DISSERTATION

The Protective Role and Underlying Mechanism of Hydrogen Sulfide against glaucomatous injuries *in vitro* and *in vivo*

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by

Hanhan Liu

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„Science is a wonderful thing.”

Albert Einstein

Declaration I

Parts of this dissertation have been published in international journals and presented at scientific conferences:

Published papers/manuscripts in revision

1) **Proteomics reveals the potential protective mechanism of hydrogen sulfide on retinal ganglion cells in an ischemia/reperfusion injury animal model**
   Liu H, Perumal N, Manicam C, Mercieca K, Prokosch V
   Pharmaceuticals (Basel) 13, no. 9, 2020, PMID: 32867129
2) **Hydrogen Sulfide and β-Synuclein Are Involved and Interlinked in the Aging Glaucomatous Retina**
   Liu H, Mercieca K, Anders F, Prokosch V
   Journal of Ophthalmology, 2020, PMID: 32351728
3) **Mitochondrial Markers in Aging and Primary Open-Angle Glaucoma**
   Liu H, Mercieca K, Prokosch V
   Journal of Glaucoma, 2020, PMID: 31977544
4) **Proteome alterations in aqueous humour of primary open angle glaucoma patients**
   Liu H, Anders F, Funke S, Mercieca K, Grus F, Prokosch V
   International Journal of Ophthalmology, 2020, PMID: 31956586
5) **Hydrogen Sulfide Protects Retinal Ganglion Cells Against Glaucomatous Injury In**
   Vitro and In Vivo
   Liu H, Anders F, Thanos S, Mann C, Liu A, Grus FH, Pfeiffer N, Prokosch V
   Investigative Ophthalmology & Visual Science, 2017, PMID: 28986598
6) **Alterations in Tight- and Adherens-Junction Proteins Related to Glaucoma Mimicked in the Organotypically Cultivated Mouse Retina Under Elevated Pressure.**
   Brockhaus K, Melkonyan H, Prokosch-Willing V, **Liu H**, and Thanos S.
   Investigative Ophthalmology & Visual Science, 2020, PMID: 32207812
7) **Elevated intraocular pressure induces neuron-specific beta-III-tubulin expression in non-neuronal vascular cells**
   Prokosch V, Brockhaus K, Anders F, **Liu H**, Mercieca K, Gericke A, Melkonyan H, Thanos S

Acta Ophthalmologica, 2019, PMID: 31885180

8) **Neuroprotective and neuroregenerative effects of CRMP-5 on retinal ganglion cells in an experimental in vivo and in vitro model of glaucoma**
Lauzi J, Anders F, **Liu H**, Pfeiffer N, Grus F, Thanos S, Arnhold S, Prokosch V
PLoS One, 2019, PMID: 30673694

9) **Relaxin 2 fails to lower intraocular pressure and to dilate retinal vessels in rats**
Hampel U, Träger K, **Liu H**, Teister J, Grus F, Prokosch-Willing V
International Ophthalmology, 2018, PMID: 29536410

10) **Morphological and Quantitative Changes in Retinal and Optic Nerve Vessels in Experimental Glaucoma Model with Elevated IOP for 7 Weeks**
Mann C, Anders F, **Liu H**, Brockhaus K, Liu A, Grus FH, Pfeiffer N, Thanos S, Prokosch V
Klinische Monatsblätter für Augenheilkunde, 2018, PMID: 29590684

11) **Femtosecond laser in refractive and cataract surgeries**
Liu H, Hu Y, Cui H
International Journal of Ophthalmology, 2015, PMID: 25938066

Poster presentations at scientific conferences:

1) Proteomics reveals the potential mechanisms hydrogen sulfide involve in to protect retinal ganglion cells in a glaucomatous animal model
Liu H, Perumal N, Manicam C, Prokosch V[1]
Deutsche Ophthalmologische Gesellschaft Kongress 2019 in Berlin

2) Analysis of mitochondrial proteomic profile to protect retinal ganglion cells from glaucomatous injury
Liu H, Perumal N, Anders F, Grus FH, Pfeiffer N, Prokosch V
Deutsche Ophthalmologische Gesellschaft Kongress 2017 in Berlin

3) Hydrogen sulfide protects retinal ganglion cells from cell death due to elevated hydrostatic pressure in vitro and in vivo
Liu H, Anders F, Thanos S, Mann C, Liu A, Grus FH, Pfeiffer N, Prokosch V
Deutsche Ophthalmologische Gesellschaft Kongress 2016 in Berlin

Declaration II

The present thesis was written under the supervision of Prof. Dr. Prokosch at the Department of Ophthalmology, University Medical Care Center, Johannes Gutenberg-University in Mainz. The doctoral thesis is designed as a cumulative dissertation and is based on the following publications and manuscripts:

1) **Hydrogen Sulfide Protects Retinal Ganglion Cells Against Glaucomatous Injury In Vitro and In Vivo**
   Liu H, Anders F, Thanos S, Mann C, Liu A, Grus FH, Pfeiffer N, Prokosch V
   Investigative Ophthalmology & Visual Science, 2017, PMID: 28986598
2) **Hydrogen Sulfide and β-Synuclein Are Involved and Interlinked in the Aging Glaucomatous Retina**
   Liu H, Mercieca K, Anders F, Prokosch V
   Journal of Ophthalmology, 2020, PMID: 32351728
3) **Proteomics reveals the potential protective mechanism of hydrogen sulfide on retinal ganglion cells in an ischemia/reperfusion injury animal model**
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   Pharmaceuticals (Basel) 13, no. 9, 2020, PMID: 32867129

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Abstract

Glaucoma, one of the leading causes of irreversible blindness worldwide and is characterized by progressive optic nerve and retinal ganglion cell (RGC) degeneration. Hydrogen sulfide (H_2S) plays a role as a potent neurotransmitter and has been proven to protect RGCs against glaucomatous injury in vitro and in vivo, although the exact mechanism is unclear.

The main objective of this doctoral thesis was to reveal the potential roles of H_2S in glaucoma pathophysiology and to better understand the mechanism of H_2S in neuroprotection.

In the process of aging, RGCs and β-synuclein (SNCB) are significantly changed in old animals. Under chronic IOP elevation there is a significant RGC loss in old animals, whereas no significant change in young animals; SNCB is significantly downregulated and 3-mercaptosulfurtransferase, a H_2S producing enzyme, showed a 3-fold up-regulation within the retina in young animals after IOP elevation, while no significant changes in old ones are notable.

GYY4137, a H_2S slow-releasing donor, was observed to protect RGC 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 ($p<0.0001$). Under acute ischemic injury, exogenous H_2S also significantly downregulated SNCB levels. Furthermore, retinal vessel diameters enlarged after intravitreal GYY4137 injection ($p<0.0001$).

As final part of this doctoral thesis, we examined the potential protective mechanisms activated by H_2S in a glaucoma animal model. We used mass spectrometry-based proteomics to elucidate how protein expression changes at the cellular level. In total 1115 proteins were identified, 48 proteins were significantly differentially expressed due to I/R. Abundance of 18 key proteins were restored by H_2S. Another 11 proteins were differentially expressed following H_2S. Proteomic and ingenuity pathway analysis (IPA) revealed a significant H_2S-mediated activation of pathways related to mitochondrial function, iron homeostasis and vasodilation.

In conclusion, the present doctoral thesis is the first study to analyse the effect of H_2S in the pathogenesis of experimental glaucoma and provide an overall insight into the

mainstay retinal proteins and pivotal signaling pathways that interact with H_2S to maintain retinal homeostasis against glaucomatous injury. These results form the basis for further research of H_2S as an innovative treatment strategy for glaucoma.

Zusammenfassung

Das Glaukom, eine der weltweit führenden Ursachen für irreversible Blindheit, ist durch eine fortschreitende Degeneration von Sehnerven und retinalen Ganglienzellen (RGC) gekennzeichnet. Schwefelwasserstoff (H_2S) spielt eine Rolle als potenter Neurotransmitter und schützt nachweislich RGCs in vitro und in vivo vor glaukomatösen Verletzungen, obwohl der genaue Mechanismus unklar ist.

Das Hauptziel dieser Doktorarbeit war es, die möglichen Rollen von H_2S in der Pathophysiologie des Glaukoms aufzudecken und den Mechanismus von H_2S bei der Neuroprotektion besser zu verstehen.

Im Alterungsprozess verändern sich RGCs und β-Synuclein (SNCB) bei alten Tieren signifikant. Bei chronischer Erhöhung des Augeninnendrucks gibt es bei alten Tieren einen signifikanten RGC-Verlust, während bei jungen Tieren keine signifikante Veränderung auftritt. SNCB ist signifikant herunterreguliert und 3-Mercaptosulfurtransferase, die H_2S-produzierendes Enzyme, zeigte bei jungen Tieren nach Erhöhung des Augeninnendrucks eine dreifache Hochregulation innerhalb der Netzhaut, während bei alten Tieren keine signifikanten Veränderungen bemerkenswert sind.

Es wurde beobachtet, dass GYY4137, ein langsam freisetzender H_2S-Donor, RGC in vitro in Abhängigkeit von der verwendeten Konzentration vor erhöhtem Druck und oxidativem Stress schützt ($p < 0,005$). In vivo bewahrte die intravitreale Verabreichung von GYY4137 RGCs vor akuten ischämischen Verletzungen und Sehnervenquetschungen ($p < 0,0001$). Bei akuten ischämischen Verletzungen regulierte exogenes H_2S auch die SNCB-Spiegel signifikant herunter. Darüber hinaus vergrößerten sich die Durchmesser der Netzhautgefäße nach intravitrealer GYY4137-Injektion ($p < 0,0001$).

