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Inhibition of excitatory neurotransmitter activity by hydrogen sulfide in bovine retina, \textit{in vitro}

by

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A THESIS

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ABSTRACT

Hydrogen sulfide (H₂S) has been known as a toxic gas and environmental pollutant for decades. However, it was recently reported to be an endogenously produced gasotransmitter and signaling molecule that can reduce intraocular pressure (IOP) in normotensive and glaucomatous rabbits in the anterior segment of the eye. Initial studies support a modulatory role for the fast H₂S donor compounds, NaHS and Na₂S on excitatory neurotransmitter release in the posterior segment of the mammalian eye, prompting further research on its pharmacological effects in these ocular tissues. So far, no studies have evaluated the pharmacological actions of H₂S donor drugs that produce the gas by different mechanisms in mammalian retina. In the present study, I investigated the effect of different categories of H₂S donor drugs, L-cysteine, a substrate for H₂S biosynthesis; N-acetyl-cysteine (NAC), a precursor to L-cysteine and GYY 4137, a slow-releasing H₂S donor on potassium (K⁺; 50 mM)-induced glutamate release (measured as [³H]D-aspartate) in bovine isolated retina using the superfusion method. Furthermore, I investigated the ability of various H₂S donor drugs to protect bovine retinal neurons from glutamate-induced toxicity using the MTT assay.

The H₂S donor drugs, L-cysteine (100 nM to 10 µM), NAC (10 µM to 1 mM) and GYY 4137 (10 nM to 10 µM) caused a concentration-dependent inhibition of K⁺-induced [³H]D-aspartate release without affecting basal tritium overflow. Interestingly, L-cysteine exhibited the highest potency on the excitatory neurotransmitter release, achieving an inhibition of 54.28 ± 3.61% (n=4, p<0.005) at the 10 µM concentration of the drug. Whereas, NAC exhibited a maximum inhibition of 29.63 ± 14.70% (n=5, p<0.01) at 1
mM concentration while GYY 4137 achieved a maximum inhibition of 21.48 ± 3.52% at the 10 µM concentration of the drug. At an equimolar concentration of 10 µM, the rank order of activity of the three H2S donor drugs was as follows: L-cysteine > GYY 4137 > NAC. Taken together, these results affirm superior modulatory role for L-cysteine, the substrate for endogenous biosynthesis of H2S over the other H2S donor drugs used in this study on the excitatory neurotransmitter release.

There is evidence that endogenous H2S biosynthesis contributes to the effect of H2S donor drugs in ocular tissues. In the present study, the Cystathionine β-synthase (CBS) inhibitor, aminooxyacetic acid (AOA; 3 mM) did not have an effect on basal K⁺-induced [³H]D-aspartate release. However, it completely reversed the inhibitory effects elicited by L-cysteine (1 µM and 10 µM) and GYY 4137 (1 µM and 10 µM) on the excitatory neurotransmitter release, suggesting that endogenous biosynthesis of H2S is involved in the inhibitory action elicited by the two H2S donor drugs. The effect of AOA on NAC was mixed. Whereas AOA partially reversed the inhibitory effect elicited by NAC (1 mM), it completely abolished NAC (10 µM)-induced inhibition of the excitatory neurotransmitter release in bovine retina. Similar to AOA, the K_ATP channel blocker, glibenclamide had no effect on K⁺-induced [³H]D-aspartate release. However, it abolished the inhibitory effects of GYY 4137 (1 µM and 10 µM) and L-cysteine (1 µM and 10 µM) but not NAC (1 mM) on K⁺-induced [³H]D-aspartate release. These data suggest that K_ATP channels are involved in the inhibitory action elicited by L-cysteine and GYY 4137 but not NAC on the excitatory neurotransmitter release in bovine retina.
The gasotransmitter, NO has been shown to interact with the pharmacological actions of H_{2}S in ocular tissues. In this study, both the non-specific inhibitor of the enzyme nitric oxide synthase (NOS), L-Arginine-N-Nitro Methyl Ester (L-NAME) and inhibitor of inducible (iNOS), aminoguanidine had no effect on K^{+}-induced [^{3}H]D-aspartate release in bovine isolate retina. However, L-NAME (300 µM) abolished the inhibitory effects of L-cysteine (1 µM and 10 µM) and GYY 4137 (1 µM and 10 µM) on the neurotransmitter release. Similarly, aminoguanidine (10 µM) reversed the inhibitory effect of L-cysteine on [^{3}H]D-aspartate release, suggesting that NO is involved in the regulation of excitatory neurotransmitter release by H_{2}S donor drugs bovine isolated retina.

Excessive levels of glutamate are associated with excitotoxicity and neuronal cell death. Since the H_{2}S donor drugs attenuate excitatory neurotransmitter release in the eye, it is conceivable that H_{2}S could confer neuroprotection to retinal neurons. In this study, I examined the effect of H_{2}S donor drugs on glutamate-induced toxicity using the MTT assay. Glutamate induced toxicity to retinal neurons in a dose dependent manner, with the 12 mM concentration of the drug eliciting about 50% of retinal neuron death. A comparison of two-hour and six-hour glutamate treatment revealed that, under my experimental conditions, the two-hour treatment period of incubation with glutamate was optimal. I further examined the effect of H_{2}S donor drugs on glutamate (12 mM)-induced toxicity in bovine isolated retina using three classes of H_{2}S donors: substrates for H_{2}S production, L-methionine, L-cysteine and its precursor NAC; the slow releasing H_{2}S donor, GYY 4137; and the fast H_{2}S releasing hybrid of latanoprost-H_{2}S donating moiety, ACS 67. In the concentration range, 10^{-8} M to 10^{-3}M, L-cysteine achieved a protective
effect of 31.2 ± 9.90%; (p<0.05; n=16) at 10^{-3} M concentration. The L-cysteine precursors, L-Methionine (10^{-8} M to 10^{-5} M) and NAC (10^{-6} to 10^{-3} M) reversed glutamate-induced toxicity by 18.43% ± 2.64 (p<0.05; n=16) at the 10^{-6} M concentration and 22.14 ± 4.25% (p<0.05; n=16) at the 10^{-8} M concentration, respectively. The slow releasing H_{2}S donor, GYY 4137 (10^{-8} M to 10^{-5} M) achieved a protective effect of 22.1 ± 3% (p<0.05; n=16) at the 10^{-8} M concentration. It was also interesting to note that the protective effect elicited by NAC (10^{-8} M) was equivalent to that of GYY 4137 (10^{-8} M). Compared to latanoprost (10^{-10} M to 10^{-7} M), ACS 67 (10^{-11} M to 10^{-7} M) reversed glutamate-induced toxicity by 28.98 ± 3.82% (p<0.05; n=16) at the 10^{-11} M concentration while latanoprost exhibited a protective effect of 18.98 ± 0.61% (p<0.05; n=16) at the 10^{-10} M. This suggests that the H_{2}S-moiety contributes to the additional neuroprotective effect of ACS 67. These data affirm the protective role for H_{2}S donors against glutamate-induced toxicity. The exact mechanism underlying the neuroprotective effect of H_{2}S donors in bovine isolated retina remains to be determined.

In conclusion, H_{2}S donor drugs attenuated K^{+}-induced [^{3}H]D-aspartate release in isolated bovine retinae with the following rank order of activity: L-cysteine > NAC > GYY 4137. Unlike NAC, the pharmacological activity of L-cysteine and GYY 4137 was dependent on the in situ release of H_{2}S and activation of K_{ATP} channels. NO was involved in the pharmacological actions of L-cysteine. Furthermore, the H_{2}S donor drugs protected retinal neurons from glutamate-induced toxicity, suggesting a potential therapeutic role for these compounds in the management of neurodegenerative conditions.
PREFACE

Bankhele, Pratik ; Salvi, Ankita; Jamil, Jamal ; Njie-Mbye, Ya Fatou ;
Kulkarni, Madhura S. ; Ohia, Sunny E. ; Opere, Catherine A. “Inhibition of
[^3H]D-aspartate release by hydrogen sulfide in isolated bovine retina”
ARVO (The Association for Research in Vision and Ophthalmology),
Seattle, held in 5-9 May 2013.

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DEDICATION

To my loved ones
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I would like to thank my mentor, Dr. Catherine Opere for her immense support, guidance and motivation throughout my research as a graduate student. I am extremely grateful to her for giving me this opportunity to be a part of her group and benefit from the enriching research experience.

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<th>Description</th>
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<tr>
<td>H₂S</td>
<td>Hydrogen Sulfide</td>
<td>IOP</td>
<td>Intraocular Pressure</td>
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<td>FBF</td>
<td>Flurbiprofen</td>
<td>AOA</td>
<td>Aminooxyacetic acid</td>
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<td>CBS</td>
<td>Cystathionine-β-synthase</td>
<td>CSE</td>
<td>Cystathionine-γ-lyase</td>
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<td>NO</td>
<td>Nitric Oxide</td>
<td>CO</td>
<td>Carbon Monoxide</td>
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<td>3-MST</td>
<td>3-Mercaptopyruvatesulfurtransferase</td>
<td>CAT</td>
<td>Cysteine aminotransferase</td>
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<td>PKA</td>
<td>Protein Kinase A</td>
<td>cAMP</td>
<td>cyclic Adenosine Monophosphate</td>
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<td>NaHS</td>
<td>Sodium hydrogen sulfide</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td>Abbreviation</td>
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<td>RGC</td>
<td>Retinal Ganglion Cells</td>
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<tr>
<td>EDRF</td>
<td>Endothelium derived relaxing factor</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>AMD</td>
<td>Age-related Macular Degeneration</td>
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<tr>
<td>DR</td>
<td>Diabetic Retinopathy</td>
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<td>GPx</td>
<td>Glutathione Peroxidase</td>
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<td>inducible Nitric Oxide Synthase</td>
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<td>Proparaglycine</td>
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<td>L-NAME</td>
<td>N-Nitro-L-Arginine Methyl ester</td>
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<td>NAC</td>
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<td>SNP</td>
<td>Sodium NitroPrusside</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>DMF</td>
<td>Dimethyl Formamide</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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INTRODUCTION
A. GENERAL CONSIDERATIONS

1. Hydrogen Sulfide

Hydrogen sulfide (H₂S) has been known as a toxic, colorless gas with a foul odor of rotten eggs for hundreds of years (Wang, 2012). Recent studies have shown that H₂S is endogenously produced in the mammalian tissues and functions as a regulatory mediator of certain physiological processes. The major mechanism responsible for its toxicity has been attributed to its inhibitory effect on cytochrome c oxidase, a mitochondrial enzyme involved in the electron transport chain and respiration (Betowski, 2007).

In addition to H₂S, two other gases, nitric oxide (NO) and carbon monoxide (CO) are known to play a physiological role in the human body. NO, the first gas identified as a gasotransmitter, was initially reported to exhibit smooth muscle relaxation properties and was designated as the endothelium-derived relaxing factor (EDRF) (Betowski, 2007). Its mechanism of action was attributed to the activation of soluble guanylyl cyclase with subsequent production of cyclic GMP (Ignarro et al., 1984; Rapoport & Murad, 1983). The well-known toxic gas, CO became recognized as the second gasotransmitter. It is produced from biliverdin by the enzyme heme oxygenase and competes with oxygen for binding to hemoglobin (Kimura & Kimura, 2010). Recent studies support the role for H₂S as the third gaseous mediator in mammals that acts as a signal molecule and a cytoprotectant in the cardiovascular and central nervous systems (Betowski, 2007; Kimura, 2011). Using chromatographic,
spectrophotometric and electrochemical methods of detection, H₂S has been localized in various biological systems (Kolluru et al, 2013). For example, its serum and brain tissue level are reported in the 50 µM to 160 µM range in rats (Betowski, 2007; Kimura et al, 2011).

1.1 Physical properties of H₂S

H₂S is a colorless, flammable and water soluble gas with a molecular weight of 34.08. One gram of H₂S dissolves in 242 mL of water at room temperature (20°C). It is a highly lipophilic molecule, with its solubility in lipophilic solvents being five times more than its solubility in water. This makes it highly permeable to the lipid bilayer of the cell membranes (Wang, 2012).

