UNIVERSITY OF CALGARY

Neurotoxicity of Sour Gas and Hydrogen Sulphide

by

Lisa M. Strosher

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

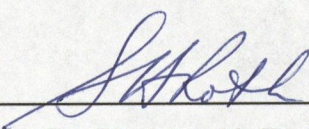DEGREE OF MASTER OF SCIENCE

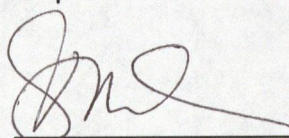DEPARTMENT OF NEUROSCIENCE

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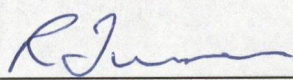**UNIVERSITY OF CALGARY**

FACULTY OF GRADUATE STUDIES

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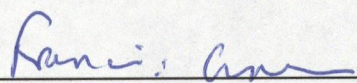
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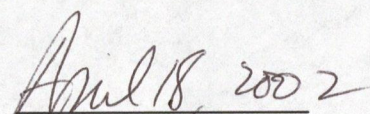
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ABSTRACT

Natural gas containing hydrogen sulphide (H_2S) is termed "sour gas", an environmental and occupational toxicant. The toxicity of sour gas has generally been attributed to the presence of H_2S. At high concentrations, H_2S causes central nervous system (CNS) effects; e.g. fatigue, dizziness, anxiety, headache, convulsions and unconsciousness. However, the effects of low concentrations of H_2S are not well understood; low-level exposures are associated with anxiety, cognitive dysfunction, fatigue and memory loss, but no direct evidence for these effects exists. The purpose of this research was to determine the effects of low concentrations of sour gas and H_2S on synaptic transmission in the *in vitro* rat hippocampal slice and examine possible mechanisms of action. Low concentrations of sour gas and H_2S cause long-lasting enhancement of synaptic transmission that is NMDA receptor-dependent. These effects may provide support for the CNS symptoms reported in humans following exposure to low-level sour gas and H_2S.

ACKNOWLEDGEMENTS

DEDICATION

This thesis is dedicated to my husband Patrick, my children Cody

and Max and my parents Mel and Sharon.

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1.0 Introduction

1.1 Sour Gas

1.1.1 Overview

Occupational and community exposure to hydrogen sulphide (H_2S), a colourless and flammable gas that is considered to be a very dangerous industrial and environmental pollutant, is a significant problem in the sour gas industry (Guidotti, 1996). "Sour gas" is a mixture of natural gas containing varying amounts of H_2S. For example, a pipeline is considered 'sour' if it contains greater than 1% H_2S or 10,000 parts per million (ppm), however a well or processing facility is a 'sour gas worksite' if it contains greater than 0.001% or 10 ppm H_2S (PCF, 2000). Approximately 30% of all natural gas produced in Canada is sour. The majority of this sour gas is found in central and western Alberta and northeastern British Columbia, with Alberta accounting for ~85% of the production (PCF, 2000).

The average H_2S content in a typical well in Alberta is 10% (10,000 ppm), although there is a large range from trace levels up to 90% (Guidotti, 1994). There are 277,000 km of oil and gas pipeline, 33,000 oil wells and 47,000 gas wells, including 5,000 sour gas wells in Alberta. These numbers are expected to increase significantly over the next two years (Suzuki, 2001). The potential for leakage and accidental release of sour gas and therefore H_2S, the major toxic

component of sour gas, from sour gas facilities has led to increased public concern over subsequent adverse health effects (Kilburn, 1999). Individuals that live in close proximity to sour gas processing facilities are confronted with the risk for repeated exposures due to atmospheric pollution (Bhambhani, 1999).

A 'critical' sour gas well (> 4% H_2S) has the potential for a large H_2S release that may affect human and animal populations and result in the emergency evacuation of communities that live in the immediate area. It may also have the potential to affect communities downwind from the site, including major urban centres. Animals in the area may be at risk and this is of great concern to ranchers. One example of a sour gas well blowout in Alberta occurred at Lodgepole in 1982 (cited in Bhambhani, 1999). This particular incident caused two deaths and various health problems in a community that was exposed to low concentrations of H_2S and sulphur dioxide over a period of 67 days (cited in Bhambhani, 1999).