Als letzten Teil dieser Doktorarbeit untersuchten wir die möglichen Schutzmechanismen, die durch H_2S in einem Glaukom-Tiermodell aktiviert werden. Wir verwendeten massenspektrometrische Proteomik, um zu untersuchen, wie sich die Proteinexpression auf zellulärer Ebene ändert. Insgesamt wurden 1115 Proteine identifiziert, 48 Proteine wurden aufgrund von I / R signifikant unterschiedlich exprimiert. Die Häufigkeit von 18 Schlüsselproteinen wurde durch H_2S wiederhergestellt. Weitere 11 Proteine wurden nach H_2S unterschiedlich exprimiert.

Die Proteom- und Einfallsreichtumsanalyse (IPA) ergab eine signifikante H_2S-vermittelte Aktivierung von Signalwegen im Zusammenhang mit Mitochondrienfunktion, Eisenhomöostase und Vasodilatation.

Zusammenfassend ist die vorliegende Doktorarbeit die erste Studie, die die Wirkung von H_2S auf die Pathogenese des experimentellen Glaukoms analysiert und einen umfassenden Einblick in die Hauptproteine der Netzhaut und die zentralen Signalwege bietet, die mit H_2S interagieren, um die Homöostase der Netzhaut gegen glaukomatöse Verletzungen aufrechtzuerhalten. Diese Ergebnisse bilden die Grundlage für die weitere Erforschung von H_2S als innovative Behandlungsstrategie für das Glaukom.

1 Introduction

1.1 Glaucoma – Pathology and treatment

Glaucoma is one of the leading causes of irreversible blindness worldwide[1]. 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[2]. Elevated intraocular pressure (IOP) is a main risk factor for glaucoma and the mainstay of treatment, but simply lowering IOP may fail to halt the disease progression in many patients despite delaying RGC death. Novel strategies which halt RGC loss are desperately needed to prolong visual function in glaucoma[3]. Various other treatment options have been examined to delay or halt RGC loss in order to preserve visual function in glaucoma patients without combating results. In recent years the role 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 has also been discussed to play a key role[4] .

1.2 Gaseous signaling molecule in the eye

The importance of NO in biology and medicine was highlighted in 1998 when the Nobel Prize was awarded in Physiology and Medicine to Robert Furchgott, Louis Ignarro and Ferid Murad for their pioneering work on the role of NO in the nervous, cardiovascular and immune systems [5]. In the same time period, carbon monoxide (CO) has also been recognized as a putative neurotransmitter [6]. They are both involved in several important aspects of neuronal function. Under physiological conditions, NO formed in the eye is beneficial in preventing the genesis of glaucoma or halting its progress; however, excessive NO would become pathogenic in the progress of glaucomatous optic neuropathy [7]. In vitro and in vivo studies have shown evidence of NO's involvement in retinal degeneration, the regulation of IOP and the production of aqueous humor [8–10]. Drugs inhibiting NO formation showed protective effects in the retina against elevated IOP in glaucoma animal models [11–13]. On the other hand, The CO production system in the retina has been highlighted as a protective mechanism, its protecting role is demonstrated in the regulation of Ischemia-reperfusion (I/R) -induced damage, protecting retinal ganglion cells against oxidative damage, increases retinal and choroidal blood flows and downregulating inflammatory response in the anterior segment of the eye [14–16].

1.3 Hydrogen sulfide- a novel endogenous gaseous signaling molecule

Hydrogen sulfide (H_2S), along with CO and NO, has been recognized as a third endogenous gaseous signaling molecule[5]. It is produced via cysteine catabolism by the cytoplasmic enzymes, cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), and also via 3-mercaptopyruvate catabolism by 3-mercaptopyruvate sulfurtransferase (3-MST) [6]. H_2S has recently been considered an endogenous gasotransmitter with protective potential at low concentrations. Alteration of endogenous H_2S levels in the retina is also linked to different pathological conditions, and its exogenous donors have been shown to exhibit potential in protecting retinal ganglion cells against insults such as diabetic retinopathy, ischemia–reperfusion injury and N-methyl-D-aspartic acid (NMDA)-induced excitatory neurotoxicity [11-13].

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 [7].

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

1.4 Neuroprotective properties of H_2S

Besides these vascular effects, neuroprotective properties have been assumed. In the central nervous system(CNS) H_2S has been found to facilitate long-term potentiation and regulate intracellular calcium concentration and pH level in brain cells. Antioxidant, anti-apoptotic, and anti-inflammatory effects of H_2S have been found e.g. in Alzheimer's disease(AD), Parkinson's disease(PD), and vascular dementia. Furthermore an abnormal generation and metabolism of H_2S has been seen involved in most of these neurodegenerative disorders [8]. Moreover, H_2S protects neurons against glutamate-mediated oxidative stress, or oxytosis, through the pleiotropic

effects of maintaining the activities of cystathionine-ɣ-lyase and cystine transport, leading to an increase in glutathione levels [9]. The neuroprotective effect of H_2S was partly attributed to its capability of vaso-relaxation, anti-oxidative stress, neuroendocrine regulation, and inflammation suppression [10-12], but our current understanding of the mechanism behind RGC apoptosis and protective properties of H_2S is far from comprehensive.

1.5 Synucleins –Structure, classes and functions

Synuclein is a family of small proteins including α (SNCA), β (SNCB) and γ (SNCG) synucleins[13,14] and is involved in various neurodegenerations in the CNS. Specifically, SNCA is a major constituent of Lewy bodies, pathological neuronal inclusion bodies found in Parkinson's disease, Alzheimer's disease, and other neurodegenerative disorders [15,16]. Mutations of SNCA play a central role in PD pathology, and misfolding and aggregation of SNCA directly linked to microglial activation, followed by inflammation and oxidative stress resulting in neurodegeneration[17]. Synucleins are present in the retina and optic nerve[18] and are associated with glaucomatous alterations in the optic nerve[19]. SNCA autoantibody was found to be downregulated in serum and upregulated in aqueous humor of glaucoma patients[20] and in our previous study, intravitreal injection of SNCA antibodies is found to be neuroprotective in glaucoma animal model[21].

β –synuclein shares a similar protein structure to SNCA [22], but lack the non-amyloid-β component domain[23]. Its expression is documented to be increased in cerebrospinal fluid in patients with neurodegenerative diseases and in neuronal retina and visual cortex of rats and nonhuman primates with age and external stress [24-26]. SNCB is thought to function as a physiological inhibitor of SNCA in neurodegenerative diseases [26,27] , it retain anti-apoptotic ability in a dose-dependent manner [13],and β-synuclein-derived peptides behave as anti-aggregating agents [15].

While SNCA and SNCB are mainly associated with diseases in the CNS, γ-synuclein is first identified as breast-specific gene protein 1[28], but it is also involved in axonal spheroid-like lesions in Parkinson’s disease, deposition in glial cells in glaucoma, and motor neuron dysfunction and death[19,29,30].

Because of the pivotal role of SNCA in neurodegeneration in CNS, it has been extensively studied in CNS. But its role in retina or glaucoma, as well as SNCB and

SNCG's roles and functions in glaucoma is still sparse and remain to be thoroughly explored.

1.6 Aims of the thesis

In first part of the study, we analyse the expression changes of H_2S in an experimental animal model of glaucoma and second H_2S's potential neuroprotective effect on RGC towards elevated pressure in glaucoma models in vitro and in vivo by addition of the slow-releasing H_2S donor GYY4137.

Studies have shown that H_2S and synucleins are involved in several mutual pathophysiological processes, such as microglia activation, p53-mediated apoptosis, inflammatory response and free radical reactions [5,31-33]. As second part of the thesis, we first elucidate the potential roles and functions of synucleins in glaucomatous neuropathy, following this, to investigate the potential of H_2S to regulate it, and to better understanding the mechanism of H_2S in neuroprotection.

At the end of the project, a mass spectrometry-based proteomics approach was employeed to analyze the retinal proteome, and bio-informatics to algorithmically generate protein connections, which allowed us to identify the most plausible signaling pathway alterations related to H_2S's neuroprotective properties against ischemia-reperfusion injury, and to provide a more precise direction for further studies.

2 Manuscripts

2.1 Publication I

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

Liu H, Anders F, Thanos S, Mann C, Liu A, Grus FH, Pfeiffer N, Prokosch V

Investigative Ophthalmology & Visual Science, 2017

PMID: 28986598, DOI: 10.1167/iovs.17-22200

2.2 Publication II

Hydrogen Sulfide and β-Synuclein Are Involved and Interlinked in the Aging Glaucomatous Retina

Liu H, Mercieca K, Anders F, Prokosch V

Journal of Ophthalmology, 2020

PMID: 32351728, DOI: 10.1155/2020/8642135

2.3 Publication III

Proteomics reveals the potential protective mechanism of hydrogen sulfide on retinal ganglion cells in an ischemia/reperfusion injury animal model

Liu H, Perumal N, Manicam C, Mercieca K, Prokosch V

Pharmaceuticals, 2020

PMID: 32867129, DOI: 10.3390/ph13090213

Glaucoma, one of the leading causes of irreversible blindness worldwide[34], is a group of disorders characterized by progressive retinal ganglion cell loss and axon atrophy, which leads to gradually visual field loss [35].

By far the only known modifiable risk factor of glaucoma is intraocular pressure, however, lowering IOP is not able to halt the deterioration of glaucoma in most patients in clinic practice, indicating again the multifactorial pathogenesis and the complexity of glaucoma [36]. The other main risk factor is age. Alternative approaches independent of IOP and probably combating ageing as well as focusing on the pathophysiological processes are in demand to ameliorate glaucoma neuropathy. Other pathophysiological processes including oxidative stress, inflammatory reaction, glial activation, vascular dysfunctions and abnormal protein accumulation are proven to be closely involved[37-40]. Various other treatment options have been examined to delay or halt RGC loss in order to preserve visual function in glaucoma patients without combating results. In recent years the role other factors like glutamate excitotoxicity, disorganized metabolism of nitric oxide (NO), overproduction of reactive oxygen species (ROS) and reduction of ocular blood flow has also been discussed to play a key role [4] .