1.2 Biosynthesis of H₂S

H₂S is produced in substantial amounts in most of the mammalian tissues. Although it is endogenously generated using both enzymatic and non-enzymatic pathways, the enzymatic biosynthetic pathways predominate in the body (Wang, 2012). H₂S is produced through the “transsulfuration pathway” from L-cysteine, the major substrate for its endogenous production (Figures 1 and 2). The two major enzymes involved in this pathway, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are dependent on pyridoxal 5’-phosphate (vitamin B₆) as a cofactor (Betowski, 2007; Wang, 2012). A third enzyme, 3-Mercaptoppyruvate sulfur-transferase (3MST) together with cysteine aminotransferase (CAT) localized in the mitochondria can produce H₂S from L-cysteine in the presence of α-ketoglutarate (H. Kimura, 2011) (Figures 1 and 2).
CBS and CSE have been identified in mammalian tissues. For instance, in the adult and developing mouse brain, Enokido et al demonstrated that CBS is mainly present in the cerebellar glial cells and astrocytes (Enokido et al., 2005). CSE activity was measured in mouse whole brain and liver (Diwakar & Ravindranath, 2007). CBS and CSE were also expressed in pancreatic islet cells in mice and in the beta cell line MIN6 (Kaneko et al, 2006). Patel et al (2009) showed that both rat and human uterine tissues expressed CBS and CSE. Moreover, H₂S was produced from these enzymes (Patel et al, 2009). In general, CBS appears to be the major H₂S-producing enzyme in the central nervous system while CSE is the primary source of H₂S in the cardiovascular system (Betowski, 2007).

**Figure 1.** Schematic representation of the biosynthetic pathway for production of H₂S from L-cysteine. Both CBS and CSE use ammonia and pyruvate for the biosynthesis of H₂S from L-cysteine. Using L-cysteine and α-ketoglutarate, CAT catalyzes the production of 3-mercaptopropionate (3-MP), which is then converted to H₂S by 3-MST. Aspartate acts as a competitor against L-cysteine for CAT enzyme.

[Source: Figure obtained from (Kimura, 2010)]
1.3 Storage and release of H$_2$S

Once H$_2$S is synthesized, it can either be released immediately or it can be stored and released in response to a physiological signal. There are two forms in which H$_2$S can be stored (as sulfur stores) in the cells:

1.3.1 Acid-labile sulphur

In this form, H$_2$S is stored in tissues as iron-sulfur complexes. These complexes play an important role in the various oxidation reduction reactions intrinsic to the
mitochondrial respiratory chain. H$_2$S can be released from the mitochondria under acidic conditions when the pH is below 5.4. (Kimura, 2010) (Figure 3). It is pertinent to note that physiological mitochondrial pH ranges between 7 and 8. Thus, H$_2$S is not released from this form under physiological conditions (Kimura, 2010). Detergents and substances which denature proteins may also cause the release of H$_2$S since the iron-sulfur complexes are unstable (Kimura, 2010)

1.3.2 Bound sulfane sulfur

H$_2$S can also be incorporated into proteins as bound sulfane sulfur (Ishigami et al., 2009). In this form, H$_2$S is stored as divalent sulfur bound to other sulfur atoms of persulfides and polysulfides and is released under reducing conditions (Kimura, 2010; Figure 3)

**Figure 3.** Two types of stored forms of sulphur that can release H$_2$S. Acid-labile sulfur consisting of iron-sulfur complexes releases H$_2$S under acidic conditions, whereas bound sulfane sulfur consisting of sulfur in form of polysulfides and persulfides releases H$_2$S under reducing conditions [Source: (Kimura, 2010)].
1.4 Metabolism and elimination of H$_2$S

Following its synthesis and release, H$_2$S is metabolized by three distinct pathways:

i. Oxidation to sulfate

ii. Methylation

iii. Reaction with metallo or disulfide containing proteins

Of these metabolic pathways, oxidation of sulfide to sulfate and subsequent excretion by the kidney is believed to be the primary metabolic and secretory pathway (Figure 4).

Figure 4. A schematic diagram summarizing the biosynthesis, storage and metabolism of endogenous H$_2$S.[Source: Figure obtained from (Tan et al, 2010)].
1.5 H$_2$S as a gasotransmitter

Gasotransmitters are gaseous substances which are produced endogenously in the body. They perform certain physiological functions in the various body/tissue systems by acting on cellular targets and regulating cell signaling pathways. H$_2$S is biosynthesized in various tissues, and is stored in the body either as acid-labile sulfur or in the bound form. In response to specific stimuli, H$_2$S released to act upon specific cellular targets and produce physiological effects in the body. Finally, it is metabolized to inactive products by different pathways. Since H$_2$S shows all the above characteristics in spite of being a gas, it is presumed to act as a gasotransmitter in the human body.

2. Neural degeneration and protection

2.1 Neurodegeneration

Neurodegeneration is an all-inclusive description for the progressive physiological and structural dysfunction that occurs in neurons, ultimately leading to cell death (Przedborski et al, 2003). Neurodegeneration is a characteristic underlying pathology in various debilitating central nervous system (e.g. Alzheimer’s, Parkinson’s and Huntington’s disease) and ocular (glaucoma, age-related macular degeneration and diabetic retinopathy) conditions. Several mechanisms have been implicated in the neurodegenerative process, including glutamate toxicity, oxidative stress, inflammatory process and protein misfolding. Below is a brief discussion pertaining to these sources of degeneration.
2.1.1 Glutamate toxicity

Excessive extracellular glutamate is known to trigger a cascade of reactions that ultimately cause neurodegeneration. Glutamate, the major excitatory neurotransmitter in the mammalian nervous system, is present in more than 50% of the nervous system and plays an important role in neuronal excitation (Mehta et al., 2013). The intra-cellular glutamate content is tightly maintained at a higher level than the extracellular concentration (Ma et al., 2012). As long as this concentration gradient is sustained, overactivation of glutamate receptors present on the outer surface of the neuronal cells is prevented (Mehta et al., 2013). Thus, mechanisms which sustain this concentration gradient are important for normal functioning of neurons (Yu et al., 2008).

![Excitotoxicity Diagram](image1)

![Oxidative toxicity Diagram](image2)

**Figure 5.** Two forms of glutamate toxicity 1. Ionotropic receptor mediated toxicity (left figure) and 2. Oxidative glutamate toxicity (right figure). Source: Figure obtained from (Kimura, 2010)
Two forms of glutamate toxicity occur in cells; ionotropic receptor-initiated excitotoxicity and non-receptor-mediated oxidative glutamate toxicity (Kimura, 2010). In the receptor-initiated excitotoxicity, excessive amounts of Ca\(^{2+}\) ions enter the cell through ionotropic glutamate receptors, leading to cell death. The second type of toxicity is a programmed pathway of cell death, that is independent of the ionotropic glutamate receptors (Murphy et al., 1989) and cannot be reversed by antagonists of these receptors. Glutamate and cysteine utilize the same amino acid transporter and compete against each other for transport into cells (Bannai & Kitamura, 1980). Consequently, elevated levels of extracellular glutamate can inhibit uptake of cysteine into the cells. Since cysteine uptake is an important source for glutathione production and glutathione serves as a major intracellular antioxidant, excess glutamate ultimately results in glutathione depletion and increase in oxidant stress (Kimura, 2010). Thus, both forms of glutamate toxicity lead to degeneration of neurons and therefore constitute potential cellular targets for neuroprotection.

### 2.1.2 Oxidative stress

Oxidative stress constitutes an important source of neuron degeneration. Oxidative stress refers to an imbalance between tissue pro-oxidant and antioxidant mechanisms in favor of pro-oxidative processes, ultimately leading to accumulation of reactive oxygen species (ROS) (Betteridge, 2000; Sies, 1997). These reactive metabolites such as hydroxyl radical, superoxide anion and
hydrogen peroxide (H$_2$O$_2$) are mostly generated as by-products of endogenous metabolic processes involving oxygen metabolism and from exogenous sources of ROS such as cigarette smoke and the environmental pollutants.

2.1.2a Oxidative stress and mitochondria

Mitochondria is one of the main sources of endogenous ROS in mammalian cells. In the mitochondria, ROS are produced when electrons escape from the respiratory chain and react with oxygen (Wang et al., 2013). Addition of a single electron to molecular oxygen results in the production of superoxide anion (O$_2^-$), which is further converted to hydroxyl anions and H$_2$O$_2$ via specific redox reactions (Wang et al., 2013). The ROS further reacts with various other biomolecules such as DNA and nucleic acids, proteins and lipids, resulting in detrimental alteration of their structure (Lobo et al., 2010) and formation of more radicals.

2.1.2b ROS and neurodegenerative diseases

Oxidative stress has been established as an underlying pathology in several neurodegenerative diseases such as Alzheimer’s disease (Lyra et al., 1997; Nunomura, 2013), Parkinson’s disease (Mandel et al., 2003) and in ocular neuropathies. For instance, oxidative stress plays a significant role in retinal damage and death of neuronal cells, including retinal ganglion cells (RGCs)
(Chen et al, 2013) and it is related to a number of ocular conditions such as age-related macular degeneration (AMD), diabetic retinopathy (DR) and glaucoma (Ohia et al, 2005). Thus, deficiency in the antioxidant defense system, disruption of electron flow or xenobiotic exposure can lead to imbalance between antioxidants and ROS production, ultimately causing tissue damage and death (Turrens, 2003).

2.1.3. Inflammatory mediators

In addition to glutamate toxicity and oxidative stress, inflammation also contributes to tissue degeneration. Emerging data supports a role for inflammatory mediators in the neurodegenerative processes. Inflammation is a defensive mechanism or a protective response from the body when there is injury to the cell or tissue (Hsieh & Yang, 2013). Many cells such as the astrocytes, microglia and endothelial cells are well known as sources of inflammatory proteins in nervous system disorders. In response to inflammation or injury, microglia undergo activation to release inflammatory mediators such as nitric oxide (NO), prostaglandins, and inflammatory cytokines (Huang et al, 2013). Cytokines include interleukins such as interleukin 1β (IL-1β) and have been implicated in many neurodegenerative disorders, in addition to their role in the etiology of inflammation (Cacquevel et al, 2004). For example, the inflammatory cytokine, transforming growth factor beta (TGF Beta) contributes to inflammatory processes associated with neuronal cell death in Alzheimer’s disease (Grammas &
Substances such as amyloid beta peptide can cause oxidative stress and indirectly lead to neuronal toxicity (Butterfield, 2002). Thus, inflammation contributes to the etiology of degeneration in tissues.

### 2.1.4. Misfolding of proteins

Protein misfolding has been linked to tissue degeneration. Protein misfolding occurs when there is a deviation from the regular protein folding process to yield a dysfunctional and often toxic protein complex. It is influenced by amino acid composition and environmental changes (Herczenik & Gebbink, 2008). Amyloidosis refers to protein misfolding diseases characterized by accumulation of protein aggregates in organs or tissues (Herczenik & Gebbink, 2008). Soluble oligomers of misfolded proteins are detrimental to cell functioning since they interfere with normal cellular processes or they trigger pathways which cause cell death (Muchowski & Wacker, 2005). Neurodegenerative disorders such as Alzheimer’s disease are associated with protein misfolding in which change in the protein conformation may lead to formation of toxic aggregates, premature degradation or inclusion of toxic components into structures (Gregersen et al, 2005). Thus, protein misfolding presents a potential target for that can be harnessed for therapeutic applications in neurodegenerative diseases.

In summary, several processes, including glutamate toxicity, oxidative stress inflammatory process and protein misfolding are involved in the etiology of neurodegeration. These processes constitute potential therapeutic targets for management of neurodegenerative conditions.
2.2. Endogenous defense mechanisms:

To counteract the deleterious neurodegenerative processes, several defensive strategies have evolved in mammalian neurons. Some of these defensive strategies include enzyme, vitamin and protein-systems that scavenge free radicals, inhibit free radical chain reaction, chelate metal ions into complexes and repair of damaged molecules (Rizzo et al., 2010). Below is a brief discussion pertaining to some endogenous major mechanisms.

