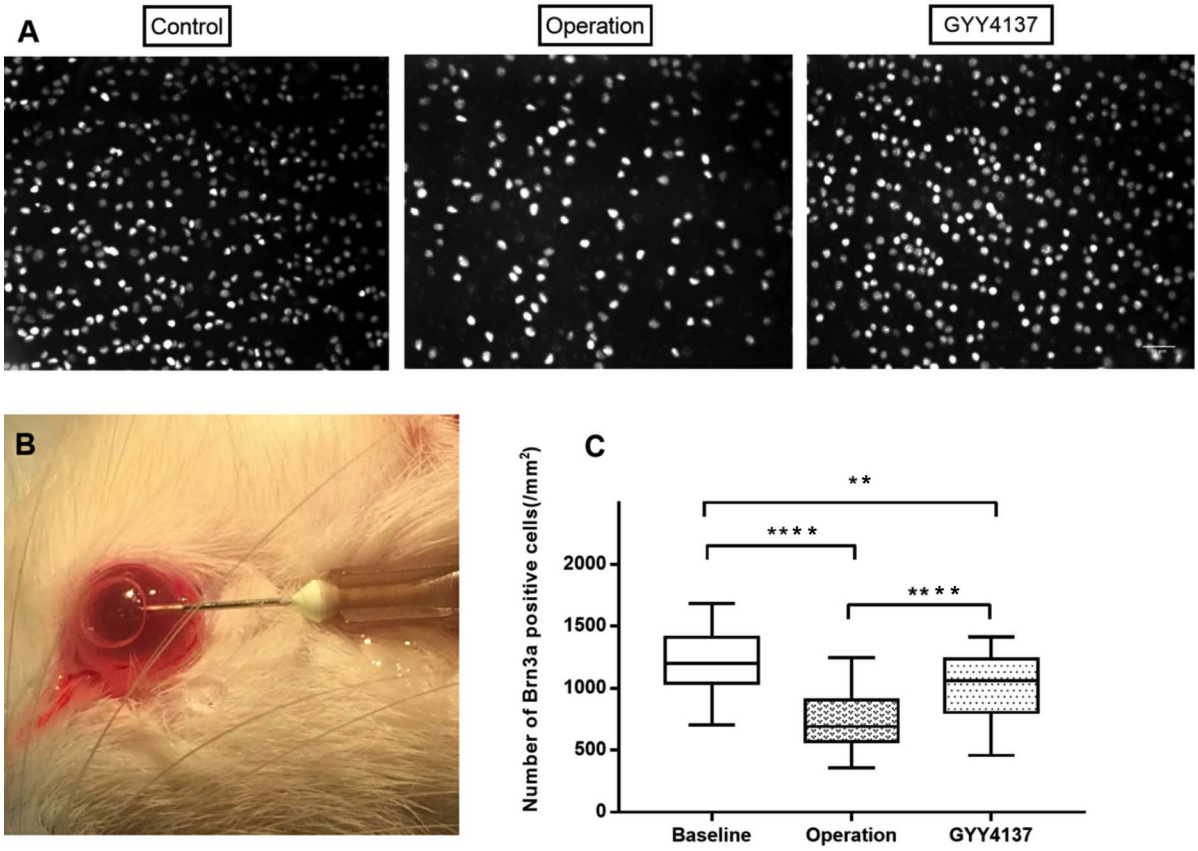


FIGURE 8. Effect of 100 nM GYY4137 (Sigma-Aldrich) on cell death induced in ischemia-reperfusion animal model in vivo. (**A**) Representative fluorescence microscopy of Brn3a staining of retinal explants 24 hours after inducing acute ischemia/reperfusion injury in vivo. (**B**) A 30-gauge needle connected to sterile saline container is inserted carefully into the anterior chamber of a Sprague-Dawley rat, puncture is made from superotemporal clear cornea, carefully avoiding injury to the lens; the bubble serves as an "air embolus" to keep anterior chamber tight while IOP was raised to 55 mm Hg. (**C**) Compared with the contralateral control (1218.8 ± 253.8 RGC/mm$^2$), 60 minutes of regional ischemia followed by 24 hours of reperfusion resulted in significant RGC loss in the experimental eye (741.3 ± 232.4 RGC/mm$^2$), pretreatment with GYY4137 showed a significant reduction of RGC loss in vivo (1035.8 ± 241.4 RGC/mm$^2$). *Vertical bars* represent means ± SD of data (****$P < 0.0001$, **$P < 0.005$, $n = 6$).