An interesting molecule in this context is H_2S. H_2S is the third identified gaseous transmitter after NO and CO and it is endogenously generated in mammalian endothelial cells [41]. It is produced via cysteine catabolism by the cytoplasmic enzymes, cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), and also via 3-mercaptopyruvate catabolism by 3-mercaptopyruvate sulfurtransferase (3-MST) [6].

Ever since H2S has been recognized as the third endogenous gaseous signaling molecule, its role in various physiological and pathological processes has been explored. As a potent reductant, H_2S plays critical roles in multiple physiological and pathological processes, it works to alleviate inflammatory responses, oxidative stress, and restores energy shortage[31,42,43]. H_2S has shown profound therapeutic efficiency potential in neurodegenerative diseases in CNS [44-47]. Researches focus on H_2S in connection with glaucoma have increasingly emerged in last few years, our knowledge on this topic is still lacking and remains to be thoroughly expanded. Alteration of endogenous H_2S level in retina is correlated with different pathological

situations, and its exogenous donors exhibited potential in protecting retinal ganglion cells against assaults, such as diabetic retinopathy, ischemia–reperfusion injury and N-methyl-D-aspartic acid (NMDA)-induced excitatory neurotoxicity[48-50].

In first part of study, covered in Publication I, endogenous H_2S synthases are observed to increase in a glaucoma animal model after seven weeks of chronic elevated IOP. Furthermore, we demonstrated that administration of GYY4137, a slow-release H_2S donor, significantly improved RGC survival under different glaucomatous injuries in vitro and in vivo[51].

The neuroprotective effect of H_2S in the retina was partly attributed to its capability of vasorelaxation, anti-oxidative stress, neuroendocrine regulation, and inflammation suppression[52] but the underlying mechanism and the specific protein interaction networks and the mechanisms involved in protective properties of H_2S remain to be elucidated.

Synuclein is a family of small proteins including α (SNCA), β (SNCB) and γ (SNCG) synucleins[13,14] and is involved in various neurodegenerations in the CNS. Synucleins are also present in the retina and optic nerve[18] and are associated with glaucomatous alterations in the optic nerve[19]. SNCA autoantibody was found to be downregulated in serum and upregulated in aqueous humor of glaucoma patients [20].

In second part of the study, covered in Publication II, we investigated the level changes of synucleins in the retina in different glaucoma animal models, a chronic progressive model of glaucoma at different age stages and an acute IOP elevation induced by ischemia-reperfusion injury. Furthermore, as synucleins and H_2S are involved in several mutual pathophysiological processes, we explored the correlation between H_2S and synucleins in order to better understand the mechanism H_2S's neuroprotective properties in experimental glaucoma.

Finally, building on previous results, we employed a mass spectrometry-based proteomics approach to analyze the retinal proteome, and bio-informatics to algorithmically generate protein connections, which allowed us to identify the most plausible signaling pathway alterations related to H_2S's neuroprotective properties against ischemia-reperfusion injury in present study. Recognition of the intricate alterations is beneficial to the future treatment in glaucoma therapy with exogenous

H_2S, and to show how these molecules play a pivotal role in restoring retinal homeostasis against I/R injury in vivo.

3.1 Hydrogen sulfide protects retinal ganglion cells against glaucomatous injury in vitro and in vivo

In recent years the role other factors like glutamate excitotoxicity, disorganized metabolism of NO, overproduction of ROS, increased endothelin-2 levels and reduction of ocular blood flow has also been discussed to play a key role in glaucoma pathology [4] .

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, anti-apoptotic, and anti-inflammatory effects of H_2S have been found e.g. in 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 [8]. However, the role of H_2S in glaucoma remains obscure.

This part of the research focused on the expression changes of H_2S in an experimental animal model of glaucoma and second its potential neuroprotective effect on retinal ganglion cells (RGC) in different glaucoma models *in vitro* and *in* vivo by addition of the slow-releasing H_2S donor GYY4137.

Firstly, by mass spectrometry, we confirmed our hypothesis, the potential correlation between H_2S and glaucoma. To do so, IOP was inducted for 7 weeks in Sprague-Dawley rats by thermic occlusion of three episcleral veins, and pressure induced RGC loss was confirmed by anti-Brn3a immunostaining retinal wholemounts, which we repeatedly used to quantify RGCs in our further study. 3-mercaptopyruvate sulfurtransferase (3-MST) is clearly upregulated (3- fold), whilst house-keeping 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,[53] which is known to synthesize H_2S in mammalian tissue through its enzymatic action[54]. It has been reported that in brain injury, 3-MST can rapidly release H_2S on stimulation[54]. We assume that in our study, 3-MST is upregulated due to the chronically elevated IOP, which suggesting H_2S may also play an important role in pathology of glaucoma.

Meanwhile, as H_2S has been excessively studied in cardiovascular system, and its vasodilating effect is widely recognized. We tested its potential to decrease IOP by dilating episcleral veins, by applying the solution of GYY4137, a slow-releasing H2S donor, as eye-drop in normotensive, untreated Sprague-Dawley rats. But it failed to decrease IOP in normotensive Sprague-Dawley rats.

Based on the mass spectrometry result, we proceeded with applying GYY4137 to retinal explants, under high-pressure condition or oxidative stress respectively. These in vitro studies proved H_2S's does-dependent protective property against glaucomatous retinal damage in vitro, it is well- known that the effect of H_2S is depending upon the concentration. High concentrations (above 250 mmol/L) are supposed to exert toxic effects, while low ones have become regarded cell protective in recent years [55]. Furthermore, these studies also gave us optimal concerntration to carry on in further in vivo studies.

With the positive results of in vitro studies, we moved on to test its function in vivo, in two different glaucomatous animal model, two different glaucomatous injuries were induced in experimental animals, by ischemia-reperfusion injury and optic nerve crush surgery. Considering the nature of two operations, GYY4137 was injected before inducing ischemia injury and immediately after optic nerve crush. Despite the differences in the mechanisms causing RGC loss in these two experimental in vivo models, both procedures caused significant RGC loss in operated eyes, but in those eyes with intravitreal injections of GYY4137, RGC survival was significantly higher than in untreated ones.

Endogenous H_2S is produced in both vascular smooth muscle cells and endothelial cells, is involved in the regulation of many physiological processes, including the vascular tone. H_2S produced in vascular smooth muscle cells can directly regulate the vascular tone in the autocrine manner. And H_2S synthesized in endothelial cells can regulate independently of smooth muscle cells by mediators binding to endothelial cell receptors. Endothelial H_2S signalling is up-regulated in some pathological conditions including ischemia-reperfusion injury [56]. 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.

As H_2S has solid vasodilating effect, we tested it further on dilating fundus vessels, GYY4137 solution was injected intravitreously in experimental animals, which we inducted their IOP by thermic occlusion of three episcleral veins. GYY4137 was injected intravitreously after IOP was elevated for 6 weeks in animals, and fundus images were monitored by Optical Coherence Tomography (OCT) for over 3 hours after injection, and repeated again one week later (7 weeks after IOP elevated), fundus images obtained before thermic occlusion surgery served as baseline. H_2S's 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 [57].

Retinal blood flow in glaucomatous condition was restricted due to optic nerve injury and vasospasms caused by elevated IOP, enlarged vessel calibres will possibly improve the retinal perfusion and RGC survival under glaucomatous conditions in the long-term. These results present the possibility that 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. Whilst 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.

3.2 Hydrogen sulfide and β-synuclein are involved and interlinked in the ageing glaucomatous retina

Synuclein is a family of small proteins including α (SNCA), β (SNCB) and γ (SNCG) synucleins[13,14] and is involved in various neurodegenerations in the CNS. Specifically, Mutations of SNCA play a central role in Parkinson's disease pathology, and misfolding and aggregation of SNCA directly linked to microglial activation, followed by inflammation and oxidative stress resulting in neurodegeneration[17]. Synucleins are also present in the retina and optic nerve[18] and are associated with glaucomatous alterations in the optic nerve[19]. SNCA autoantibody was found to be downregulated in serum and upregulated in aqueous humor of glaucoma patients [20] and intravitreal injection of SNCA antibodies is found to be neuroprotective in glaucoma animal model[21].Because of the pivotal role of SNCA in neurodegeneration

in CNS. But its role in retina or glaucoma, as well as SNCB and SNCG's roles and functions in glaucoma is still sparse and remain to be thoroughly explored.

As synucleins and H_2S are involved in several mutual pathophysiological processes, such as microglia activation, p53-mediated apoptosis, inflammatory response and free radical reactions. In second part of this doctoral thesis, we explore the correlation between H_2S and synucleins in order to better understand the mechanism H_2S's neuroprotective properties in experimental glaucoma.

In first part of the research, we chronically elevated IOP in adult female Sprague-Dawley rats for 7 weeks by cauterization of 3 episcleral veins. The IOP of all operated eyes increased significantly three weeks after the episcleral vein occlusion(EVO). Subsequent loss of RGCs is in agreement with thinning of the retinal nerve fiber layer, which suggests that EVO is a sufficient glaucoma model. As predominantly elder people are affected in glaucoma, older animals also showed a higher susceptibility to IOP elevation resulting in significant loss of RGCs and retinal nerve fiber layer (RNFL) thickness, while younger animals seemed to show resistance against mildly elevated IOP.

Secondly, we employed label-free quantification process following LC-ESI-LTQ-Orbitrap mass spectrometry to measure the abundance of synucleins in the retina. We found that SNCB has significantly more abundant in retina than other family members, furthermore, its level is significantly altered due to aging and elevated IOP, while the other two family members didn't show noticeable changes, which indicates that SNCB might have a more pivotal role to play in neurodegenerations in retina than other family members.