2.2.1 Enzymes

Several enzymes detoxify and protect tissues from ROS-induced damage, thereby serving as endogenous neuroprotectants. The superoxide dismutase enzymes constitute the initial step of detoxification of two superoxides by converting them into hydrogen peroxide (H$_2$O$_2$) and oxygen (Kannan & Jain, 2000). These enzymes contain metals such as zinc, manganese or copper that facilitate their free radical scavenging action. Catalase converts H$_2$O$_2$ to water and oxygen using two concentration-dependent mechanisms. Whereas it degrades H$_2$O$_2$ to water and oxygen at high peroxide levels, at low peroxide levels, it exerts a peroxidatic reaction in the presence of a hydrogen donor (Scibior & Czeczot, 2006). Glutathione peroxides (GPx) are a family of widely distributed cytosolic enzymes, GPx1-8, that detoxify peroxides. The majority of GPxs contain selenium. In addition to degrading H$_2$O$_2$ to water, GPxs convert organic peroxides to corresponding alcohols (Kayanoki et al., 1996). Glutathione-S-transferase
catalyzes the conjugation of glutathione with oxidation end products to protect tissues from toxic substances produced by ROS-mediated reactions (Masella et al., 2005). **Glutathione reductase** reduces intracellular anti-oxidant glutathione from its oxidized disulfide form to its reduced sulfhydryl form. It has been shown to have cytoprotective effects against oxidative stress in human hepatoma cells (Kim et al., 2010).

### 2.2.2. Antioxidants

In addition to enzymes, antioxidants play a protective role in tissues against oxidative damage. Cellular antioxidants include glutathione, ascorbate (Vitamin C), Vitamin E, coenzyme Q, uric acid, bilirubin, L-carnitine and melatonin (Puglia & Powell, 1984, Rizzo et al., 2010). **Glutathione** is a water-soluble tripeptide consisting of glutamate, cysteine and glycine that is capable of scavenging reactive species such as ROS and RNS and can cleave disulfide linkages formed inside and between proteins due to reaction with oxidants (Rizzo et al., 2010). **Reduced glutathione (GSH)** competes with oxidants and preferentially gets oxidized to glutathione disulfide (GSSG), thereby acting as an antioxidant. **Coenzyme Q** is the only endogenously synthesized lipid soluble antioxidant that exerts a protective effect on lipids, proteins and DNA in the cell (Rizzo et al., 2010). **L-Carnitine** is a naturally occurring biomolecule which has protective effect on the antioxidant defense system comprising of enzymes such as catalase and superoxide dismutase (Binienda & Ali, 2001). Additionally, the
amino acid **methionine** residues can act as important endogenous antioxidants due to their high susceptibility to oxidation (Levine et al., 1996).

Taken together, these endogenous defense mechanisms protect and preserve neurons from both exogenous and endogenous deleterious stimuli. Use of exogenous neuroprotective mechanisms is warranted when defensive endogenous mechanisms are overcome by the deleterious stimuli.

### 2.3. Neuroprotection

Neuroprotection refers to the attenuation and/or prevention of neuron degeneration and preservation of neuron structure and function (Chang & Goldberg, 2012). The most common neuroprotective mechanisms target **glutamate-induced toxicity** and **oxidative stress**.

#### 2.3.1. Glutamate receptor antagonism

Glutamate excitotoxicity is one of the main targets for neuroprotection in mammalian CNS. Memantine, an NMDA glutamate receptor antagonist, was the first drug used as a neuroprotective agent to treat moderate to severe Alzheimer’s dementia (Chang & Goldberg, 2012). There is evidence that it significantly attenuates loss of RGCs in glaucoma rat model, thus serving as a neuroprotective function in ocular tissues (Hare et al., 2001). Due to its potential beneficial ocular effects, an expensive clinical trial was conducted on the potential application of memantine as a neuroprotectant in glaucoma patients. In the a phase 3 clinical
study, the disease progression was slowed in patients ingesting higher doses of the
drug compared with those taking lower doses (Osborne, 2009). Since the second
phase 3 trial did not affirm the previous phase 3 findings, the clinical trial for use
of memantine in glaucoma patients was terminated (Osborne, 2009). In general,
memantine did not show any significant benefit over patients who received
placebo. In addition to memantine, several drug candidates have exhibited
neuroprotective actions. Interestingly, the non-competitive NMDA-receptor
antagonist, bis(7)-tacrine, has also been shown to protect rat RGC from
glutamate-induced damage, in vitro and in vivo experiments (Fang et al, 2010)
but has not been translated into human for therapeutic use.

In addition to targeting the NMDA receptors, additional neuroprotective strategies
have been examined. Caspase inhibitors have been shown to increase survival of
the retinal neurons against glutamate excitotoxicity model (Chang & Goldberg,
2012). Copolymer-1 is an anti-inflammatory drug which targets the TNFα
signaling pathway and triggers a neuroprotective autoimmune response (Chen et
al, 2013) and it has also shown to protect the rat RGCs against glutamate-induced
excitotoxicity(Schori et al., 2001). Brimonidine, an α2 adrenergic receptor agonist
protected RGCs a rat model of ocular hypertension (WoldeMussie et al, 2001).
Other drug candidates including latanoprost (Drago et al., 2001), melatonin (Siu
et al, 2004), co-enzyme Q (Nakajima et al., 2008) have exhibited potential
neuropotective actions in RGCs as well.

In summary, the most common pathway for neuroprotection targets the glutamate
receptor mediated excitotoxicity. However, glutamate receptor antagonists are
incapable of protecting the cells against oxidative glutamate toxicity. Therefore, the ability of H2S to decrease oxidative glutamate toxicity may open up a superior therapeutic strategy in ocular neuropathies

2.4. H2S and neuroprotection:

Emerging data supports a possible neuroprotective role for H2S in neurons. So far, several mechanisms are implicated in the neuroprotective effect of H2S:

2.4.1. Increase in antioxidant levels

H2S has been reported to increase the concentrations of glutathione, a major intracellular antioxidant in neurons by several mechanisms. It increases the activity of cystine/glutamate antiporter, thereby elevating the concentration of cysteine required for glutathione production (Kimura & Kimura, 2004). Additionally, H2S is reported to facilitate transport of cysteine into the cell (Kimura, 2010). It is has been proposed that once the highly permeable H2S is synthesized in the cells, it is released into the extracellular space where it causes the reduction of cystine to cysteine. Cysteine is then taken up by its transporters that are present in abundance in a wide variety of cells to be used for the synthesis of glutathione (Kimura, 2010). Thus, an increase in intracellular glutathione content is thought to account for the protective action of H2S against oxidative glutamate toxicity in primary culture of neurons (Y. Kimura et al., 2010).
2.4.2. Protection from oxidative stress

Although mitochondria are the storehouse of energy, they constitute a primary source of oxidative stress in neurons. H$_2$S-producing enzymes 3MST and CAT are mainly present in the mitochondria of cells (Kimura, 2010). Mitochondrial dysfunction resulting from oxidative stress is associated with various neurodegenerative disorders. However, neuronal cells which express 3MST and CAT show substantial resistance to oxidative glutamate toxicity (Kimura, 2010). It is conceivable that that H$_2$S synthesized by the mitochondrial enzymes, 3MST and CAT play a protective role in these neurons by scavenging the mitochondrial ROS.

2.4.3. Action on ion channels

Using cloned hippocampal neuronal HT22 cell line, it was shown that H$_2$S is able to protect the cells by increasing the activity of K$_{ATP}$ and Cl$^-$ channels (Y. Kimura, Dargusch, Schubert, & Kimura, 2006). Besides its action on K$_{ATP}$ channels, H$_2$S also activates L-type (García-Bereguaiain et al., 2008) and T-type Ca channels (Kawabata et al., 2007). The activation of CFTR Cl$^-$ channels in the HT22 neuronal cell lines by H$_2$S is postulated to be a possible mechanism for neuroprotection during oxytosis (Kimura et al., 2006).
2.4.4. Reaction with ROS and RNS species

H$_2$S is a highly reactive molecule that can easily react with other molecules like the ROS and RNS (Betowski, 2007). It reacts with at least four different reactive species: superoxide anion (Mitsuhashi et al., 2005), H$_2$O$_2$ (Geng et al., 2004), peroxynitrate (Whiteman et al., 2004) and hypochlorite (Whiteman et al., 2005). Confirming this mechanism of H$_2$S protection, is reported to suppress generation of ROS and protects against hypoxia-induced cell injuries (Chen et al, 2010).

2.4.5. Role in transcriptional regulation

H$_2$S also has been shown to have an effect on transcriptional regulation. Its interaction with intracellular transcription factors, mostly those associated with its role in inflammation and tissue ischemia/reperfusion injury has been extensively studied in literature. The H$_2$S donors, NaHS and GYY 4137 were found to inhibit the activation of LPS-induced nuclear factor κB (NF-κB) in cultured RAW 246.7 macrophages and mouse macrophages respectively (Li et al., 2011), leading to the downregulation of a number of proinflammatory genes such as those encoding inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) enzymes. In addition to decreasing the number of inflammatory cytokines and chemokines, H$_2$S was found to activate a transcription factor called as signal transducer and activator of transcription 3 (STAT 3). STAT 3 is important since it is involved in regulation of genes which control processes such as cell survival, proliferation and angiogenesis (Li et al, 2011).
3. Mammalian eye

3.1 Anatomy of the mammalian eye

The eye is a specialized sensory organ that facilitates visual function of the body (Figure 6). The anterior segment makes up the third front of the eye and consists of the cornea, iris, ciliary body and lens. The posterior segment, consisting of vitreous humor, retina, choroid and the optic nerve makes up the back two-thirds of the eye. (Cassin, B. and Solomon, S. *Dictionary of Eye Terminology*. Gainsville, Florida: Triad Publishing Company, 1990). The retina is the light sensitive layer of neurons that forms the inner-most layer of the posterior segment. Light enters the eye through the cornea into the lens which focuses images the retina. The retina encodes these images into electric signals that are transmitted to the brain via the optic nerve (“Retina,” 2011). The vertebrate retina is composed of three layers of cell bodies of neurons (Klob, 2014) (Figure 7). The first or outer layer contains cell bodies of the photoreceptor cells i.e. the rods and the cones; the middle layer contains cell bodies of the horizontal, bipolar and the amacrine cells while the inner or ganglion cell layer contains the cell bodies of all the ganglion cells (Klob, 2014). The axons from the ganglion cell layer converge to make the optic nerve which transmits information into the brain (Liesegang, 1996). In humans, the optic nerve consists of approximately one million axons, whose cell bodies are mostly located primarily in the ganglion cell layer.
Figure 6. Anatomy of the human eye. [Source: Figure obtained from www.ezcontactusa.com]

Figure 7: Structure of the retina [source: (Klob, 2014)]
3.2 Optic neuropathies and glaucoma

Optic neuropathies are a group of disorders associated with dysfunction of the optic nerve along with and compromised visual function (Osborne et al., 2004). A common characteristic of all types of optic neuropathy is the degeneration of RGCs (Osborne et al., 2004). Hence, RGC death represents a common pathway for most of the optic nerve disorders including glaucomatous optic neuropathy (Shahsuvaryan, 2013) and a potential target for therapeutic intervention.

Glaucoma, a progressive neurodegenerative disorder of the eye, is one of the leading causes of blindness worldwide. It is characterized by the death of RGCs, atrophy of the optic nerve and subsequent loss of visual field function. Elevated intraocular pressure (IOP) does not constitute a positive diagnosis for glaucoma but remains one of the major, modifiable risk factors for the disease (Law, 2007). Since neurodegeneration and RGC may be an important cause of glaucoma (Liesegang, 1996; Shahsuvaryan, 2013), prevention of RGC death presents a significant therapeutic rationale in the treatment of glaucoma and other ocular disorders.

3.3 H2S and the Eye

There is evidence supporting the regulatory function for H2S in the anterior segment of the eye. Perrino et al reported that ACS 67, a hybrid of latanoprost and an H2S-donating moiety (Figure 8) was significantly more potent than latanoprost alone in lowering IOP after topical administration to glaucomatous pigmented
rabbits. Moreover, the incremental IOP-lowering response correlated well with the marked increase in GSH levels in the aqueous humor of ACS 67-treated rabbits when compared to the values in latanoprost-treated rabbits (Perrino et al., 2009). The observed increase in GSH suggested a neuroprotective role for ACS 67 in the ocular tissues (Perrino et al., 2009).

Figure 8. Structures of latanoprost and its hybrid, ACS 67. ACS 67 is an ester of latanoprost acid containing dithiolethione (ACS1) and is likely to undergo hydrolysis either \textit{in vitro} or \textit{in vivo} to the parent compound. [Source: Figure obtained from (Perrino et al., 2009)].