1.1.2   Properties of Sour Gas

Sour gas is a mixture of substances present in varying concentrations that are source dependent. Components of these mixtures can include hydrocarbons (methane, ethane, propane, butane), H_2S, carbon dioxide, and water vapour (PCF, 2000). Sour gas is highly flammable and toxic and has a strong "rotten egg" odour characteristic of the H_2S component.

1.1.3   Formation of Sour Gas

A simple anaerobic process is responsible for the presence of small amounts of H_2S and other sulphur compounds in many oil and gas reservoirs in the world today (PCF, 2000). The process is similar to the anaerobic decomposition of organic materials that produces H_2S itself (Beauchamp et al., 1984; Roth, 1993; PCF, 2000). This decomposition process occurs in sewers, manure piles, swamps, bogs and compost heaps, yielding H_2S gas (Beauchamp et al., 1984; Roth, 1993; PCF, 2000). Chemical reactions within sedimentary rock produce H_2S, sometimes in large volumes (up to 90% in some reservoirs). These deposits of H_2S, buried deep in sedimentary rock, are similar to those found in the foothills of the Canadian Rockies (PCF, 2000). However, many "petroleum-bearing rock formations" contain iron that interacts with the sulphur of H_2S, forming a precipitate, iron sulphide, and "sweet gas" or natural gas that does not contain significant amounts of H_2S (PCF, 2000).

1.1.4   By-products of Sour Gas Production

Sour gas is mined for its great economic value, and through subsequent processing yields natural gas (once H_2S is removed) that can be separated into three marketable products:

> 1) Sales gas (largely methane) sold to consumers for purposes such as home heating;

2) Natural gas liquids (ethane, propane, butane) used in manufacturing petrochemicals and for heating and transportation fuel;

3) Condensate (pentane and heavier hydrocarbons) used for making gasoline and jet fuel (PCF, 2000).

Sour gas processing plants convert almost all of the H_2S produced (> 97%) into elemental sulphur that is subsequently used to manufacture fertilizers, pharmaceuticals, plastics and matches (PCF, 2000). The remaining H_2S (< 3%) is combusted in incinerators or flares converting H_2S primarily into water vapour and sulphur dioxide (SO_2), which is less odourous than H_2S and not flammable. SO_2 is also considered to be toxic and strict guidelines for exposure have been established (PCF, 2000). The majority of SO_2 produced is generally released by dispersion into the atmosphere using tall incinerator stacks to minimize ground level impact (Strosher, 2000). A small amount of H_2S escapes directly into the air due to small leaks referred to as "fugitive emissions", and less frequently due to accidents and equipment failures at wells, pipelines and plants (PCF, 2000).

There are other emissions from sour gas facilities that are released along with H_2S due to the combustion process. These include very small quantities of carbon disulphide (CS_2), carbonyl sulphide (COS), nitrogen oxides (NOx) and volatile organic compounds (Strosher, 2000). CS_2 has a strong odour and is

neurotoxic, but to a lesser degree than H_2S; COS is less toxic than H_2S but can have a depressant effect at high concentrations. NOx include nitric oxide (NO), nitrogen dioxide (NO_2) and nitrous oxide (N_2O), the so-called greenhouse gases that cause ground-level ozone (smog) and acid deposition (PCF, 2000). Inefficient combustion of hydrocarbons present in sour gas can produce volatile organic compounds including the "BTEX" compounds: benzene, toluene, ethylbenzene and xylene, all considered to be toxic and contributing to smog (PCF, 2000; Strosher, 2000).

1.1.5   Toxicology of Sour Gas

The toxic effects of sour gas mixtures have not been previously studied. H_2S, however, is generally regarded to be the major toxic chemical component of sour gas and its toxic effects have been extensively studied. Subsequent guidelines for exposure to sour gas are based on H_2S toxicology (see Introduction Section 1.2.7). While the single exposure of greatest interest in the sour gas industry has been focused on H_2S, numerous other chemicals are present in sour gas as a mixture or are associated with sour gas processes. Exposures to any or all of these products of sour gas production present a potential problem with respect to toxicology (Guidotti, 1994). Individuals can be exposed to these aforementioned substances, along with an exhaustive list of numerous other compounds, including agents used in the drilling, recovery, processing and separation processes associated with the oil and gas industry (Guidotti, 1994). Due to the

incredible number and wide variety of chemicals with different toxicities present in the sour gas industry, it is possible that significant interactions, or "cumulative" effects may result from sour gas operation exposures (Guidotti, 1994).