In physiological aging, the abundance of SNCB declines, which is correlated with the decreased RGC density and increased susceptibility to IOP. As under physiological conditions, SNCB is thought to be neuroprotective by functioning as a physiological inhibitor of SNCA and behaving as anti-aggregating agents. Studies in autopsy brains of PD, dementia with Lewy bodies and AD suggest that decreased amount of SNCB may lead to relative loss of protective functions of SNCB against neurotoxicity caused by SNCA[58]. Furthermore, downregulation of SNCB could occur not only in aggregation of SNCA, but also in other types of neurodegenerative disease[59].

SNCB's downregulation with aging increases RGC's susceptibility to glaucomatous assaults secondary to elevated IOP, such as elevated mechanic stress, insufficient retinal perfusion, and increased oxidative stress.

Under pathological conditions—chronic elevated IOP, SNCB in juvenile animals is downregulated, and the downregulation of SNCB is correlated with reduced RGC loss. While in aged animals, there is no significant alteration of SNCB in respond to assault, but more significant RGC loss.

According to mass spectrometry results, the amount of 3MST in rat retina is not significantly altered through the process of aging. In juvenile animals, 3MST was significantly upregulated in retina when exposed to elevated IOP over a period of 7 weeks, while no significant change is observed in old animals. Associating with the data from immunofluorescence staining of RGC, it suggests that upregulation of 3MST, a key H_2S producing enzyme, is correlated with reduced RGC loss induced by elevated IOP. The self-regulation of H_2S is decreased with aging. Therefore, we assume that downregulating SNCB and upregulating endogenous H_2S level are neuroprotective against elevated IOP, and the function of regulating them is weakened with aging, which renders RGC's vulnerability.

Furthermore, acute IOP elevation is induced in juvenile animals, which led to significant RGC loss and SNCB downregulation. Although juvenile animals are more resilient than old animals to mildly elevated IOP (ca. 18mmHg), but when it reaches a threshold, acute IOP elevation (IOP ca. 55mmHg) led to a significant RGC loss. Downregulation of SNCB might be therefore a self-protective mechanism presenting from the beginning of the IOP elevation, but an exhaustion of the functional reserve eventually led to RGC loss.

Our data on SNCB abundance and RGC loss agrees with recent studies in retina, showing that the protective property of SNCB is exerted in a dose-dependent manner[13], which means overexpression and accumulation of SNCB increases oxidative stress and inflammatory responses, and furthermore promote the apoptosis, while lower concentrations of SNCB shows anti-apoptotic effect[26,60]. In various aspects of neurodegeneration, accumulation of SNCB is present, such as in dystrophic neurites in the hippocampal region in brains from PD and DLB patients, which suggest that accumulation of SNCB is involved in the axonal pathology[29]. SNCB was found to form toxic cytosolic inclusions in a similar manner to SNCA, and shares similar

toxicity mechanisms, including vesicular trafficking impairment and induction of oxidative stress[61]. Overexpression of SNCB in cultured primary cortical neurons led to cell loss and signs of metabolic impairment, in a similar manner to overexpressing SNCA neurons[62].

Treatments targeting SNCA to reduce its levels and toxicity have shown positive results in rescuing neuronal cells and halting the neurodegeneration process in preclinical studies[59,63]. For example, in our previous study, intravitreal injection of SNCA antibodies is found to be neuroprotective in glaucoma animal model [21].

Thus, it is reasonable to target the pathogenic SNCB and to decrease the intracellular SNCB as novel strategies for therapeutic intervention in neurodegeneration. Removal of pathogenic SNCB or reduce its abundancy may be effective to rescue neuron and halt the progression of glaucoma.

H_2S has shown profound involvement in various retinal neuropathy processes, in previous studies by different groups including us, exogenous donors exhibited therapeutic potential in conditions of several retinal diseases[31,51]. The underlying mechanism, through which H_2S exerts its neuroprotection, was partly attributed to its capability of vasorelaxation, anti-oxidative stress, neuroendocrine regulation, and inflammation suppression[10-12]. Nevertheless, H_2S is involved in several mutual pathophysiological processes with SNCB[5,31-33].

Quantification of Brn3a positive RGCs showed that administration of exogenous H_2S correlated positively with RGC survival improvement in acute IOP elevation. The mass spectrometric–assisted proteomics analysis of the retinal tissue demonstrated that administration of H_2S also further downregulated SNCB.

Overall, the work described in this part of the dissertation suggested that downregulating SNCB partly contributes to the neuroprotection by H_2S under glaucomatous condition. The extent to which internal mechanism and/or inflammatory factors, signalling pathways or the disruption of vascular function participate in the process is to be elucidated.

3.3 Proteomics reveals the potential protective mechanism of hydrogen sulfide on retinal ganglion cells in an ischemia/reperfusion injury animal model

Ever since H_2S has been recognized as the third endogenous gaseous signalling molecule and its role in various physiological and pathological processes has been explored. Building on previous results, we employed a mass spectrometry (MS)-based proteomics approach to analyze the retinal proteome, and bio-informatics to algorithmically generate protein connections, which allowed us to identify the most plausible signalling pathway alterations related to H_2S's neuroprotective properties against ischemia-reperfusion injury in present study. Addressing the intricate alterations at the protein level can be attributed to the treatment with exogenous H_2S, and to show how these molecules play a pivotal role in restoring retinal homeostasis against I/R injury in vivo.

We elevated the IOP of the Spargue-Dawley to 55mmHg for 60min to induce regional ischemic injury, followed by 24 hours of reperfusion. Compared to the contralateral control, I/R injury resulted in significant reduction in the number of RGC in the operated eyes, injection of GYY4137 prior to I/R injury significantly improved RGC survival.

MS-based discovery proteomics was utilized to identify and distinguish proteome profiles of rat retina undergoing I/R injury with or without pre-treatment of H_2S. The overview of sampling and proteomics workflow used in this study is presented in Figure 1.

In total 1115 retinal proteins were identified with a false discovery rate (FDR) of 1%. Following I/R injury, the abundance of 48 proteins was significantly altered. Changes of the signaling pathways involved in mitochondrial homeostasis and function, calcium dysregulation, cytotoxicity regulation, ROS scavenging, neural transduction and vascular function were found. The proteins, which were regulated by H_2S were categorized into two clusters: 1) a cluster 18 proteins, the abundance of which was significantly altered in I/R, and was then restored to normal or near normal level by H_2S; 2) a cluster of 11 proteins, the abundance of which was significantly altered due to H_2S administration compared to the CTRL group. Based on the results from the ingenuity pathway analysis, the signaling pathways regulated by H_2S comprised iron homeostasis and ROS regulation, mitochondrial homeostasis and function, vasodilation and DNA repairing.

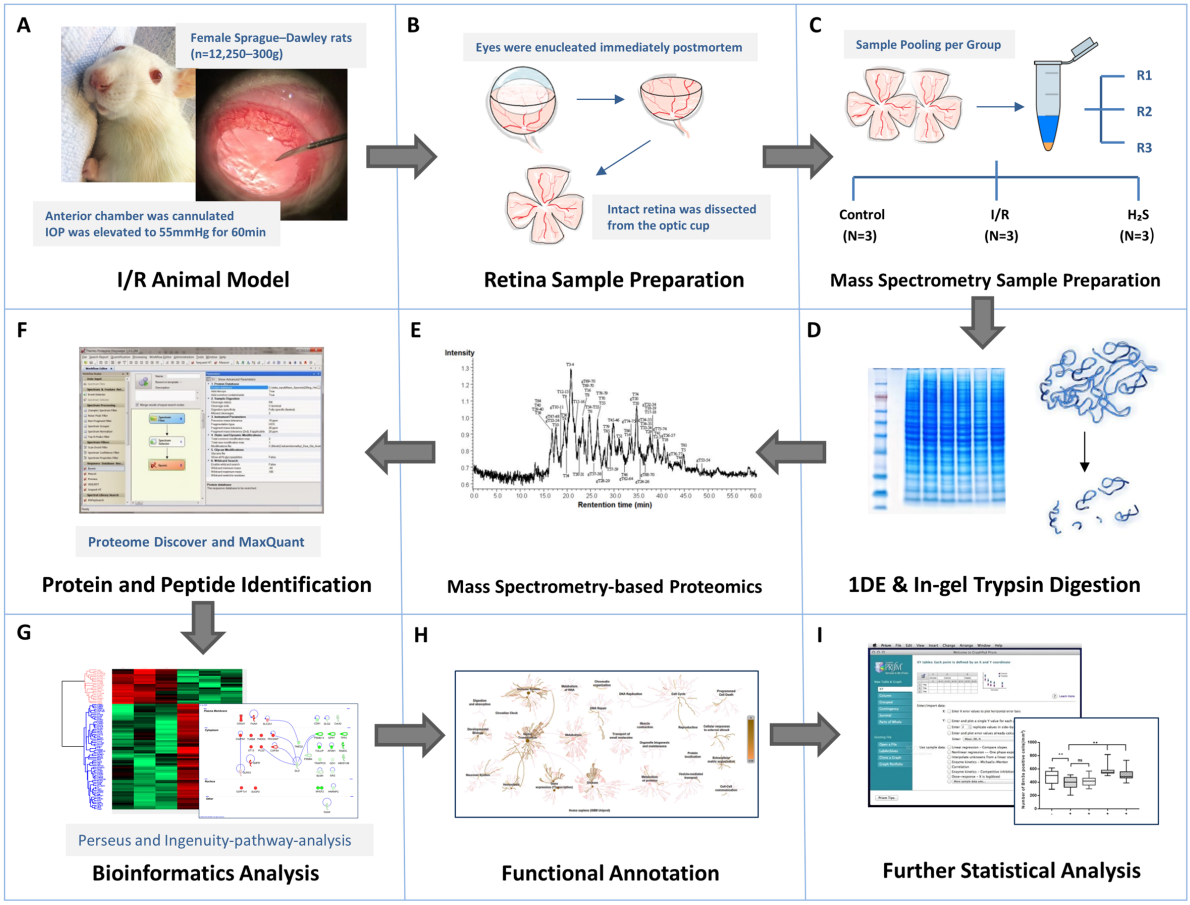

Figure 1. Workflow overview. I/R injury was induced in the left eyes of adult female Sprague-Dawley rats (n=12), six of which received intravitreal injection of GYY4137, a H2S slow-releasing precursor, shortly before intervention. Animals were executed 24h after intervention, retinae were harvested immediately postmortem. Retinae from contralateral eyes were designated as controls. Retinal samples were immediately weighed and lysed by T-PER Tissue Protein Extraction Reagent and Bullet Blender Storm. Six samples of retinal protein from respective groups were pooled equally into three biological replicates after protein measurements, represented by RI, R2 and R3, and subsequently subjected to first dimensional gel electrophoresis. The protein bands were sliced and digested by trypsin prior to proteomic analysis by LC - ESI - MS/MS. The emerging datasets were subjected to robust bioinformatics analyses and functional annotations to identify the differential protein expressions and protein interaction networks.