In addition to its IOP-lowering effect, H$_2$S exerts pharmacological actions in isolated mammalian iris-ciliary bodies in the anterior uvea (Kulkarni et al., 2009). Kulkarni and coworkers showed that H$_2$S donors, NaHS and Na$_2$S elicited a concentration-dependent inhibition of norepinephrine release from isolated porcine iris-ciliary bodies by mechanisms that were sensitive to inhibition by the CBS inhibitor, aminooxyacetic acid (AOA) and CSE inhibitor propargylglycine (PAG) (K. H. Kulkarni et al., 2009). Moreover, these H$_2$S donors (Monjok et al., 2008) and L-cysteine, the substrate for endogenous production of H$_2$S (Ohia et al., 2010) inhibited the contraction of isolated porcine irides induced by muscarinic receptor stimulation by mechanisms that were dependent on
endogenous production of H$_2$S from the biosynthetic enzymes CBS and CSE (Ohia et al., 2010). Taken together, these studies suggest a role for H$_2$S and its endogenous biosynthesis in the regulation of sympathetic neurotransmitter release in the anterior uvea. Although the pharmacological actions of H$_2$S in the anterior uvea have been studied, the pharmacological actions of this gasotransmitter on retinal neurons have not been completely delineated. Therefore, this study focused on the pharmacological actions of H$_2$S donor drugs on retinal neurons as hypothesized below:
Hypothesis

H₂S donor drugs can reduce excitatory glutamate (measured as [³H]D-aspartate) neurotransmitter release and protect retinal neurons from glutamate-induced toxicity in isolated bovine retinas and act as potential neuroprotective agents.

To test this hypothesis, the following specific aims were proposed:

Specific Aims

Specific Aim 1:

To investigate the effects of H₂S donor drugs on the excitatory neurotransmitter release

To examined the effect of three drugs: GYY 4137, a slow-releasing H₂S donor; L-cysteine, an endogenous substrate for H₂S synthesis and NAC, a precursor to L-cysteine on K⁺-induced [³H]D-aspartate release in isolated bovine retina.

Specific Aim 2:

To investigate the mechanism of action of H₂S donor drugs

To investigate the mechanism of action of H₂S donor drugs, I investigated the effect of AOA, a CBS enzyme inhibitor (Xia, Chen, Muh, Li, & Li, 2009) and glibenclamide, a K_{ATP} channel blocker (Zhao et al., 2001) on responses elicited by H₂S donors on K⁺-induced [³H]D-aspartate release in isolated bovine retina.
Specific Aim 3:

To determine the role of the gasotransmitter, nitric oxide (NO) in the regulation of H$_2$S activity

To investigate role of the gasotransmitter, NO on the regulation of H$_2$S activity, I examined the effect of L-NAME, a NO synthase inhibitor (Pfeiffer, Leopold, Schmidt, Brunner, & Mayer, 1996) and aminoguanidine (Corbett & McDaniel, 1996), the inducible NO inhibitor on H$_2$S-mediated responses on K$^+$-induced [$^3$H]D-aspartate release in isolated bovine retina.

Specific Aim 4:

To investigate the protective effect of H$_2$S donor drugs against glutamate-induced toxicity in isolated bovine retinae

To investigate the protective effect of H$_2$S donor drugs from glutamate-induced toxicity in isolated bovine retina, I examined the effect of three categories of H$_2$S donor: substrates for endogenous production of H$_2$S, L-cysteine, NAC and methionine, slow-releasing H$_2$S donor drug, GYY 4137 and fast releasing H$_2$S hybrid of latanoprost and H$_2$S-donating moiety, ACS 67 on cell survival using the MTT assay.
Literature Review of Specific Aims

Specific Aim 1:

To investigate the effects of H$_2$S donor drugs on the excitatory neurotransmitter release

In addition to serving as the major excitatory neurotransmitter in brain and retina, glutamate, is involved in several physiological and pathological processes in neurons (Meldrum, 2000; Platt, 2007). Thus, the interaction between H$_2$S donors and glutamate could have significant pathophysiological implications. In ocular tissues, the fast releasing H$_2$S donors, NaHS and Na$_2$S have been reported to attenuate the release of excitatory neurotransmitter in the mammalian isolated retina (Opere et al., 2009). So far, no studies have compared the pharmacological actions of drugs that produce H$_2$S by different mechanisms on the excitatory neurotransmitter release. Therefore, in this specific aim, I will investigate the effect of L-cysteine, an endogenous substrate for H$_2$S synthesis; NAC, a precursor to the synthesis of L-cysteine and GYY 4137, a slow-releasing donor of H$_2$S, on K$^+$-stimulated $[^3]$H]-D-aspartate release on isolated bovine retina.

Specific Aim 2:

To determine the mechanism of action of H$_2$S donor drugs

There is evidence that in situ H$_2$S biosynthesis contributes to the pharmacological actions of H$_2$S donor drugs in ocular tissues (Kulkarni et al., 2011). Indeed, the H$_2$S biosynthetic
enzymes, CBS and CSE have been localized retina (Pong et al., 2007). Moreover, the CBS inhibitor, AOA) (Asimakopoulou et al., 2013) has been reported to reverse the inhibitory effects of H$_2$S donor, NaHS on sympathetic neurotransmitter release in isolated porcine iris-ciliary body (Kulkarni et al., 2009). Similarly, AOA and CSE inhibitor propargylglycine (PAG) significantly attenuated H$_2$S-induced cyclic AMP production in rat retinal pigment epithelial cells (Njie-Mbye et al., 2012). Taken together, these studies suggest that in situ H$_2$S-production contributes to the pharmacological actions of H$_2$S donors in ocular tissues. Therefore, in this aim, I investigated the role of endogenous production of this gasotransmitter on the inhibitory action of H$_2$S donor drugs on K$^+$-induced [$^3$H]D-aspartate release using CBS enzyme inhibitor, AOA.

Activation of K$_{ATP}$ channels has been implicated in the pharmacological actions of H$_2$S donor drugs in various biological systems. For instance, H$_2$S suppressed glutamate toxicity in mouse hippocampal neuronal cell line (Kimura et al., 2006) via K$_{ATP}$ channel-dependent mechanisms (Wang, 2012). In the cardiovascular system, K$_{ATP}$ channels were reported to partially regulate H$_2$S-induced vasorelaxation in the rat aorta and vascular smooth muscle cells (Kimura, 2010; Zhao & Wang, 2002; Zhao et al., 2001). Glibenclamide, a competitive blocker of the K$_{ATP}$ channels (Pompermayer et al., 2005), reversed the vasodilatory effects of H$_2$S on the piglet cerebral arteriole smooth muscles (Liang et al., 2011). Similarly, glibenclamide attenuated NaHS-induced increase in cyclic AMP formation in rat retinal pigment epithelial cells (Njie-Mbye et al., 2012). Therefore, in this aim, I also investigated the role of K$_{ATP}$ channels in the inhibitory effect of H$_2$S donor drugs on K$^+$-induced [$^3$H]D-aspartate release in bovine isolated retina.
Specific Aim 3:

To determine the role of gasotransmitter, nitric oxide (NO) on H2S-response

H2S is now established as the third gasotransmitter (following NO and CO) in biological systems. Recent studies have attempted to investigate the interaction between H2S and NO with mixed results. Although both gases independently relax vascular tissues, the interaction between them appear complex (Wang, 2012). For example, at a concentration of 60 µM, H2S decreased the NO-donor, sodium nitroprusside (SNP)-induced vasorelaxation in rat aorta (Zhao et al, 2001). Li et al (1997) reported that L-cysteine and L-homocysteine, endogenous precursors of H2S, inhibited both the NO-induced vasorelaxation and NO-induced enhancement of cyclic GMP in rabbit aorta (Li et al 1997). These studies suggest an inhibitory effect of H2S on the vasorelaxant effect of NO. On the contrary, Hosoki et al (1997) observed a synergistic relaxant action between SNP and NaHS in rat aorta (Hosoki, Matsuki, & Kimura, 1997). These contrasting effects could be attributed to difference in the experimental conditions used by the two groups of researchers. Thus, in order to delineate the role of NO on H2S activity in the retina, I investigated the effect of two NOS inhibitors, the non-specific NO inhibitor, L-NAME and the specific iNOS inhibitor, aminoguanidine on the inhibitory effect of H2S on excitatory neurotransmitter release.
Specific Aim 4:

To investigate the protective effect of H$_2$S donor drugs from glutamate-induced toxicity in isolated bovine retinae

Excessive elevation of glutamate is associated with NMDA-dependent and NMDA-independent neuronal toxicity. Thus, drug candidates that reduce glutamate release can potentially attenuate glutamate-induced neurotoxicity. The ability of H$_2$S donor drugs to attenuate glutamate release suggests a neuroprotective role for H$_2$S in neurons. Corroborating this hypothesis, mounting data supports a neuroprotective role for H$_2$S in neurons. For instance, H$_2$S has been reported to protect neurons from toxic effects of high levels of glutamate via an increase in the concentration of intracellular anti-oxidant glutathione (Betowski, 2007). Moreover, H$_2$S protected cultured neuroblastoma (SH-SY5Y cells) from peroxynitrite-induced and hypochlorous acid-induced toxicity (Whiteman et al., 2004, 2005) In in vivo studies, H$_2$S decreased the damage elicited by recurrent febrile seizures whereas the CBS inhibitor, hydroxylamine increased the neuronal damage in rat models (Han et al., 2005). In ocular tissues, the latanoprost-H$_2$S donor hybrid, ACS67 attenuated retinal ischemia and reduced the oxidative stress to RGCs in culture (Osborne et al., 2010). Taken together, these studies support a neuroprotective function for H$_2$S in neurons. In this aim, I sought to delineate the role of H$_2$S on glutamate-induced toxicity on bovine isolated retina using the MTT assay.
MATERIALS AND METHODS
METHODS

1. Neurotransmitter Release
   1.1 Preparation of Buffer Solutions
   1.2 Tissue Isolation
   1.3 Measurement of [³H]D-Aspartate Release
   1.4 Data Analysis

2. Tissue culture studies (MTT assay)
   2.1 Preparation of glutamate stock solutions
   2.2 Preparation of MTT reagent solution and lysing buffer
   2.3 Isolation of tissue
   2.4 Treatment
   2.5 Post-treatment

3. Sources of drugs and chemicals
1. NEUROTRANSMITTER RELEASE

Neurotransmitter release studies were conducted using oxygenated Krebs buffer solution containing ascorbic acid (1 µM) and flubiprofen (FBF; 3 µM). The Krebs buffer solution had the following composition (mM): NaCl 118; KCl 4.8; CaCl₂ 2.5; KH₂PO₄ 1.2; NaHCO₃ 25; MgSO₄ 2.0; dextrose 10 (pH 7.4) and was prepared as follows:

1.1 PREPARATION OF BUFFER SOLUTIONS

Initially, the stock solutions, A, B, C, and modified A (high KCl 50 mM) were prepared as indicated on Tables 1, 2, 3 and 4 below:

Table 1: Composition of Krebs buffer solution A

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<th>GRAM/%</th>
<th>mM</th>
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<td>1.2</td>
<td>1.6</td>
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<tr>
<td>MgSO₄, 7H₂O</td>
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<td>2.0</td>
<td>3.0</td>
<td></td>
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<tr>
<td>QS</td>
<td></td>
<td></td>
<td></td>
<td>QS to 1000 mL with double distilled water</td>
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</table>
Table 2: Composition of Krebs buffer solution B

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<th>mM</th>
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<td>21</td>
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<td>QS</td>
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Table 3: Composition of Krebs buffer solution C

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<th>SALT</th>
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<tr>
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<td>QS to 1000 mL with double distilled water</td>
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Table 4: Composition of Krebs modified buffer solution A (High K⁺ buffer solution)

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<tr>
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<th>mM</th>
<th>GRAMS</th>
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<tr>
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<td>1.6</td>
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<tr>
<td>MgSO₄, 7H₂O</td>
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<tr>
<td>q.s.</td>
<td></td>
<td></td>
<td></td>
<td>QS to 1000 mL with double distilled water</td>
</tr>
</tbody>
</table>

Ascorbic acid (Mol.wt 176.12) stock solution (10 mg/ml; 5.67 x10⁻² M), prepared by dissolving 500 mg of ascorbic acid in 50 mL distilled water and FBF (Mol.wt. 244.3) stock solution (10⁻² M) was prepared by dissolving 48mg FBF in 20 mL ethanol.