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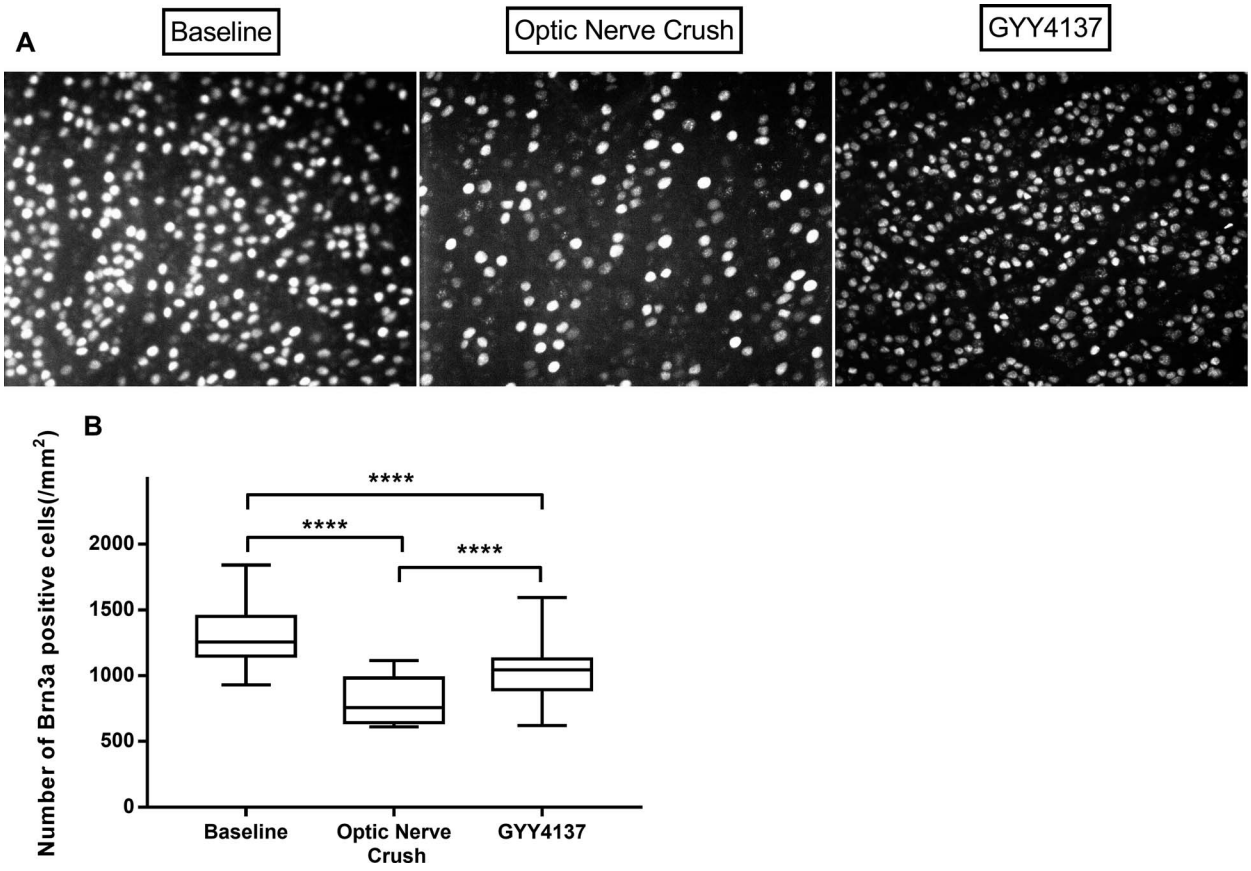


FIGURE 9. Effect of 100 nM GYY4137 (Sigma-Aldrich) on cell death induced by ONC in Sprague-Dawley rats in vivo. (**A**) Representative fluorescence microscopy of Brn3a staining of retinal explants 24 hours after ONC in vivo. (**B**) Compared with the contralateral controls (1314.5 ± 238.7 RGC/mm$^2$), ONC caused significant RGC loss in the experimental eye (815.2 ± 170.8 RGC/mm$^2$). Intraocular injection with GYY4137 following ONC showed a significant reduction of RGC loss in vivo (1013.2 ± 206.2 RGC/mm$^2$). *Vertical bars* represent means ± SD of data (****$P < 0.0001$, $n = 6$).