3.3.1 Changes in Iron Homeostasis and ROS Regulation

Redox mechanisms are known to partially contribute to the protective properties of H_2S in various tissues. Firstly, we observed that *Slc2a1*level is significantly downregulated in I/R and restored by H_2S. *Slc2a1* facilitates docosahexaenoic acid (DHA), the oxidized form of vitamin C, transport across the inner blood-retina barrier and convert DHA to ascorbic acid and accumulates as ascorbic acid in the retina [64]. As *Slc2a1*

level is downregulated following I/R injury, Vitamin-C transport is downregulated accordingly. Vitamin C is the primary circulatory antioxidant to be quickly used and depleted when excessive ROS is generated by the pathophysiological pathways triggered by I/R injury, therefore sparing other endogenous antioxidants[65]. Oxidative stress promotes the oxidation of ascorbic acid to DHA, which is shown by studies to promote neuronal death under oxidative stress [66]. Downregulated Vitamin-C transport indicates that I/R injury impaired anti-oxidative property of the retina. H_2S increased Vitamin-C transport, subsequently strengthening the anti-oxidative property of the retina as opposed to the ROS generated due to I/R.

Secondly, several proteins involved in iron homeostasis, such as biliverdin reductase A (*Blvra*), *Cyb5r3* and Glrx3, were differentially abundant due to I/R and/or H_2S administration. Iron metabolism and regulation is crucial in mammals and is essential for physiological neuronal functions such as neural respiration and metabolic activities, myelin synthesis, neurotransmitter production and synaptic plasticity[67]. However its overload triggers axonal degeneration and neuronal cell death[68]. Excessive iron may catalyze the formation of highly reactive hydroxyl radicals, which eventually induce the accumulation of ROS [69,70]. Nevertheless, it is well known that the disproportionally increased brain Fe level is correlated with neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease and neurodegeneration with brain Fe accumulation.

Most of the body Fe is contained within the protoporphyrin ring of heme [70]. Heme is essential for the survival of organisms, while being potentially toxic. The excessive release of heme from hemoproteins can lead to the generation of unfettered oxidative stress and programmed cell death [67]. Heme degradation is therefore important for maintaining iron homeostasis and preventing its cytotoxicity and oxidative stress [71].

The abundance of *Blvra*, the main isoform of biliverdin reductase [72], is significantly increased due to I/R injury. Increased *Blvra* level corresponds to upregulated heme degradation.

Blvra is a pleiotropic enzyme primarily known for reducing heme-derived biliverdin into the powerful antioxidant and anti-nitrosative molecule bilirubin [73,74]. Bilirubin is able to inhibit free radical chain reactions and protects against oxidant-induced damage [75]. Studies reported that reducing *Blvra* level in cell or knockout *Blvra* gene in mice both led to increased oxidative stress [75,76]. On the other hand, increasing the levels

of *Blvra* is beneficial through an increase in intracellular bilirubin generation and direct signaling through cytoprotective pathways [72,77,78].

Cyb5r3, the principal reductase in mitochondria, is also significantly upregulated due to I/R injury, and can be restored by H_2S. *Cyb5r3* is also a heme reductase and recycles oxidized heme (Fe3+) back to its reduced form (Fe2+) [79]. Upregulation of *Blvra* and *Cyb5r3* level following I/R injury is likely to confer self-protection against oxidative stress.

Glrx3 level was the most significantly modulated by H_2S when compared to the control group, while it was not impacted by I/R injury. Depletion of *Glrx3* in mammalian cells was associated with moderate deficiencies of cytosolic Fe-S cluster enzymes and evidence of altered iron homeostasis [80]. Overexpressed *Glrx3* can compensate for the lack of other reducing equivalents [81].

Glrx3 belongs to the glutaredoxin family, it utilizes the reducing power of glutathione to maintain and regulate the cellular redox state and is essential for iron-sulfur (Fe-S) cluster assembly [82,83]. Iron-sulfur clusters are ancient, ubiquitous cofactors composed of iron and inorganic sulfur which participate in numerous biological processes [84-87].

In mammalian cells, *Glrx3* localizes to the cytosol and it has been proposed that it plays dual roles in iron trafficking and regulation [88,89]. *Glrx3* is a monothiol glutaredoxin, being able to form [2Fe-2S] cluster-bridged dimers and deliver Fe-S clusters to recipient proteins[90,91].

Detoxification of neural Fe overload is a potential therapeutic approach in the treatment of neurodegenerative disease. Based on the results of proteomic and IPA analysis, we assume that, when exposed to oxidative stress, cells increase their capability to reduce oxidized heme and to degrade it, which partially contributes to a self-protective mechanism that reduces the deleterious effects of free heme. H_2S plays a similar reducing role as *Cyb5r3* in recycling oxidized heme to its reduced state; the abundance of *Cyb5r3* was thus not increased when H_2S was present. Nevertheless, H_2S has shown a strong association with *Glrx3*, which is able to regulate cellular iron homeostasis [92,93].

3.3.2 Changes in Retinal Metabolism, Mitochondrial Homeostasis and Function

As mentioned above, the abundance of *Slc2a1* is significantly downregulated in I/R and restored by H_2S to near normal level, which also corresponds to increased HIF1α signaling. *Slc2a1* abundance in HIF1α signaling plays a key role in promoting neuronal survival by mediating the endogenous protective responses after hypoxia-ischemia. In glaucoma mice, decreased HIF1α expression is observed to be correlated with RGC loss[94]. H_2S regulates HIF1 factors in different patterns, depending on different cell type and experimental conditions. It increases expression of the HIF1α in rat brain capillary endothelial cells and in mouse spinal-cord primary culture [95,96], and inhibits HIF1 activation in human hepatoma Hep3B cells, cervical carcinoma HeLa cells, and aortic smooth-muscle cells [97,98].

Our proteomic data indicate that H_2S administration increased HIF1α signaling. Upregulating expression of HIF1α induces an increase of aerobic glycolysis, which transforms glucose to lactate and generates nicotinamide adenine dinucleotide (NAD+) [99]. HIF1 also directly induces pyruvate dehydrogenase kinase 1 [100], which phosphorylates the pyruvate dehydrogenase (PDH) complex, and consequently inhibits PDH complex from catalyzing pyruvate to form acetyl CoA. This fuels the mitochondrial tricarboxylic acid cycle (TCA) cycle, which provides the mitochondrial NADH needed to power electron transport. Hence, HIF1α actively represses mitochondrial respiration.

Furthermore, in this study, H_2S reduced the abundance of NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5 (*Ndufa5*), which subsequently also decreased mitochondrial respiration. *Ndufa5*, localizes to the inner mitochondrial membrane and is an accessory sub-unit of complex I [101]. Reducing *Ndufa5* suppresses complex I activity [102,103]. It is generally believed, that mitochondrial complex I deficiency contributes to the process of neurodegeneration because of reduced adenosine triphosphate (ATP) production, oxygen consumption and increased ROS production. However, complex I has been reported as a site of mitochondrial ROS generation [104,105], and actively suppressing its activity may actually be protective. Neuronal complex I deficiency is not correlated with the neurodegeneration, mitochondrial DNA damage in Parkinson's disease brain, nor in mice with a central nervous system knockout of *Ndufa5* [102,106]. It has been shown in neurodegenerative disorders that reversible inhibition of complex I can be protective against ischemia-reperfusion injury by modifying mitochondrial ROS production [107,108].

Studies strongly suggest that active suppression of the TCA cycle, mitochondrial respiration and ROS production, as well as augmentation of ATP levels, are crucial for the cell survival under I/R injury [100,109]. We assume that by suppressing mitochondrial respiration through upregulating HIF1-α and downregulating *Ndufa5* levels, H_2S reduces mitochondrial oxidative phosphorylation, thereby repressing mitochondrial oxygen consumption and resulting in the relative increase of intracellular oxygen tension and limited ROS production under I/R.

As discussed above, mitochondrial PDH activity is attenuated by HIF1α following H_2S administration. Neurons usually prefer glucose for energy, but when mitochondrial metabolism of pyruvate is limited, ketone bodies can be oxidized and release the only alternative source of acetyl CoA for neurons [110]. Using ketone bodies as an alternative energy source has shown neuroprotective effects in Alzheimer's disease, Parkinson's disease and ischemic and traumatic brain injury [111-116]. The metabolic pathway that produces ketone bodies is ketogenesis. In this study, the signaling pathways most significantly downregulated by I/R injury and then restored by H_2S were ketogenesis, ketolysis and leucine degradation.