RECONSTITUTION OF BUFFER SOLUTIONS

a. Normal Krebs buffer solution

To reconstitute the normal Krebs buffer solution, stock solutions were mixed as follows: 100 mL of solution A, 100 ml of solution B, 100 mL of solution C, 1.8 mL of ascorbic acid and 300 µL of FBF. The final volume was made up to 1 liter.
with distilled water and pH adjusted to 7.45 using either HCl or NaOH solution. The buffer solution was then oxygenated and warmed to 37°C in a water bath for the duration of the experiment.

b. *Iso-osmotic, high potassium (KCl; 50mM) buffer solution*

To reconstitute the iso-osmotic, high potassium buffer solution, 100 mL of high potassium solution A, 100 ml of solution B, 100 mL of solution C, 1.8 mL of ascorbic acid and 300 µL of FBF stock solutions were mixed together and the final volume was made up to 1 liter with distilled water. The pH adjusted to 7.45 using either HCl or NaOH solution. The buffer solution was oxygenated and warmed to 37°C in water bath for the duration of the experiment.

1.2. TISSUE ISOLATION

Bovine eyes were obtained either from O’Neil Packing or Greater Omaha Packing Co., Omaha, NE within 4 hrs following enucleation and transported to the laboratory in an ice box. To isolate the retina, an incision was made along the equator of each eye followed by the removal of vitreous humor and lens. The retina was then isolated from the posterior segment and immersed in warm (37°C), oxygenated (95% O₂: 5% CO₂) Krebs buffer solution for Superfusion studies.
1.3 MEASUREMENT OF [³H]D-ASPARTATE RELEASE

The method used for assessment of [³H]D-aspartate release was essentially the same as previously described (Opere et al., 2009), with minor modifications. Isolated bovine retina were incubated in fresh oxygenated Krebs buffer solution containing 200 nM [³H]D-aspartate and FBF (300 µM) at 37°C for 1 hour. Following incubation, tissues were rinsed twice with 25 mL of non-radioactive, oxygenated Krebs buffer for 5 min each to remove excess tritium. Tissues were then mounted between nylon mesh and placed in thermostatically-controlled Superfusion Chambers, taking care to eliminate any residual air bubbles and superfused at a rate of 1.5 ml/min for the duration of the experiment. Following a 12-minute priming stimuli using the iso-osmotic, high potassium buffer solution, tissues were washed for one hour in order to establish a stable baseline. After the one hour washout period, fractional collection of the superfusates was initiated at 6 min.-intervals and analyzed for radioactivity by liquid scintillation counter.

To induce neurotransmitter release, iso-osmotic K⁺ (50 mM) buffer solution was applied at 80–88 min (S₁) and 114–124 min (S₂) after start of superfusion. Release of tritium caused by chemical stimulation was estimated by subtraction of the extrapolated basal tritium efflux from total tritium released during the 20 min period after the onset of stimulation. It was assumed that basal tritium efflux declined linearly between pre-stimulation and post-stimulation fractions. To examine the effects of the drug on K⁺-evoked [³H]D-aspartate release, the drugs were applied 12 min before S₁ and during S₂. To determine the effect of the
antagonist on responses to the drug, the antagonist was present in the buffer 30 min before the onset of S₁ and also during S₂. K⁺-evoked [³H]D-aspartate release during S₁ and S₂ was determined by comparing the areas of the two peaks (S₂/S₁ ratios).

1.4 DATA ANALYSIS

Results obtained from the [³H]D-aspartate release studies were expressed as counts (disintegrations per minute) or as absolute S₂/S₁ ratios of the K⁺-evoked [³H]D-aspartate release in control and treated preparations. Values given are the arithmetic means of the experimental values obtained ± standard error of the mean (SEM). Significance of differences between control and test values was determined using the analysis software by analysis of variance (ANOVA) test followed by Dunnett’s test (Graph Pad Prism software San Diego, CA). The level of significance selected was P<0.05.

2. TISSUE CULTURE STUDIES (MTT ASSAY)

2.1 Preparation of glutamate stock solutions

Glutamate-media stock solution was prepared by dissolving 146.8 mg glutamic acid hydrochloride (Molecular weight 183.59) in 40 mL of RPMI 1640 media (containing glutamine) to make 40 mL (20 mM) stock solution. This solution was used in preparation of other stock solutions and subsequent serial dilutions conducted as indicated on Table 2.
### Table 5: Preparation of glutamate solutions

<table>
<thead>
<tr>
<th>Aliquots used</th>
<th>Final volume and Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ml (of 20 mM stock) + 5 ml media</td>
<td>10 ml of 10 mM glutamate</td>
</tr>
<tr>
<td>6 ml (of 20 mM stock + 4 ml of media)</td>
<td>10 ml of 12 mM glutamate</td>
</tr>
<tr>
<td>8 ml (of 20 mM stock) + 2 ml of media</td>
<td>10 ml of 16 mM glutamate</td>
</tr>
<tr>
<td>5 ml (of 16 mM ) + 5 mL of media</td>
<td>10 ml of 8 mM glutamate</td>
</tr>
<tr>
<td>5 ml (of 8 mM) + 5 ml of media</td>
<td>10 ml of 4 mM glutamate</td>
</tr>
</tbody>
</table>

#### 2.2 Preparation of MTT reagent solution and lysing buffer

MTT stock solution (5mg/mL) was prepared by dissolving 30 mg of the MTT reagent i.e. (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) in 6 mL of the oxygenated Krebs buffer solution. Care was taken to protect MTT by covering the solution with an aluminium foil because MTT is light-sensitive and can undergo degradation. A mixture of Sodium Dodecyl Sulfate (SDS) and DiMethyl Formamide (DMF) was used as the lysing buffer for extraction. To prepare this lysing buffer, 20% of SDS in distilled water was mixed with an equal amount of DMF solvent (i.e. 20% SDS, 50% DMF).

#### 2.3 Isolation of tissue

Bovine eyeballs were dissected as described above on an ice-bath and vitreous humor removed to expose the retinal tissue. Each retina was punched using the
diameter of the back of a 1 mL pipette tip to make circular pieces of retinal tissues, weighing approximately 14-19 mg. The exact weight of each piece of circular tissue was recorded. Each tissue was then placed in a well (48-well plate) containing 500 µL of freshly prepared and oxygenated Krebs buffer solution. The well plate was kept on ice bath during the entire process of dissection, separation and weighing of retinal tissues.

2.4 Treatment

**Effect of glutamate on retinal neuron survival**

The well plate was transferred to a cell culture hood and the Krebs buffer solution was aspirated and discarded by removing 450 µL into a waste container. The first column of the well plate (8 cells) was treated with plain RPMI 160 media and used as control. The subsequent columns of wells were treated with 700 µL of varying concentrations of glutamate-media solution (4 mM, 8 mM, 10 mM, 12 mM and 16 mM). The well plate was the placed in an incubator 2 hours at 37°C. After 2 hours of incubation, 100 µL of the MTT reagent was aseptically added to each well in culture hood. The well plate was incubated for another 2 hours at 37°C.
**Effect of H₂S donors on glutamate-induced toxicity**

To determine the effect of H₂S donor drugs, tissues were treated with the different concentrations of drug solution (prepared as indicated above) for 1 hour prior to exposure to glutamate-enriched media.

**2.5 Post-treatment**

After the incubation period was completed, the well plate was placed in an ultracentrifuge and balanced with another similar plate containing water. The well plates were centrifuged at 3000 rpm for 6 minutes. After centrifugation, retinal tissues were settled to the bottom, facilitating removal of the supernatant to be discarded. 750 μL of SDS-DMF stock solution was then added to each of the well and the well plate wrapped with a parafilm and kept overnight in an incubator shaker at 37°C and 180 rpm for 20 hours. After 20 hours, 250 μL of the supernatant from each well was transferred to another well plate (same labeling maintained). For the five-fold dilution, 50 μL of the supernatant form each well was transferred to another well plate and each well was further diluted with 200 μL of SDS-DMF lysing buffer solution. Absorbance of each well was obtained at 540 nm and % absorbance was calculated. A graph was plotted for % absorbance (Y-axis) vs. glutamate concentrations (X-axis).
3. SOURCES OF DRUGS AND CHEMICALS

In this study, I will determine the effect of three classes of drugs: L-cysteine, a substrate for endogenous H$_2$S synthesis (Kimura, 2011); NAC, a prodrug which gets converted to L-cysteine and GYY 4137, a slow-releasing H$_2$S donor drug (Lee et al., 2011). A fast-releasing H$_2$S donor hybrid, ACS67 (Osborne et al., 2010) was used in the tissue culture studies. L-cysteine is an amino acid which is present in the human body whereas, GYY 4137 and ACS 67 are donors of H$_2$S. The antagonists used for the release study experiments were AOA, glibenclamide and L-NAME.

ACS 67 and GYY 4137 were purchased from Cayman Chemical Co., Ann Arbor, MI. L-cysteine, AOA and glibenclamide were obtained from Sigma Chemical Co., St.Louis, MO.

NAC was obtained from Alfa Aesar whereas L-methionine was obtained from Acros Organics. FBF was obtained from Fisher Scientific. Stock solutions of L-cysteine and AOA were made in water, whereas, those of GYY 4137, ACS 67 and glibenclamide were made in organic solvent dimethyl sulfoxide (DMSO).
RESULTS
Specific Aim # 1: To investigate the effects of \( \text{H}_2\text{S} \) donor drugs on the excitatory neurotransmitter release

1.1. Effects of agonist on \( \text{[^3H]}\text{D-aspartate release} \)

Application of high potassium (KCl; 50 mM) buffer stimuli to superfused retinal neurons incubated in \( \text{[^3H]}\text{D-aspartate} \) elicited tritium efflux over basal levels. In control experiments in which no drug was present, application of two subsequent stimuli during the fraction collection yielded two peaks of \( \text{[^3H]}\text{D-aspartate} \) overflow, as depicted in Figure 9. The area under curve (AUC) calculated for both the peaks \( S_1 \) and \( S_2 \) was almost the same. Thus, the \( S_2/S_1 \) ratios obtained from several control experiments in which no drug was added was almost equal to unity (1.033 ± 0.03; \( n=12 \)).

As illustrated in Figure 9, GYY 4137 (10 \( \mu \text{M} \)) inhibited the chemically-evoked \( \text{[^3H]}\text{D-aspartate} \) release without affecting the basal efflux of tritium. The drug was administered 12 min prior to application of the second chemical stimulus (\( S_2 \)). The slow-releasing \( \text{H}_2\text{S} \) donor, GYY 4137 attenuated efflux of tritium after the application of \( S_2 \), yielding an \( S_2/S_1 \) ratio less than one.
Figure 9. Effect of H$_2$S donor, GYY 4137 (10 µM) on KCl (K$^+$, 50 mM)-induced release of [³H]D-aspartate from isolated, superfused bovine retina. K$^+$ stimuli were applied at fractions 5/6 (S$_1$) and 13/14 (S$_2$). Left Panel, control (no agent present); Right Panel, GYY 4137 (10 µM) in presence of flurbiprofen (FBF; 3 µM), applied 12 min before S$_2$. Fractions of the superfusate were collected at 6 min. intervals and analyzed for radioactivity as described under Methods.

1.2. Effects of H$_2$S donor drugs on [³H]D-aspartate release

There is evidence that the fast-releasing H$_2$S donors, NaHS and Na$_2$S inhibit both sympathetic (Kulkarni et al., 2009) and excitatory neurotransmitter release (measured as [³H]D-aspartate) (Opere et al., 2009) in isolated bovine iris-ciliary bodies and retina, respectively. Since this effect has been described for the fast-releasing H$_2$S donors (NaHS and Na$_2$S) only, I examined the effect of different categories of H$_2$S donors on [³H]D-aspartate release in isolated bovine retina. The H$_2$S donor drugs tested in this aim fall under two categories:

1. Substrates for endogenous H$_2$S production
   a. L-cysteine (an endogenous substrate used in the production of H$_2$S)
   b. NAC (precursor to L-cysteine)
2. Slow-releasing H₂S donor drug

a. GYY 4137

As demonstrated in Figure 10, the slow-releasing H₂S donor, GYY 4137 (10⁻⁸M to 10⁻⁵M) exhibited a dose-dependent inhibition of [³H]D-aspartate release in superfused, isolated bovine retina. Within the concentration range tested, the 10⁻⁵M concentration eliciting the maximum inhibitory effect of 21.48 ± 3.52%.