To best of our knowledge, this is the first study analyzing the effect of $H_2S$ on RGCs in in vitro and in vivo animal models of glaucoma.

Our first finding is that 3-MST is clearly upregulated (3-fold) in the mass spectrometric–assisted proteomics analysis of retinal tissue of experimental glaucoma animals, while housekeeping proteins remained unchanged in the proteomics approach, indicating some relation between experimental glaucomatous injury, neuronal impairment and the protein 3-MST. 3-MST is a mitochondrial protein,[17] which is known to synthesize $H_2S$ in mammalian tissue through its enzymatic action.[18] It has been reported that in brain injury, 3-MST can rapidly release $H_2S$ on stimulation. It is possible that $H_2S$ produced by 3-MST is readily stored as bound sulfane sulfur.[18] $H_2S$ has been recognized as a novel gas neurotransmitter and plays a protective role in diverse mammalian systems; however, to the best of our knowledge, there are no former studies on the regulation of $H_2S$-producing enzymes in glaucoma. We can assume that in our study, 3-MST is upregulated due to the chronically elevated IOP we induced in experimental animals, which suggests $H_2S$ may also play an important role in pathology of glaucoma.

There are different $H_2S$ donors available; the most widely used donors are inorganic sulfite salts, such as NaHS and $Na_2S$. The reason we chose GYY4137 is that it seems more beneficial to expose cells to low concentrations of the gas over a longer period. So we took the slow-releasing $H_2S$ donor GYY4137. GYY4137 slowly releases low but consistent concentrations of $H_2S$ over several hours in aqueous solutions at physiologic pH and temperature. GYY4137 therewith mimics the time course of the physiologic $H_2S$ release in vivo. Additionally, as known so far, GYY4137 does not cause any significant cytotoxic effects.

In our study, GYY4137 showed protective effects on RGCs against elevated hydrostatic pressure and oxidative stress in vitro. We found that the slow-releasing $H_2S$ donor GYY4137 exerts dose-dependent neuroprotective effects against glaucomatous retinal damage in vitro due to both elevated oxidative stress and hydrostatic pressure. It is well-known that the effect of $H_2S$ depends on the concentration. High concentrations (above 250 mmol/L) are supposed to exert toxic effects, whereas low ones have become regarded as cell protective in recent years.[19]

However, in our study, in terms of oxidative stress, higher concentrations were needed as if RGCs were exposed to elevated hydrostatic pressure. The effect was furthermore dose-dependent as well. Overall concentrations of 100 nM seem to be most effective against both oxidative stress and hydrostatic pressure, which both occur in glaucoma.

In previous studies, it has been assumed that the reduction of oxidative stress is one of the possible mechanisms of the protective effect of $H_2S$. Kimura et al.[20] showed that $H_2S$ can protect cells from oxidative stress by enhancing the production of glutathione by enhancing cysteine/cysteine transporters and redistributing glutathione to mitochondria. Furthermore, $H_2S$ produced in mitochondria may also directly