In comparison to glucose, ketone bodies have a higher inherent energy[117]. As ketone bodies provide more energy per unit of oxygen than glucose [118,119], in states of metabolic stress such as I/R injury, ketone bodies are more energy-efficient fuel for neurons. They can also bypass the inhibited PDH complex and maintain the metabolites of TCA cycle, therefore permitted continued ATP production under ischemia.

Administration of H_2S has shown evidence of actively suppressing oxidative phosphorylation and limiting the utilization of glucose as the energy source, therefore increasing intracellular oxygen tension under ischemia and limiting ROS production during reperfusion. Furthermore, H_2S promotes the utilization of ketone bodies as an alternative energy source, which is more energy-efficient than glucose and maintains ATP production. H_2S therefore enhanced ability of retinal neurons to withstand metabolic stress induced by I/R, which would normally deplete the resilience of the neurons and result in neuronal cell loss (see in Figure 2).

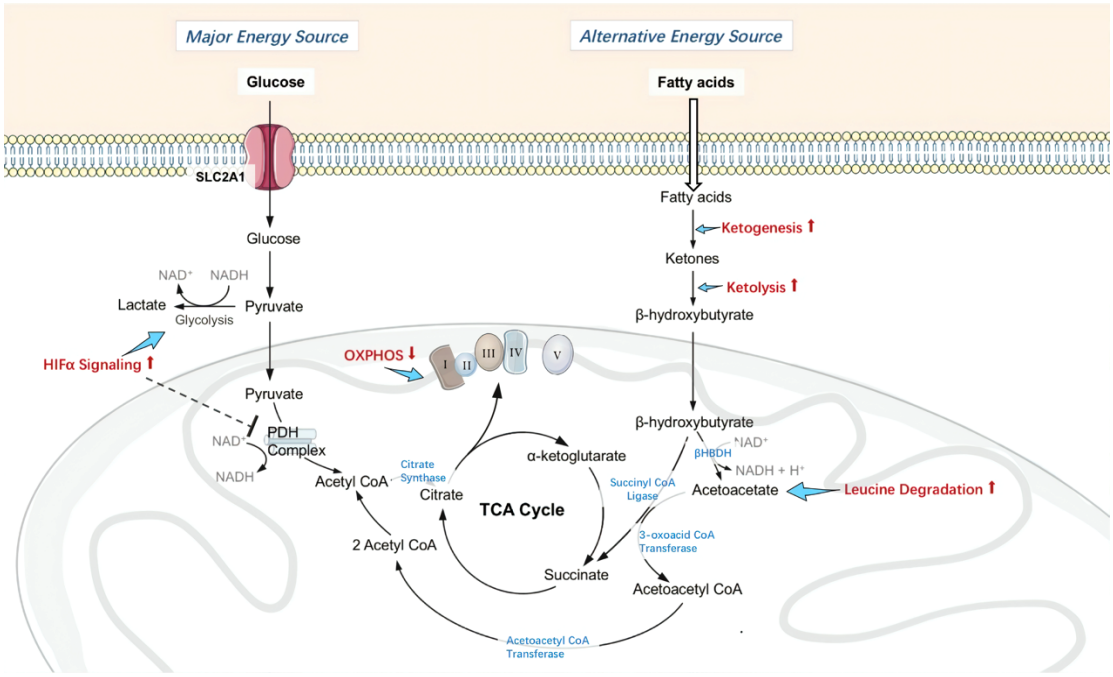

Figure 2. Changes in retinal metabolism, mitochondrial homeostasis and function. Administration of H_2S actively inhibited pyruvate dehydrogenase (PDH) complex activity by upregulating HIF1α signaling, therefore limited using glucose as energy source, and suppressed oxidative phosphorylation by inhibiting Complex I activity, consequently increased intracellular oxygen tension under ischemia and limited ROS production during reperfusion. Furthermore, H_2S promotes the utilization of ketone bodies as an alternative energy source, which is more energy-efficient than glucose, thus permitted continuous adenosine triphosphate (ATP) production. H_2S enhanced the ability of retinal neurons to withstand metabolic stress induced by I/R, therefore less neuronal cell loss.

3.3.3 Changes in Retinal Vascular Function

Decreased average blood flow in the retinal, optic nerve head and choroidal circulations is also demonstrated in glaucoma patients [120-122]. The “vascular theory” of glaucoma hypothesizes RGC loss as a consequence of insufficient blood supply [123]. Vasospasm and autoregulatory dysfunction have been postulated to reduce ocular blood flow[124]. Increased IOP has also been shown to reduce vascular caliber in rat retina while H_2S acted as a vasodilator in our previous studies [51]. H_2S can be endogenously generated in vascular smooth muscle cells. Its vasorelaxant potency is partially mediated by a functional endothelium [125].

However, I/R is known to cause endothelial dysfunction. In this study, eNOS signaling is downregulated following I/R injury. Endothelium-derived NO is a critical regulator of vascular homeostasis and tone [126]. NO continually regulates the diameter of blood vessels and maintains an anti-apoptotic environment in the vessel wall[127].

Downregulation of eNOS signaling is an indicator of impaired endothelial function, which results in diminished microcirculation and reduced response to endothelial-dependent vasodilators and vasoconstrictors. This causes dysregulated blood flow and loss of the endothelial barrier.

While eNOS signaling is downregulated, protein kinase A (PKA) signaling is significantly upregulated following I/R. PKA represents a signaling hub for a large variety of hormones, neurotransmitters and cytokines [128]. PKA has been shown to regulate different aspects of endothelial cell physiology [129-131], and to inhibit angiogenesis when activated [132,133]. Upregulation of PKA is likely a self-protective mechanism.

Furthermore, one of the most significantly upregulated proteins I/R, which can be restored by H_2S to normal level, is *Cyb5r3*. It is the principal reductase involved in the mitochondrial amidoxime reducing component (mARC)-containing enzyme system [134]. In vascular smooth muscle, *Cyb5r3* functions as a soluble guanylyl cyclas (sGC) heme iron reductase, and is critical for vasodilation [135]. Through its reductase activity, *Cyb5r3* maintains NO-sGC-cGMP function [136]. The NO-sGC-cGMP pathway in aortic smooth muscle cell (SMC) is known to relax SMC and dilate vessels [136].

Hydrogen sulfide and the −SH anion reduce a variety of organic substrates [137]. It is reasonable to assume that H_2S plays a similar reducing role as *Cyb5r3* in maintaining vascular relaxation and enable a less constricted vascular environment due to I/R.

Although the abundance of *DDX5* was not influenced by I/R injury, it is significantly more abundant following the administration of H_2S. As *DDX5* regulates the expression of a number of key regulators upstream of the estrogen-receptor [138], estrogen-receptor signaling was also significantly upregulated in our PPI results.

Increasing evidence suggests that estrogen exposure may have a neuroprotective effect on the progression of glaucoma and may alter its pathogenesis [139]. The vasodilation caused by estrogen and its effects on aqueous humor outflow may contribute [140]. These estrogen receptors are abundantly expressed throughout the eye, and in particular the retina and more specifically in the RGC [141,142]. In various CNS injuries and diseases, the activation of estrogen receptor signaling has shown neuroprotective effects via attenuation of neuro-inflammation and neurodegeneration

[143]. In different animal models of RGC degeneration, activation of estrogen receptor attenuated RGC apoptosis under acute elevated IOP [144]; impaired estrogen receptor expression is observed in a mouse model with early RGC apoptosis [145]; and in Leber's hereditary optic neuropathy cells activating estrogen receptors improve cell viability by reducing apoptosis [146]. Moreover, inhibition of estrogen synthesis is associated with IOP elevation and reduced RGC counts in female mice [147] and blockage of estrogen receptor signaling in rats intensifies impairment in visual function and retinal structure after ocular hypertension [148]. Although estrogen receptor signaling was not impacted by I/R injury in our study, activating estrogen receptor signaling via upregulating *DDX5* is still one of the possible pathways through which H_2S exerts protective effects and partially contributes to its capability of vasorelaxation, anti-oxidative stress and inflammation suppression.

H_2S also significantly downregulated annexin A6 compared to the control group, which is a calcium dependent phospholipid binding protein [149].

Annexins are important in Ca(2+)-induced neurotoxicity or neuroprotection[150]. Annexin A6 has been shown to play a central role in vascular remodelling processes[151]. It has been demonstrated that annexin A6 accumulates and converts exosomes in vascular smooth muscle cells into calcifications under calcium stress, which is known to be induced by I/R[151]. Vascular smooth muscle cells contribute significantly to physiological regulation of vascular tone and arterial blood pressure [152]. The presence of coronary artery calcification is significantly associated with raised IOP regardless of conventional cardiovascular risk factors [153]. By reducing the abundance of annexin A6, H_2S potentially makes the calcification in vascular smooth muscle cells subside due to I/R, thus maintaining vascular autoregulation.

PKA signaling and *Cyb5r3* level is upregulated, likely serving as a self-protective mechanism to maintain endothelial function and vasodilation against I/R injury, while eNOS signaling is downregulated as an indication of endothelial dysfunction. Administration of H_2S regulated the abundance of *Cyb5r3* and activated estrogen receptor signaling to facilitate vascular relaxation. H_2S also reduced the abundance of annexin A6, which plays a central role in artery calcification. Combined together, H_2S enabled a less constricted vascular environment in the retina, thereby resulting in better retinal perfusion to counteract I/R injury (see Figure 3).