![Figure 10: Effect of the slow H₂S donor, GYY 4137 (0.01-10 μM) on high K⁺-evoked [³H]D-aspartate release in isolated bovine retina in vitro. Vertical bars represent means ± SEM. Number of observations is in parenthesis. * P<0.05 significantly different from the control.](image)

Next, I examined the effect of L-cysteine, a substrates for endogenous production of H₂S via the enzymes, CSE and CBS on [³H]D-aspartate release. In the concentration range, 10⁻⁷M to 10⁻⁵M, L-cysteine inhibited K⁺-induced [³H]D-aspartate release in a dose-dependent manner, achieving a maximum inhibitory effect of 54.28 ± 3.61%, at the 10⁻⁵M concentration of the drug (Figure 11).
I further examined the effect of NAC, an acetylated derivative of the amino acid L-cysteine and a precursor to L-cysteine on the neurotransmitter release. Like L-cysteine, this substrate for endogenous production of H$_2$S exhibited a concentration-dependent inhibition of $[^3]$H]-aspartate release in isolated retinal tissue. Contrary to L-cysteine, NAC achieved a maximum inhibitory effect of 29.63%; p<0.05; n=5 at the $10^{-3}$M concentration (Figure 12).
Figure 12: Effect of the L-cysteine precursor, NAC(0.01-1 mM) on high K⁺-evoked [³H]D-aspartate release in isolated bovine retina *in vitro*. Vertical bars represent means ± SEM. Number of observations is in parenthesis. * P<0.05 significantly different from the control.

A comparison of the inhibitory action the three drugs (Figure 13, Table 6) revealed that at an equimolar concentration of 10⁻⁵M, the rank order of activity was as follows: L-cysteine > GYY 4137 > NAC. It is also pertinent to note that although NAC effect at 10⁻⁵M was minimal, its effect at 10⁻³M was significant (29.63%; p<0.05; n=5).
Figure 13: A comparison of the inhibitory effects of the slow H$_2$S donor, GYY 4137 (0.01-10 μM); endogenous substrate for H$_2$S synthesis, L-cysteine (0.1-10 μM) and the L-cysteine precursor NAC (0.01-1 mM) at equimolar concentration of 10$^{-5}$M on high K$^+$-evoked $[^3]$H]-D-aspartate release in isolated bovine retina in vitro. Vertical bars represent means ± SEM. Number of observations is in parenthesis. * P<0.05 significantly different from the control.
Specific Aim # 2: To investigate the mechanism of action of H$_2$S donor drugs

Having established the inhibitory effect of H$_2$S donor drugs on [$^3$H]D-aspartate release, I next determined the possible mechanisms of action of underlying the effect of H$_2$S donor drugs on the excitatory neurotransmitter release.

2.1. Role of H$_2$S-biosynthetic enzyme, CBS

There is evidence that CBS enzyme regulates the effect of H$_2$S donors on neurotransmitter release in ocular tissues (Kulkarni et al., 2009). For instance, the CBS inhibitor AOA reversed the effect of NaHS on sympathetic release in isolated
iris-ciliary bodies. (K. H. Kulkarni et al., 2009). Therefore, in this aim, I tested the effect of AOA on the inhibitory action of the H_{2}S producing compounds, L-cysteine, NAC and GYY 4137. Whereas AOA (3 mM) had no effect on K^{+}-induced [^{3}H]D-aspartate release, it reversed the inhibitory action of L-cysteine, NAC and GYY 4137 on neurotransmitter release (Figure 14, 15 and 16).

**Figure 14:** Effect of the CBS inhibitor AOA (3 mM) on L-cysteine (1-10 μM) mediated [^{3}H]D-aspartate release inhibition in isolated bovine retina *in vitro*. Vertical bars represent means ± SEM. Number of observations is in parenthesis. * P<0.001 significantly different from the control.
**Figure 15**: Effect of the CBS inhibitor AOA (3 mM) on NAC (0.1-1 mM) mediated [³H]D-aspartate release inhibition in isolated bovine retina *in vitro*. Vertical bars represent means ± SEM. Number of observations is in parenthesis. * P<0.05 significantly different from the control.

**Figure 16**: Effect of the CBS inhibitor AOA (3 mM) on GYY 4137 (1-10 μM) mediated [³H]D-aspartate release inhibition in isolated bovine retina *in vitro*. Vertical bars represent means ± SEM. Number of observations is in parenthesis. * P<0.05 significantly different from the control.
2.2. Role of potassium $K_{ATP}$ channels

In the next series of experiments, I examined the involvement of $K_{ATP}$ channels on the inhibitory action of the $H_2S$ donor drugs on excitatory neurotransmitter release. There is evidence that glibenclamide, a $K_{ATP}$ channel inhibitor antagonizes the relaxant action of NaHS in porcine irides (Monjok et al., 2008). Therefore, in this aim, I examined the effect of glibenclamide (300 $\mu$M) on $H_2S$-donor induced-inhibition of excitatory neurotransmitter release in bovine retina. Although glibenclamide (300 $\mu$M) had no effect on $K^+$-induced $[^3H]$D-aspartate release, it reversed the inhibitory action of L-cysteine and GYY 4137 on neurotransmitter release (Figure 17, 19). Interestingly, glibenclamide had no effect on the inhibitory action of NAC on the neurotransmitter release (Figure 18).

![Graph showing the effect of glibenclamide on $[^3H]$D-aspartate release](image)

**Figure 17**: Effect of the $K_{ATP}$ channel blocker, glibenclamide (300 $\mu$M) on L-cysteine (1-10 $\mu$M) mediated $[^3H]$D-aspartate release inhibition in isolated bovine retina *in vitro*. Vertical bars represent means ± SEM. Number of observations is in parenthesis. * P<0.001 significantly different from the control.
Figure 18: Effect of the K<sub>ATP</sub> channel blocker, glibenclamide (300 μM) on NAC (1 mM) mediated [3H]-D-aspartate release inhibition in isolated bovine retina in vitro. Vertical bars represent means ± SEM. Number of observations is in parenthesis. * P<0.05 significantly different from the control.

Figure 19: Effect of the K<sub>ATP</sub> channel blocker, glibenclamide (300 μM) on GYY 4137 (1-10 μM) mediated [3H]-D-aspartate release inhibition in isolated bovine retina in vitro. Vertical bars represent means ± SEM. Number of observations is in parenthesis. * P<0.05 significantly different from the control.
Specific Aim # 3: To determine the role of gasotransmitter, nitric oxide (NO) in the regulation of H₂S activity

3.1. Effect of NOS inhibitors L-NAME and aminoguanidine

The gasotransmitter, NO has been shown to interact with H₂S in various tissues (Li et al., 1997). Therefore, in the next series of experiments, I sought to determine the possible interaction between H₂S and NO in isolated bovine retina. It was interesting to note that the non-specific inhibitor of NOS, L-NAME (300 µM) had no effect on K⁺-induced [³H]D-aspartate release (Figure 20). However, L-NAME (300 µM) completely reversed the effects of L-cysteine and GYY 4137 on [³H]D-aspartate release (Figure 20; Figure 21).

![Figure 20: Effect of the non-specific NOS inhibitor L-NAME (300 µM) on L-cysteine (1-10 µM) mediated [³H]D-aspartate release inhibition in isolated bovine retina in vitro. Vertical bars represent means ± SEM. Number of observations is in parenthesis. * P<0.01 significantly different from the control.](image)
Figure 21: Effect of the non-specific NOS inhibitor L-NAME (300 µM) on GYY 4137 (1-10 µM) mediated [³H]D-aspartate release inhibition in isolated bovine retina in vitro. Vertical bars represent means ± SEM. Number of observations is in parenthesis. * P<0.05 significantly different from the control.

To further delineate the role NO in the inhibitory effect of H₂S donors, I investigated the effect of the specific inhibitor of inducible NOS (iNOS), aminoguanidine (Corbett & McDaniel, 1996) H₂S-induced inhibition of the excitatory neurotransmitter release. Although aminoguanidine (10 µM) had no effect on the neurotransmitter release, it reversed the inhibitory effects of L-cysteine on [³H]D-aspartate release (Figure 22).
Figure 22: Effect of the specific iNOS inhibitor aminoguanidine (10 μM) on L-cysteine (1-10 μM) mediated [3H]D-aspartate release inhibition in isolated bovine retina in vitro. Vertical bars represent means ± SEM. Number of observations is in parenthesis. * P<0.05 significantly different from the control.

Specific aim # 4: Role of H₂S donors in neuroprotection

To investigate the ability of H₂S donor drugs to protect retinal neurons from glutamate-induced toxicity, cell survival was assessed by the MTT assay following exposure to glutamate.

4.1. Determination of experimental conditions

Initially, I sought to optimize experimental parameters for this study by determining concentration of glutamate that could induce retina neuron death by about 50% and determine the appropriate treatment time of exposure that induced sub-maximal retinal survival. As depicted in Figure 23, in preliminary experiments, exposure of
retinal neurons to glutamate (0.5 mM to 32 mM) for two hours induced cell death in a concentration-dependent manner.

**Figure 23:** A preliminary dose-response curve of glutamate (0-32 mM) showing the toxic effects of glutamate on retinal neuronal tissue survival. Vertical bars represent means ± SEM. * P<0.001 significantly different from the control.

There was a tremendous drop in magnitude of retinal neuron survival when glutamate concentration was increased from 8 mM to 16 mM. Therefore, in subsequent experiments, lower concentrations of glutamate were used. Figure 24 depicts the dose-response curve to lower concentrations of glutamate. Interestingly, a 12 mM concentration of glutamate achieved approximately 50% retinal neuron death.
Figure 24: A time-dependent study involving a dose-response curve of glutamate (0-16 mM) with 2 hours incubation period showing the toxic effects of glutamate on retinal neuronal tissue survival. Vertical bars represent means ± SEM. * P<0.001 significantly different from the control.

To confirm that 2-hours period of incubation with glutamate was sufficient for the experiment, I further compared the effect of incubating retinal neurons for 2-hours to that of 6-hours. As shown in Figure 25, a 6-hour incubation period enhanced neuronal death, achieving about 75% cell neuron death at 12 mM glutamate concentration. Further experiments were therefore conducted using the 2-hour incubation period with glutamate (12 mM).
Figure 25: A time-dependent study involving a dose-response curve of glutamate (0-16 mM) with 6 hours incubation period showing the toxic effects of glutamate on retinal neuronal tissue survival. Vertical bars represent means ± SEM. * P<0.001 significantly different from the control.

4.2. Effect of H₂S donors on glutamate-induced toxicity

Next I examined the effect of H₂S donors on glutamate-induced toxicity. Retinal neurons were pretreated with the H₂S donor drugs for one-hour prior to incubation with glutamate. In addition to GYY 4137, L-cysteine, NAC, other H₂S donor drugs tested included L-methionine, another substrate for endogenous production of H₂S, ACS 67, a hybrid of latanoprost and a H₂S-donating moiety and latanoprost which was used as a control for ACS 67 response (Figure 28).
As shown in Figure 26 and Table 7, I investigated the effect of the substrates for endogenous production of H$_2$S, L-cysteine ($10^{-8}$ M to $10^{-3}$M), NAC ($10^{-6}$ to $10^{-3}$M), L-methionine ($10^{-8}$M to $10^{-5}$M) on glutamate-induced toxicity. It was interesting to note that the protective action of the H$_2$S producing compounds did not exhibit a dose-dependent action on glutamate-induced toxicity. Thus, L-cysteine significantly reversed glutamate-induced toxicity by 31.2 ± 9.90%; (p<0.05; n=16) at $10^{-3}$M concentration (Figure 27 and Table 7), while NAC and L-methionine significantly
reversed glutamate-induced toxicity by 18.43% ± 2.64 (p<0.05; n=16; 10⁻⁸M) and 22.14 ± 4.25% (p<0.05; n=16; 10⁻⁸M), respectively (Table 7).

![Graph showing survival rates with different treatments.]

**Figure 27:** Effect of L-cysteine (0.1 μM-1 mM) treatment on glutamate (12 mM)-induced retinal neuronal toxicity. Vertical bars represent means ± SEM. ** P<0.05 significantly different from the glutamate treatment.