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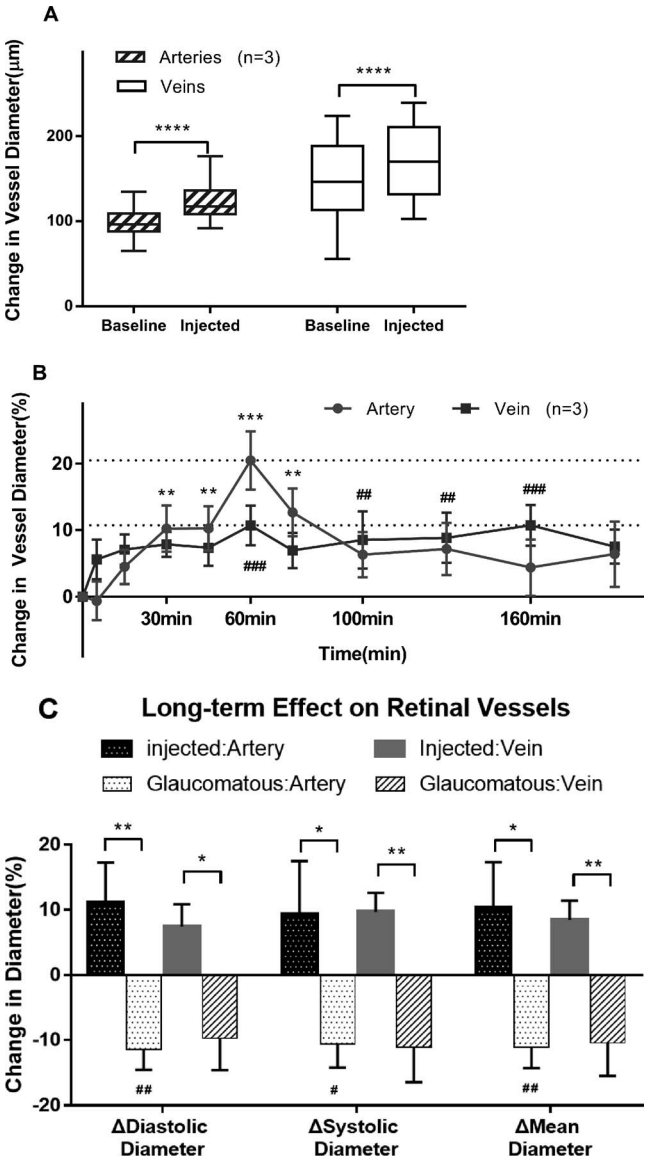


FIGURE 10. (**A**, **B**) Short-term effect of GYY4137 on retinal vessel in vivo. (**A**) During the period of observation after intravitreal injection of GYY4137 (final intraocular concentration 100 nM), retinal vessels significantly enlarged. Arterial diameter increased 25.5%, veins increased 14.2%. (*Vertical bars* represent means ± SD of data, $^{****}P < 0.0001$.) (**B**) Vessels enlarged shortly after the injection, arterial calibers enlarged significantly after 30 minutes. One hour after the injection, arterial calibers increased 20.46% compared with baseline ($^{***}P < 0.001$), then gradually narrowed down. Veins are significantly enlarged after 1 hour, and remained significantly enlarged over 3 hours. (*Vertical bars* represent mean ± SEM of data, $^{**}P < 0.005$, $^{***}P < 0.001$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.) (**C**) Long-term effect of 1 nM GYY4137 on retinal vessel diameter in glaucomatous Sprague-Dawley rats. Six weeks after IOP elevation, retinal arterial mean diameter is 9.80% smaller than in baselines ($^{\#\#}P < 0.005$, $^{\#}P < 0.01$), meanwhile 1 week after GYY4137 injection, arterial diameter increased 10.26% compared with baseline, arterial diameter during diastolic period increased 11.07%, venous diameter increased 8.95%, venous diameter during the systolic period increased 10.71%; compared with those without injection, vessel diameter is significantly enlarged ($^{**}P \leq 0.005$, $^{*}P < 0.01$). Data are represented as mean ± SEM.

suppress oxidative stress. In the presence of GYY4137, a much higher-level cell viability was observed against $H_2O_2$;-induced cell damage.[21]

Besides the in vitro effects, we could prove neuroprotective effects in vivo. Transient IOP elevation was induced in Sprague-Dawley rats through the insertion of a needle into the anterior chamber connected to a saline container, and elevated IOP at 55 mm Hg. This procedure caused significant RGC loss in operated eyes, but in those eyes pretreated with intravitreal injections of GYY4137, RGC survival was significantly higher than in untreated ones.

The retinal ischemia-reperfusion injury model mimics clinical situations like acute glaucoma.[22–24] Biermann et al.[25] reported that rapid preconditioning with inhaled $H_2S$ mediates antiapoptotic and thus neuroprotective effects in the rat retina after ischemia-reperfusion injury. Sakamoto et al.[26] reported that $H_2S$ protects retinal neurons against injury induced by intravitreal N-methyl-D-aspartate in rats in vivo. Lu et al.[27] found that $H_2S$ reversed $H_2O_2$-induced cellular injury in a concentration-dependent manner.