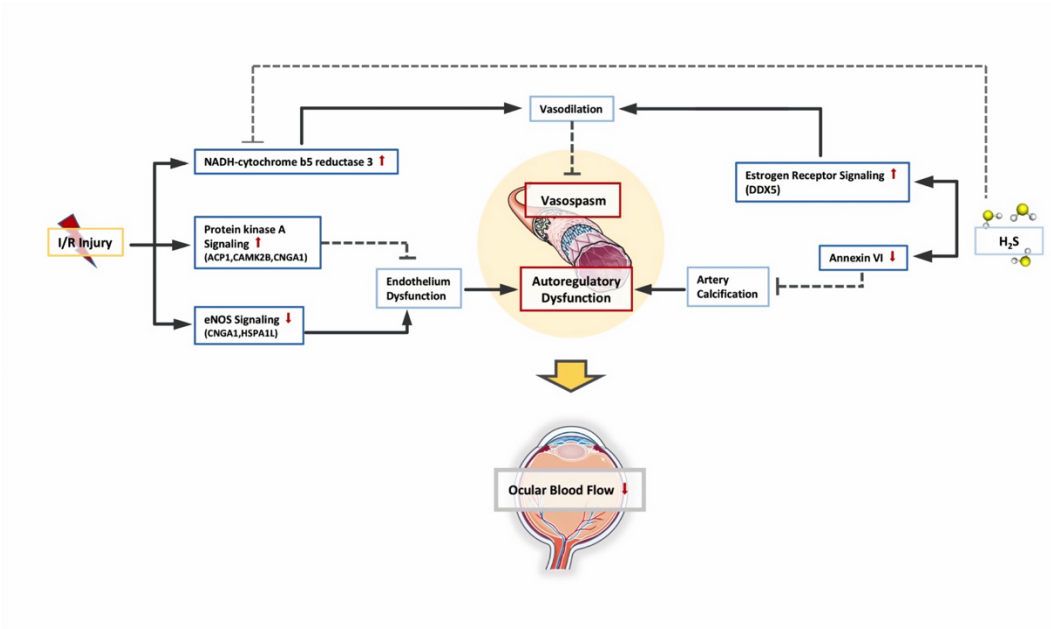

Figure 3. Changes in retinal vascular function. Due to I/R, PKA (protein kinase A) signaling and NADH-cytochrome b5 reductase 3(*Cyb5r3*) is upregulated as self-protective mechanism to maintain endothelial function and vasodilation, while eNOS signaling is downregulated as an indication of endothelial dysfunction. Administration of H_2S activated estrogen receptor signaling to facilitate vascular relaxation. H_2S also reduced the abundance of annexin A6, which plays a central role in artery calcification. Combined together, H_2S protected the blood flow regulatory mechanisms and enabled a less constricted vascular environment in retina against I/R injury.

3.3.4 Changes in GABA Receptor Signaling

GABA receptor signaling is the most impacted signaling pathway by I/R and this can be restored by H_2S. The *AP2M1* and *SLC32A1* levels are upregulated due to I/R, which correspond to upregulated GABA receptor signaling. Gama-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian brain[154]. In the retina, GABA is similarly important in neural inhibition with approximately 40% of all retinal cells having been described as GABAergic [155]. GABA-containing vesicles release the neurotransmitter into the synaptic cleft to bind with GABA receptors present on the post-synaptic neuron [156].

SLC32A1, solute carrier family 32 (GABA vesicular transporter) member 1, is a transmembrane transporter which is responsible for the storage of GABA or glycine in synaptic vesicles [157]. *AP2M1* is highly expressed in the central nervous system [158,159], and encodes the μ-subunit of the adaptor protein complex 2 (*AP-2*) [160]. AP-2 regulates the neuronal surface levels of GABA and glutamate receptors [161,162]. An imbalance between excitatory and inhibitory synaptic transmission is

thought to contribute to excitotoxicity and neuronal cell death during ischemic insult. AP-2 was found to be crucial in reducing the loss of synaptic GABA receptors during simulated ischemia in the rat brain [163]. Increased abundance of *SLC32A1* and *AP2M1* suggests raised retinal GABA levels following I/R injury, which may occur in order to maintain excitatory/inhibitory balance, thereby improving the survival of RGCs, the latter being most susceptible to extracellular glutamate.

However, similar to glutamate, the functions of GABA are also controversial. There is evidence that the retina is extremely sensitive to abnormally accumulated GABA and its resulting toxicity, particularly in the presence of ischemia [164-166]. An antiepileptic drug, vigabatrin (Gama-vinyl GABA), exerts its effect by increasing GABA levels [167]; as the drug increases retinal GABA levels much more significantly than in the brain and retina has a lower tolerance to GABA toxicity than brain [165,166], one third of patients receiving vigabatrin present binasal visual field loss [168].

GABA also has a role in the regulation of vascular tone [165]. GABA receptors are known to interact with perivascular astrocytes which synthesise vasoactive materials [169,170]. In the rat retina, GABA is shown to be capable of eliciting both vasodilator and vasoconstrictor responses but endogenous GABA is unlikely to be an important regulator of resting vascular diameter and blood flow in the retina [171].

The exact role of GABA receptor signaling in this study is unclear but the results certainly raise intriguing questions about the involvement of GABA receptor signaling in neurodegeneration and the interaction between GABA signaling and H_2S.

3.3.5 Changes in DNA Repair

The nucleotide excision repair (NER) pathway is at the top of the canonical pathways modulated by H_2S, with a particularly significant upregulation of *COPS6* level. In combination with the ubiquitin (Ub) proteasome system (UPS) the COP9 signalosome controls the stability of cellular regulators [172] and regulates several important intracellular pathways, including DNA repair, cell cycle, developmental changes, and some aspects of immune responses [173-175].

COPS6 has been shown to be cleaved by caspase 3 during apoptosis in vitro and in vivo [176] and this cleavage can be completely blocked by specific caspase 8 inhibitor [177]. Upregulation of *COPS6* led to upregulation of the NER pathway, one of the major

cellular DNA repair pathways for the removal of bulky helix lesions [178,179]. Enhancement of the NER pathway has shown protective effects in peripheral neurons both in vitro and in vivo[180]. The roles of DNA repair in neuronal cell survival and the response to aging and ROS such as that generated by mitochondrial respiration in glaucoma is of particular interest. The promotion of DNA repair by activation of the NER pathway through COP9 Signalosome Subunit upregulation could be an interesting aspect to explore in future studies.

Neurodegenerative diseases are characterized by decades of apparent normality, during which local deficits are compensated. Eventually the deficits become too accentuated and/or the compensatory mechanisms fail[181]. From the results we obtained in this study, I/R injury led to changes in ROS regulation, retinal metabolism, mitochondrial homeostasis and function, retinal vascular function and metal homeostasis, with over half of the altered pathways in the I/R group being self-protective mechanisms. H_2S provided additional support where self-protective mechanisms fell short and demonstrated DNA repair properties, eventually providing neurons a better outcome against I/R injury.

3.4 Critical discussion

Admittedly there are limitations in this study. There are limitations of the study. Unfortunately, there is no proper glaucoma model, neither *in vitro* nor *in vivo*. Therefore, in the first part of this thesis, we used very different kind 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. But however within the framework of those limitations mentioned, we could see neuroprotective properties and vasodilatative effects, which are in accordance with previous findings.

Furthermore, in the second part of this doctoral thesis (manuscript II) we could confirm that downregulating SNCB partly contributes to the neuroprotection by H_2S under glaucomatous condition. However, the extent to which internal mechanism and/or inflammatory factors, signaling pathways or the disruption of vascular function participate in the process is to be elucidated

The last part of this theses provides for the first time an overall insight into the mainstay retinal proteins and pivotal signaling pathways that interact with H2S to maintain retinal homeostasis against I/R injury.

Firstly, the results attained from the ischemia/reperfusion model are not necessarily valid in other glaucomatous models. There are various experimental models of glaucoma in the marketplace; each of them has specific pros and cons. It seems that degeneration caused by chronic elevated IOP mimics the characteristics of glaucoma better than I/R model. Clinical observations have demonstrated retinal vascular abnormalities and impaired blood flow at the optic nerve head which suggest ischemia plays a key role in the pathogenesis of glaucoma[182,183]. A pure ischemic lesion certainly cannot represent the glaucomatous damage, reperfusion injury is also present in glaucoma patients [184]. It is evident that IOP fluctuations are more damaging than a stabled increased IOP, and reduced circulation due to vascular dysregulation is more damaging than reduced circulation due to arteriosclerosis[185].

Furthermore, individual retinal samples were deliberately pooled into biological replicates to minimize inter-individual variations [186,187]. Due to the limitation of sample material and the variety of the signaling pathways H_2S is involved in, the differentially expressed proteins could not be confirmed by a second technique in the present study design.

4 Conclusion and outlook

The main objective of the present doctoral thesis was to evaluate the potential roles of H_2S against glaucomatous injuries in retina and to unravel the underlying mechanisms of its neuroprotective effects. To answer these questions, we firstly employeed a state-of-the-art LC-MS-based proteomic approach to analyse the expression changes of H_2S in an experimental animal model of glaucoma and secondly H_2S's potential neuroprotective effect on RGC towards elevated pressure in glaucoma models in vitro and in vivo by addition of the slow-releasing H_2S donor GYY4137(manuscript I). As second part of the thesis, we elucidate the potential roles and functions of synucleins in glaucomatous neuropathy, following this, to investigate the potential of H_2S to regulate it, and to better understanding the mechanism of H_2S in neuroprotection (manuscript II). As the final part oft he thesis, a mass spectrometry-based proteomics approach was employeed to analyze the retinal proteome, and bio-informatics to algorithmically generate protein connections, which allowed us to identify the most plausible signaling pathway alterations related to H_2S's neuroprotective properties against ischemia-reperfusion injury (manuscript III).

In the first part of the thesis, the results present the possibility that H_2S plays a role in the pathology of glaucoma. And it exerts protective effect against glaucomatous injuries in different glaucoma animal models in vitro and in vivo. Furthermore, H_2S may exert its protective effect in glaucoma, by stabilizing ocular perfusion and easing ischemia-reperfusion injury. Whilst 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.