In the next series of experiments, I examined the effect of the slow releasing H₂S donor, GYY 4137 (10⁻⁸M to 10⁻⁵M) on glutamate-induced toxicity. As depicted in Table 7, GYY 4137 (10⁻⁸M) significantly reversed glutamate-induced toxicity by 22.1 ± 3% (p<0.05; n=16).

I further examined the effect of the latanoprost-H₂S donating moiety, ACS 67 (10⁻¹¹M to 10⁻⁷M) and compared it to that of latanoprost (10⁻¹⁰M to 10⁻⁷M; used as a control) on glutamate-induced toxicity in retinal neurons. ACS 67 (10⁻¹¹M) reversed glutamate-induced toxicity by 28.98 ± 3.82% (p<0.05; n=16) while latanoprost reversed glutamate-induced toxicity by 18.98 ± 0.61% (p<0.05; n=16), suggesting
that the H$_2$S-moiety contributes to the additional effect of ACS 67 (Figure 28, Table 7).

**Figure 28:** Effect of ACS 67 (0.1 μM, 1 nM and 10 pM) treatment on glutamate (12 mM)-induced retinal neuronal toxicity. Vertical bars represent means ± SEM. * P<0.05 significantly different from the glutamate treatment.
Table 7: A comparison of the effects of various H₂S donors on glutamate-induced cell death in the MTT assay

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. Range tested</th>
<th>Max. Effective drug conc.</th>
<th>Max. Magnitude of reversal (±sem)</th>
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</thead>
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<tr>
<td>L-cysteine</td>
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<td>10⁻³M</td>
<td>31.17 ± 9.90</td>
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<tr>
<td>N-acetyl-L-cysteine</td>
<td>10⁻⁶M to 10⁻³M</td>
<td>10⁻⁶M</td>
<td>18.43 ± 2.64</td>
</tr>
<tr>
<td>L-methionine</td>
<td>10⁻⁶M to 10⁻⁵M</td>
<td>10⁻⁸M</td>
<td>22.14 ± 4.25</td>
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<td>GYY 4137</td>
<td>10⁻⁶M to 10⁻⁵M</td>
<td>10⁻⁸M</td>
<td>22.10 ± 3.00</td>
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<td>ACS 67</td>
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<td>28.98 ± 3.82</td>
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<tr>
<td>Latanoprost</td>
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<td>10⁻¹⁰M</td>
<td>18.98 ± 0.61</td>
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</table>
DISCUSSION
H₂S, a gaseous molecule that has been known as an industrial toxicant for over a century was recently accepted as the third gasotransmitter in mammalian tissues. It is endogenously synthesized from its major substrate, L-cysteine via the “transsulfuration pathway” using the pyridoxal-5-phosphate-dependent enzymes, CBS and CSE (Abe & Kimura, 1996; H. Kimura, 2011). A third enzyme, 3-MST, acting together with CAT has been reported to generate H₂S from L-cysteine in the presence of α-ketoglutarate (Kimura, 2011). Current research suggests that CBS is predominantly localized in the CNS while CSE in the cardiovascular system. H₂S acts on various cellular targets, where it modulates several physiological functions of the body, including the CNS and CVS. In ocular tissues, evidence suggests the presence of a functional transsulfuration pathway (Persa et al, 2004). Indeed, this gasotransmitter is present in ocular tissues, including iris, retina and the cornea in detectable concentrations (Kulkarni et al., 2011). Moreover, both CBS and CSE have been localized in ocular tissues (Pong et al., 2007). In spite of data supporting a physiological function for H₂S in ocular tissues, its exact role has not been fully elucidated, warranting further investigation.

Effect of H₂S donor drugs on [³H]D-aspartate release

There is evidence that H₂S donor compounds accurately and sufficiently generate H₂S in biological systems in a reproducible manner (Abe & Kimura, 1996). Thus, H₂S donor drugs emulate the effect of H₂S gas in biological experiments. A majority of studies of ocular effects of H₂S reported in literature utilized fast H₂S donor drugs such as NaHS or Na₂S as the H₂S source (Kulkarni et al., 2009; Njie-Mbye et al., 2010; Ohia et al., 2010;
Opere et al., 2009). For instance, the fast H₂S donor drugs, NaHS and Na₂S have been shown to attenuate electrically-induced [³H]NE and potassium-induced [³H]D-aspartate release in mammalian irides (K. H. Kulkarni et al., 2009) and retinae (Opere et al., 2009). So far, no studies have compared the effect of H₂S donors that generate the gasotransmitter by a variety of mechanisms in mammalian retina. Therefore, I compared the pharmacological actions elicited by three categories of H₂S donor drugs on K⁺-induced [³H]D-aspartate release in isolated bovine retina: L-cysteine, a substrate for in situ biosynthesis of H₂S by CBS and CSE enzymes (H. Kimura, 2011); NAC, an acetylated derivative of L-cysteine which is a prodrug for L-cysteine and an endogenous precursor to glutathione (http://www.drhoffman.com/page.cfm/326) and GYY 4137, a slow-releasing/slow donor of H₂S (Lee et al., 2011). Similar to the aforementioned fast H₂S donor drugs, all the three H₂S donors elicited a concentration-dependent inhibitory effect on the [³H]D-aspartate release in isolated bovine retina without affecting the basal tritium efflux. At an equimolar concentration of 10 µM, the drugs exhibited the following rank order of activity: L-cysteine > GYY 4137 > NAC. It was interesting to note that L-cysteine, the substrate for in situ enzymatic production of H₂S elicited the highest activity (IC₅₀ value of 9.2 µM) of all the three H₂S donor drugs used in the study. I also found that it was necessary to pre-treat tissues with the L-cysteine for a longer period of time (18 mins as opposed to 12 mins) to facilitate biosynthesis of H₂S. This increase in exposure time could account for the higher level of activity exhibited by L-cysteine. The acetylated derivative of L-cysteine, NAC, is more stable than L-cysteine and acts as a prodrug for L-cysteine to facilitate its systemic delivery into the body. Like L-cysteine, NAC was exposed to retinal tissues for 18 mins before the chemical stimulus was
applied. However, NAC exhibited the least inhibitory action of all the H₂S donors tested (IC₂₅ value of 843.5 µM). Since NAC is an acetylated prodrug for L-cysteine, conversion to L-cysteine is necessary to elicit a pharmacological action. This conversion may account for its low level of activity among the H₂S donor drugs tested. GYY 4137 is a slow H₂S donor which releases the gas at a slow and sustained rate without the burst release phenomenon (Lee et al., 2011). Compared to L-cysteine and NAC, GYY 4137 elicited a moderate (IC₂₅ value of 35.34 µM) inhibitory effect on the neurotransmitter release. The absence of burst release could account for the modest inhibitory response exhibited by GYY 4137 on neurotransmitter release. It is also possible that a longer duration of treatment time prior to stimulation would have supported a more potent action of this H₂S donor drug on the neurotransmitter release. Under similar experimental conditions, Opere et al reported IC₅₀ values for NaHS and Na₂S on excitatory neurotransmitter release in bovine retina of 0.006 µM and 6.0 µM, respectively, suggesting that these fast H₂S donor drugs are more potent than the drugs used in this study (Opere et al., 2009). It is conceivable that the burst release of H₂S contributes to the apparent superior potency exhibited by fast H₂S donor drugs. Taken together, this study supports an inhibitory action of H₂S donor drugs on excitatory neurotransmitter release. Furthermore, the magnitude of activity of H₂S donors is partially dependent on the burst release of H₂S.
Determination of mechanism of action of H$_2$S donor drugs

a. Role of endogenous biosynthesis of H$_2$S

Having established the inhibitory effect of H$_2$S donor drugs on excitatory neurotransmitter release, I next sought to determine the mechanism action underlying this inhibitory response elicited by the H$_2$S donor drugs. There is evidence that endogenous synthesis of H$_2$S plays a role in the pharmacological actions of H$_2$S donor drugs in biological systems. For instance, Zhang and coworkers (2011) reported that AOA, a CBS inhibitor reversed the protective effects of H$_2$S to radioresistance caused by hypoxia on human hepatoma cells (Zhang et al., 2011). Similarly, in ocular tissues, AOA reversed the inhibitory effect NaHS on sympathetic neurotransmitter release (Kulkarni et al., 2009). In this study, AOA completely antagonized the inhibitory effect of L-cysteine on K$^+$-induced [$^3$H]D-aspartate release in the bovine retina. Since L-cysteine is a substrate for endogenous biosynthesis of H$_2$S, it was not surprising that its activity was nullified by the CBS inhibitor, AOA. Corroborating this observation, AOA attenuated the inhibitory effect of L-cysteine in isolated porcine irides (Ohia et al., 2010). Similarly, it reversed the L-cysteine mediated increase in cAMP production in rat retinal pigment epithelial cells, (Njie-Mbye et al., 2012). Together, these studies affirm the involvement of in situ H$_2$S biosynthesis in the pharmacological effects elicited by L-cysteine in these tissues.

It was surprising to note that AOA completely reversed the inhibitory action elicited by GYY 4137 on K$^+$-induced [$^3$H]D-aspartate release in the bovine retina. Consistent with my observations, Chitnis et al (2013) recently reported that AOA (30 µM)
reversed the inhibitory effects of GYY 4137 on phenylephrine-induced tone in isolated bovine ciliary artery (Chitnis et al., 2013). Contrary to L-cysteine which is a substrate for endogenous biosynthesis of H₂S, GYY 4137 releases H₂S slowly in aqueous medium (Lee et al., 2011). Therefore, its ability to generate H₂S should be independent of in situ biosynthesis of H₂S. It is conceivable that under my experimental conditions, endogenous biosynthesis of H₂S contributes the observed GYY 4137 response. It is possible to speculate that either H₂S-biosynthetic enzymes and/or the presence of high potassium (K⁺; 50 mM) stimulus facilitate the release of H₂S from GYY 4137. In support of this hypothesis, Teague et al (2002) reported that application of electrical stimulus could trigger endogenous biosynthesis of H₂S in mouse cerebral cortical slices treated with H₂S donor drugs (Teague et al., 2002). Clearly, more studies are necessary to delineate the exact role of H₂S biosynthesis on effects elicited by GYY 4137.

NAC is an acetylated prodrug for L-cysteine that facilitates systemic delivery of L-cysteine into the body. Following its absorption into the tissues, NAC is deacetylated to release L-cysteine which then participates in various physiological processes, including biosynthesis of H₂S gasotransmitter. In this study, the effect of AOA on NAC-induced inhibition of excitatory neurotransmitter release was mixed. Whereas it completely reversed the inhibitory effect elicited by NAC (10⁻⁴M), AOA only partially antagonized the effect elicited by the higher concentration of NAC (10⁻³M) on the neurotransmitter release. It is possible that at higher concentrations, NAC inhibits excitatory neurotransmitter release by recruiting other mechanisms, in addition to release of H₂S. In summary, these studies demonstrate that, although all
the three H₂S donor drugs examined in this project generate H₂S by different mechanisms, endogenous biosynthesis of H₂S plays an important role in their inhibitory activity on excitatory neurotransmitter release in bovine retina.

b. Role of K<sub>ATP</sub> channels

There is evidence that K<sub>ATP</sub> channels are involved in the pharmacological actions elicited by H₂S donor drugs in various biological systems (Wang, 2012). For example, the role of H₂S in neuroprotection against glutamate toxicity has been reported to correlate to its K<sub>ATP</sub> channel-opening activity (Wang, 2012). In ocular tissues, the K<sub>ATP</sub> channel inhibitor, glibenclamide reversed the inhibitory effects of NaHS on sympathetic release in porcine irides (Monjok et al., 2008). In the present study, glibenclamide completely reversed the inhibitory effects of [³H]D-aspartate release mediated by L-cysteine and GYY 4137, suggesting the involvement of K<sub>ATP</sub> channels in the action of these H₂S donors drugs on K⁺-induced [³H]D-aspartate release. Consistent with these observations, glibenclamide abolished the gastroprotective effects of L-cysteine against ethanol-induced toxicity in mice (Medeiros et al., 2009). Similarly, it antagonized GYY 4137-induced relaxation in isolated bovine ciliary artery (Chitnis et al., 2013). Interestingly, this K<sub>ATP</sub> channel inhibitor had no effect on NAC-induced inhibition of excitatory neurotransmitter release, further supporting the notion that NAC activity involves other mechanisms, in addition to release of H₂S. Taken together, these observations suggest that unlike L-cysteine and GYY 4137 whose activity is mediated by K<sub>ATP</sub> channels, NAC inhibits excitatory neurotransmitter activity by K<sub>ATP</sub>-channel-independent mechanisms.
3. Role of gasotransmitter NO in H₂S activity