The ONC injury is another important experimental disease model for glaucoma; the crush injury to the optic nerve leads to gradual RGC apoptosis. In this study, we could show that GYY4137 intravitreally injected after ONC in Sprague-Dawley rats significantly improved RGC survival evaluated 24 hours after the procedure.

Thus, despite the differences in the mechanisms causing RGC loss in these two experimental in vivo models, GYY4137 decreased cell loss significantly in both cases, showing its potential to protect RGCs under glaucomatous conditions in vivo.

Although the mechanisms underlying optic nerve injury in glaucoma remain obscure, vascular, mechanical theories have been widely recognized. The mechanisms involved in vascular and mechanical theories are similar, pressure-induced distortions of the lamina cribrosa result in shearing and compressive forces that decrease choroidal blood flow and also act directly on the ganglion cell axons,[28,29] as well as vascular dysregulation.

We could observe that the intravitreal injection of GYY4137 showed a vasodilative effect on retinal vessels. Retinal vessel calibers started enlarging shortly after the injection, and the effect lasted 1 week. It possibly improved blood flow significantly, which was restricted due to optic nerve injury and eased vasospasms caused by elevated IOP, which will possibly improve the retinal perfusion and RGC survival under glaucomatous conditions in the long term. These results present the possibility that besides its antioxidant properties, $H_2S$ may act as a vasoregulator. $H_2S$ may exert its protective effect in glaucoma, by stabilizing ocular perfusion and easing ischemia-reperfusion injury. Although optic nerve injury has a direct impact on ganglion cell axons, this part of the mechanism by which $H_2S$ resulted in protection of RGCs against optic nerve injury remains unclear.

In accordance with this, its action in the cardiovascular system has been excessively studied. Endogenously generated and exogenously administered $H_2S$ exerts a wide range of actions in vascular and myocardial cells, including vasodilator/vasoconstrictor effects via modification of the smooth muscle tone. It has been reported that GYY4137 has protective effects against myocardial ischemia and reperfusion injury by attenuating oxidative stress and apoptosis.[30]

Endogenous $H_2S$ is produced in both vascular smooth muscle cells and endothelial cells, and is involved in the regulation of many physiological processes, including the vascular tone. $H_2S$ produced in vascular smooth muscle cells can directly regulate vascular tone in an autocrine manner,

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and $H_2S$ synthesized in endothelial cells can regulate independently of smooth muscle cells by mediators binding to endothelial cell receptors. Endothelial $H_2S$ signaling is upregulated in some pathologic conditions, including ischemia-reperfusion injury.[31] As there is evidence suggesting that the major cause of blood flow reduction in glaucoma patients is rather a vascular dysregulation, $H_2S$ may have a specific protective potential by maintaining the regulation of the vascular tone.

However, there are limitations of the study. Unfortunately, there is no proper glaucoma model, neither in vitro nor in vivo. That is why we used very different kinds of glaucoma models to cover it all. Furthermore, the measurement of the vessels by means of OCT might be affected by not spotlessly clean measurements. However, within the framework of those limitations mentioned, we could see neuroprotective properties and vasodilative effects, which are in accordance with previous findings.

## Conclusions

To best of our knowledge this is the first study to analyze the effect of $H_2S$ in the pathogenesis of experimental glaucoma with respect to vasodilative effects. $H_2S$ is specifically regulated in experimental glaucoma. By scavenging ROS and dilating retinal vessels, $H_2S$ protects RGCs from elevated pressure and oxidative stress in vitro and in vivo. Therefore, $H_2S$ might be a novel neuroprotectant in glaucoma.

### *Acknowledgment*

Supported by Deutsche Forschungsgemeinschaft PR 1569 1/1.

Disclosure: **H. Liu**, None; **F. Anders**, None; **S. Thanos**, None; **C. Mann**, None; **A. Liu**, None; **F.H. Grus**, None; **N. Pfeiffer**, None; **V. Prokosch-Willing**, None

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