Based on the previous results, in the second part of the doctoral thesis, we explore the underlying mechanism of H_2S's neuroprotective property in relation with synucleins. Our data demonstrated that SNCB's downregulation with aging increases RGC's susceptibility to glaucomatous assaults secondary to elevated IOP, such as elevated mechanic stress, insufficient retinal perfusion, and increased oxidative stress.

Under pathological conditions—chronic elevated IOP, SNCB in juvenile animals is downregulated, and the downregulation of SNCB is correlated with reduced RGC loss. While in aged animals, there is no significant alteration of SNCB in respond to assault, but more significant RGC loss.

The self-regulation of H_2S is decreased with aging. Therefore, we assume that downregulating SNCB and upregulating endogenous H_2S level are neuroprotective against elevated IOP, and the function of regulating them is weakened with aging, which renders RGC's vulnerability.

Our data on SNCB abundance and RGC loss agrees with recent studies in retina, showing that the protective property of SNCB is exerted in a dose-dependent manner[13], which means overexpression and accumulation of SNCB increases oxidative stress and inflammatory responses, and furthermore promote the apoptosis, while lower concentrations of SNCB shows anti-apoptotic effect[26,60]. In various aspects of neurodegeneration, accumulation of SNCB is present.

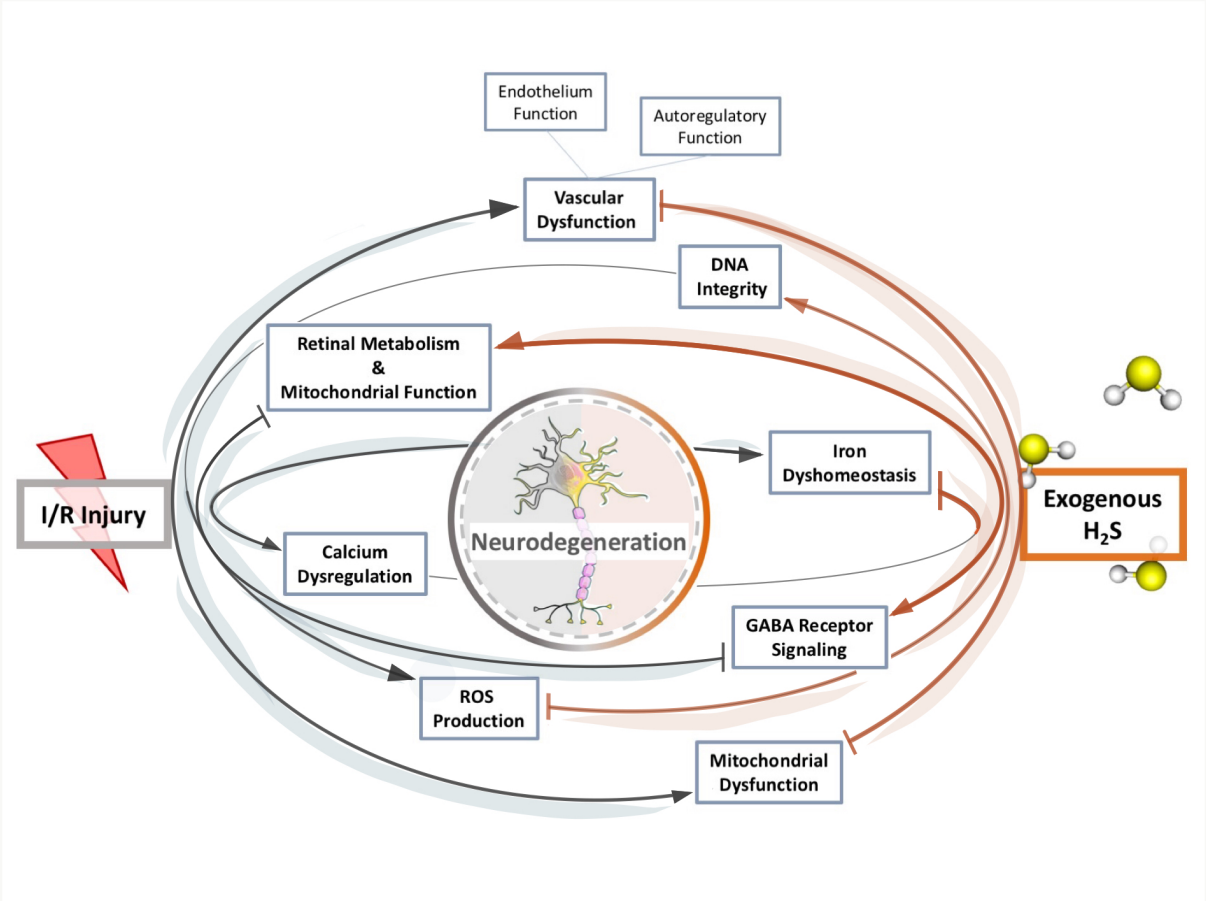

Figure 4. Overview of altered signalling pathways due to I/R injury and H_2S treatment.

It is reasonable to target the pathogenic SNCB and to decrease the intracellular SNCB as novel strategies for therapeutic intervention in neurodegeneration. Removal of pathogenic SNCB or reduce its abundancy may be effective to rescue neuron and halt the progression of glaucoma.

Overall, this part of the thesis suggested that downregulating SNCB partly contributes to the neuroprotection by H_2S under glaucomatous condition. The extent to which internal mechanism and/or inflammatory factors, signalling pathways or the disruption of vascular function participate in the process is to be elucidated.

As final part of the present doctoral thesis, a state-of-the-art LC-MS-based proteomic approach is employeed to provide a thorough overview of the retina proteome changes related to neuroprotective properties of H_2S in retina, the main focus was to acquire a comprehensive perspective of the complex interaction between different proteins. But the findings presented here point to directions for further research, for example exploring the significance of these identified proteins and pathways and investigating related data from other glaucoma models. From the results we obtained from this part of the study, I/R injury led to changes in ROS regulation, retinal metabolism, mitochondrial homeostasis and function, retinal vascular function and metal homeostasis, with over half of the altered pathways in the I/R group being self-protective mechanisms. H_2S provided additional support where self-protective mechanisms fell short and demonstrated DNA repair properties, eventually providing neurons a better outcome against I/R injury (Figure 4).

In conclusion, the present thesis provided important new insights into the complex neuroprotective effects of H_2S in glaucoma and emphasized H_2S as a promising and effective therapeutic tool for glaucoma.

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6 Appendix

6.1 Contributions to the manuscripts

Publication I:

Hanhan Liu developed the study design, organized and performed the experimental experiments and wrote the manuscript. Fabian Anders participated in the study design, assisted with the mass spectrometric measurements, proofread the manuscript and contributed important intellectual content. Carolina Mann and Aiwei Liu participated in sample recruitment, reviewed the manuscript. Solon Thanos, Norbert Pfeiffer and Franz H. Grus critically reviewed the manuscript and provided important intellectual input. Verena Prokosch performed the study coordination as well as the study design and participated in review and approval of the manuscript.

Publication II:

Hanhan Liu developed the study design and experimental set-up, organized and performed the experimental experiments and wrote the manuscript. Fabian Anders performed the MS-based affinity capture experiments as well as data analysis. Karl Mercieca aided in the study coordination, reviewed the manuscript and provided important intellectual knowledge. Verena Prokosch performed the study coordination and reviewed as well as approved the manuscript.

Publication III:

Hanhan Liu participated to write the manuscript, developed the study design, performed the animal experiments and assisted in the mass spectrometric measurements as well as data analysis. Natarajan Perumal and Caroline Manicam assisted in the development of the study design, performed the MS-based affinity capture experiments as well as data analysis and proofread the manuscript. Karl Mercieca reviewed the manuscript and provided important intellectual knowledge. Verena Prokosch participated in the study coordination as well as study design and critically reviewed the manuscript.

6.2 List of abbreviations

RGC	retinal ganglion cells	**OCT**	Optical Coherence Tomography
IOP	intraocular pressure	**EVO**	episcleral vein occlusion
ROS	reactive oxygen species	**RNFL**	retinal nerve fiber layer
NO	nitric oxide	**MS**	mass spectrometry
H_2S	hydrogen sulfide	**FDR**	false discovery rate
CO	carbon monoxide	**DHA**	docosahexaenoic acid
CBS	cystathionine-β-synthase	**NAD+**	nicotinamide adenine dinucleotide
CSE	cystathionine-γ-lyase	**PDH**	pyruvate dehydrogenase
3MST	3-mercaptopyruvate sulfurtransferase	**TCA**	tricarboxylic acid cycle
NMDA	N-methyl-D-aspartic acid	**ATP**	adenosine triphosphate
AD	Alzheimer's disease	**PKA**	protein kinase A
PD	Parkinson's disease	**sGC**	soluble guanylyl cyclase
SNCA	synuclein α	**SMC**	smooth muscle cell
SNCB	synuclein β	**AP-2**	adaptor protein complex 2
SNCG	synuclein γ	**NER**	nucleotide excision repair (NER)
CNS	central nervous system	**UPS**	ubiquitin proteasome system
IPA	ingenuity pathway analysis		

6.3 List of figures

6.5 Declaration of academic honesty

I hereby declare that I wrote the dissertation submitted without any unauthorized external assistance and used only sources acknowledged in the work. All textual passages which are appropriated verbatim or paraphrased from published and unpublished texts as well as all information obtained from oral sources are duly indicated and listed in accordance with bibliographical rules. In carrying out this research, I complied with the rules of standard scientific practice as formulated in the statutes of Johannes Gutenberg-University Mainz to insure standard scientific practice. This is my first attempt to promotion.

Mainz, den 24.09.2020

(Hanhan Liu)