The relationship between the two gasotransmitters, NO and H₂S is reportedly complex and contradictory, being both antagonistic and additive in various tissues (Wang, 2012). For example, Li et al (1997) reported an NO-mediated relaxation and increase in cGMP production in rabbit aorta that was inhibited by L-cysteine and L-homocysteine, substrates for H₂S production (Li et al., 1997). This study suggested that H₂S nullifies the relaxant effect elicited by NO on vascular tissues. On the contrary, Hosoki and coworkers demonstrated that SNP, a NO donor and NaHS, the fast H₂S donor drug exhibited additive relaxant effects on rat aortic tissue (Hosoki et al., 1997). Moreover, treatment of cultured vascular smooth muscle cells with a NO donor upregulated the expression transcriptional levels of CSE, a H₂S producing enzyme found predominantly in the cardiovascular system (Zhao et al., 2001). Although conflicting in nature, these reports collectively affirm the complex pharmacological interactions between H₂S and NO gasotransmitter pathways. Furthermore, a majority of these studies focused on the interaction between H₂S with NO in the vascular system. In the present study, I investigated the role of NO on inhibitory action elicited by H₂S donor drugs on retinal neurons in bovine retina. It was interesting to note that L-NAME, a potent inhibitor of the constitutive NOS (consisting of eNOS and nNOS isoforms), reversed the inhibitory effects of L-cysteine and GYY 4137 on [³H]D-aspartate release. Moreover, the potent and selective inhibitor of the inducible NOS (iNOS) (Corbett & McDaniel, 1996) abolished the inhibitory effects elicited by L-cysteine on [³H]D-aspartate release. Thus my study supports an inhibitory interaction between H₂S and NO.
gasotransmitters. Together, my data suggests that NO generated from different isoforms of NOS (nNOS, iNOS and eNOS) modulate the pharmacological activity of H\textsubscript{2}S on excitatory neurotransmitter release. However, the current study does not reveal the predominance of individual NOS isoforms on the regulation of neurotransmitter release. Clearly, more studies are necessary to delineate the exact role of NOS each NOS isoform on the inhibitory effects of H\textsubscript{2}S donor drugs on excitatory neurotransmitter release.

4. Role of H\textsubscript{2}S donor drugs in neuroprotection

It is well established that excessive elevation of glutamate leads neuronal toxicity. Consequently, this excitatory neurotransmitter is routinely used to induce experimental toxicity in biological systems (Pereira & Oliveira, 2000). Thus, the ability of H\textsubscript{2}S donor drugs to inhibit glutamate release suggests a potential neuroprotective role for H\textsubscript{2}S in neurons. In support of this hypothesis, several studies have demonstrated the neuroprotective effect of H\textsubscript{2}S in biological tissues (Whiteman et al., 2004, 2005). For instance, H\textsubscript{2}S protected neurons from toxic effects of high levels of glutamate (Betowski, 2007) and cultured human neuroblastoma (SH-SY5Y) cells from peroxynitrite-induced and hypochlorous acid-induced toxicity (Whiteman et al., 2004, 2005). Therefore, in this aim, I sought to determine the role of H\textsubscript{2}S on glutamate-induced toxicity on bovine isolated retina using the MTT assay. The MTT assay is based on the fact that living tissues contain mitochondrial dehydrogenase enzyme, which can cleave the tetrazolium ring in the MTT reagent {3-(4,5-


Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide) to produce purple formazan crystals. Therefore, quantification of formazan crystals so formed presents an accurate assessment for survival in tissue viability experiments.

In order to determine the optimal concentration of glutamate that induces about 50% retinal neuron death, tissues were treated with glutamate (0-32 mM) for two hours and neuron survival assessed by the MTT method. This yielded a steep decrease in the tissue survival for glutamate concentrations between 8 mM to 16 mM, prompting me to conduct further experiments using a shorter range of glutamate concentration (4, 8, 10, 12 and 16 mM). Under my experimental conditions, the EC₅₀ (concentration of glutamate inducing about 50% retinal death) was approximately 12 mM. To further refine my experimental conditions, I conducted time-dependent experiments by pretreating tissues with glutamate for six instead of two hours. This yielded EC₅₀ value of approximately 10 mM while the 12 mM concentration of glutamate induced about 75% tissue death. Thus there was no apparent advantage of conducting these experiments using a longer duration of glutamate-pretreatment time. Subsequent experiments were therefore conducted using the two-hour glutamate pretreatment time. Interestingly, other investigators have reported a glutamate-induced, concentration-dependent neurotoxicity in neurons. For example, in embryonic rat cerebral cortex cultures, glutamate elicited a concentration-dependent increase in the release of lactate dehydrogenase (LDH), with an EC₅₀ = 50 μM (Amin & Pearce, 1997). It is pertinent to note that these researchers used neuronal cell cultures and assessed cell death by measuring release of LDH while in this study, I utilized MTT assay to measure survival in whole retinal neurons.
Having defined my experimental conditions, I next determined the effect three different categories of H₂S donor drugs on glutamate-induced neuron toxicity:

1. Substrates for H₂S synthesis such as L-cysteine, NAC and L-methionine
2. Slow H₂S-releasing drug, GYY 4137
3. Fast H₂S-releasing hybrid, ACS 67 (the prostaglandin latanoprost was used as control).

It was surprising to note that the protective effect of H₂S donor drugs was not concentration-dependent. Although a wide range of concentration of H₂S donor drugs (L-cysteine [10⁻⁹M to 10⁻³M]; NAC [10⁻⁶M to 10⁻³M]; L-methionine [10⁻⁸M – 10⁻⁵M]) were examined, this protective effect was very discrete and concentration-specific. It was even more surprising that both NAC and L-methionine that require conversion to L-cysteine prior to biosynthesis of H₂S were more potent that L-cysteine. Specifically, a higher concentration of L-cysteine (10⁻³M) reversed neuron death by 31.17 ± 9.90%. On the contrary, NAC and L-methionine conferred protection from glutamate-induced toxicity at lower concentrations 10⁻⁶M (18.43 ± 2.64%) and 10⁻⁸M (22.14 ± 4.25%) respectively, but with a lower magnitude of protection. It is possible to speculate that both NAC and L-methionine are more hydrophobic than L-cysteine and can thus display superior access to retinal tissues than L-cysteine, accounting for the higher potency of action. The extra conversion step necessary to convert these H₂S donors to L-cysteine prior to biosynthesis of H₂S could be lead to a delay in production of H₂S, thereby leading to the lower magnitude of action, compared to L-cysteine. It is also possible that these H₂S producing drugs
act exert their protective effect by mechanisms that are independent of H₂S biosynthesis.

The protective effect exhibited by slow H₂S donor drug, GYY 4137 (range tested, 10⁻⁸M to 10⁻⁵M; 10⁻⁸M produced 22.1 ± 3.00%) was comparable to that of L-methionine. Moreover, its protective effect was much more potent than that displayed by both L-cysteine and NAC. The rank order of potency based upon the neuroprotective concentration was as follows: GYY 4137 (10⁻⁸M) > NAC (10⁻⁶M) > L-cysteine (10⁻³M). This was contrary to the pattern observed for inhibition of excitatory neurotransmitter release in specific aim #1 above (L-cysteine > GYY 4137 > NAC), suggesting that the mechanisms underlying excitatory neurotransmitter release are distinct from those conferring protection from glutamate-induced toxicity in retinal neurons. It is also possible that the mechanism of H₂S production from these H₂S donors contribute to their pharmacological actions in these tissues. The hybrid of latanoprost and a H₂S-donating moiety, ACS 67 exhibited the highest potency, protecting retina neurons from glutamate-induced toxicity in the picomolar range (28.98% at 10⁻¹¹M). Moreover, this effect was 10-fold higher than that of latanoprost (18.98% at 10⁻¹⁰M), suggesting that the H₂S-donating moiety contributes to the additional neuroprotective effect of this hybrid compound. The superior potency displayed by ACS 67 is consistent with that of latanoprost, a prostaglandin FP analogue that is used for treatment of glaucoma at very low concentrations (50mcg/mL). Interestingly, these H₂S producing compounds have been shown to protect tissues from noxious stimuli in other biological systems (Osborne et al., 2010). For example, L-cysteine conferred neuroprotection from lanthanum induced
damage in adult rat brains by restoring the whole brain total antioxidant status (TAS) (S & Department of Pharmacology, Medical School, University of Athens, Athens, 2009). NAC protected spinal motor neurons following spinal cord injury (Karaliija et al., 2012). Similarly, GYY 4137 attenuated neuronal toxicity in Parkinson’s disease (Vandiver et al., 2013) while ACS 67 attenuated peroxide-induced retinal ischemia and oxidative toxicity in RGC-5 cells i.e. retinal ganglion cells (Osborne et al., 2010). Collectively, these studies affirm the protective effect of these H2S donors in biological systems. It is also apparent that the mechanisms underlying the neuroprotective effect of these H2S donor drugs is complicated and merits further research.

**Significance of study**

Previous literature reports on H2S were conducted using inorganic, fast-releasing H2S donors such as NaHS and Na2S. So far, no studies have ranked the pharmacological effects of H2S donors. To the best of my knowledge, this is the first study that has categorically compared the pharmacological effects of various H2S donor drugs (substrates, slow-releasing and fast-releasing donors) on the same tissue. Furthermore, these studies affirm the pharmacological role of H2S in ocular tissues.

Optic neuropathies are a group of disorders associated with dysfunction of the optic nerve along with and compromised visual function (Osborne et al., 2004). RGC degeneration is a common characteristic in optic neuropathies (Osborne et al., 2004). Therefore, RGC degeneration presents a common pathway for most of the optic nerve
disorders, including glaucomatous optic neuropathy (Shahsuvaryan, 2013). More specifically, glaucoma, a progressive neurodegenerative disorder of the eye, is one of the leading causes of blindness worldwide. It is characterized by the death of RGCs, atrophy of the optic nerve and subsequent loss of visual field function. Therefore, prevention of RGC death presents a significant therapeutic rationale in the treatment of glaucoma and other ocular disorders. Since H$_2$S donors have been shown to decrease IOP (Perrino et al., 2009), and my study has confirms the protective role of in retinal neurons, H$_2$S donor drugs could present a new class of drugs for management of glaucoma and other ocular neuropathies. Moreover, these drugs could play a neuroprotective role in central nervous system neurodegenerative conditions such as Parkinson’s disease and Alzheimer’s disease as well.

**Conclusion**

In conclusion, all the three H$_2$S donors attenuated K$^+$-induced [$^3$H]D-aspartate release in isolated bovine retinae with the following rank order of activity: L-cysteine > NAC > GYY 4137. Unlike NAC, the pharmacological activity of L-cysteine and GYY 4137 was dependent on the on the in situ release of H$_2$S and activation of K$_{ATP}$ channels. NO was involved in the pharmacological actions of L-cysteine. Furthermore, the H$_2$S donor drugs protected retinal neurons from glutamate-induced toxicity, suggesting a potential therapeutic role for H$_2$S donor drugs in management of neurodegenerative conditions.
Future Directions

The present study demonstrates the attenuation of excitatory neurotransmitter release and subsequent neuroprotection by H$_2$S donor drugs, *in vitro*. Further studies are needed to affirm the neuroprotective role of H$_2$S donors in an in animal *in vivo* model of ischemia/oxidative stress. This could have a significant impact in the treatment of glaucoma, since retinal neurodegeneration is one of the characteristic pathology in glaucoma. Since H$_2$S has been reported to decrease IOP, these drugs could offer a potential therapeutic advantage over the current drugs used in management of glaucoma. Additionally, the role of NO and other gasotransmitters like CO on the activity of H$_2$S merit further evaluation. Experiments should be conducted using NO donor and specific NOS inhibitors to determine the exact role played by the different NOS isoforms in the retina. Further studies are needed to clarify other mechanisms underlying the pharmacological actions of H$_2$S in ocular tissues.
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