1.2   Hydrogen Sulphide

1.2.1   Overview

H_2S is an environmental and industrial pollutant with the potential to cause a multitude of effects in both humans and animals, depending on the duration and concentration of exposure.   The majority of information regarding the human health effects as a result of H_2S exposure originates from studies of uncontrolled occupational exposures or communities living in the vicinity of industrial sources of H_2S (Bhambhani, 1999).   At high levels, it is an extremely toxic substance with deadly consequences.   At low levels, it is regarded more as a nuisance due to its pungent odour.   However the possibility that low-level H_2S exposure can cause serious health effects in humans and animals does exist and is of great concern to the general public and medical community.

1.2.2   History of Research

Research on the toxicology of H_2S has a very long and convoluted history. Despite almost 300 years of documentation, research and reviews, the effects and mechanisms of toxicity of H_2S are still not understood.   Most research in the past has focused on effects of acute high-level sublethal or lethal exposures to

H_2S. However, current interests appear to be shifting to the effects of low-level H_2S exposures.

In 1713, Bernardino Ramazzini, an Italian physician and the father of occupational medicine, presented what became the first published account of H_2S poisoning in his book "De Morbis Artifacum" (cited in Smith, 1989; Glass, 1990; Roth, 1993). He reported ocular effects, including painful irritation and inflammation of the eyes of cleaners of privies and cesspits (cited in Smith, 1989; Roth, 1993). The inflammation subsequently led to secondary ocular effects including bacterial infection and even total blindness in some workers (cited in Smith, 1989). Ramazzini postulated that the symptoms were caused by a volatile acid (now known to be H_2S) released during the cleaning process (cited in Smith, 1989). This first documented symptom of eye irritation due to H_2S exposure became the basis for the 10 ppm H_2S threshold limit value (TLV) in the workplace that is still in place today (Smith, 1989).

In 1775, a Swedish chemist, Carl Wilhelm Scheele, discovered H_2S by treating ferrous sulphide with a mineral acid or by heating sulphur in hydrogen gas (cited in Beauchamp et al., 1984; Smith, 1989; Roth 1993). He termed the resultant odour "Schwefelluft" (sulphur air), or more precisely "Stinkende" (stinking or fetid) (cited in Smith, 1989). His master, Torben Bergman, demonstrated the presence of this malodourous gas in some mineral springs (cited in Smith, 1989). In 1796,

Berthollet analysed the gas and termed it "hydrogen sulphide" or sulphuretted hydrogen (cited in Beauchamp et al., 1984).

The effects of H_2S were first studied in animals at the end of the 19th century. In 1863, Felix Hoppe-Seyler observed changes in blood pigment that he referred to as "sulfhemoglobin" when a stream of pure H_2S was passed through a blood sample, and incorrectly concluded that H_2S was a "blood poison" (cited in Smith, 1989) similar to carbon monoxide. This hypothesis was disregarded in 1938 when Belgian physiologists, Corneille and Jean Heymans, discovered that sulphide, cyanide and azide affected the respiratory system via stimulation of chemoreceptors in the carotid body (cited in Smith, 1989); high, lethal doses of sulphide produced central respiratory paralysis.

Recent research has focused on enzymatic mechanisms of toxicity and high-level H_2S has been found to react primarily with cellular respiratory enzymes, resulting in cellular hypoxia or anoxia (Beauchamp et al., 1984). *In vitro* experiments have uncovered a possible biochemical mechanism of action common to H_2S and cyanide: potent inhibition of cytochrome oxidase (Nicholls, 1975; Nicholls et al., 1976; Roth et al., 1997; Nicholson et al, 1998). However, there are many differences that exist between H_2S and cyanide. For example, H_2S is more irritating to the lung than cyanide (Beauchamp et al., 1984; Smith,

1989), with a greater prevalence in the incidence of pulmonary edema (tissue damage induced directly by sulphide), suggesting that the two are different.

Other research implicates other enzymes such as carbonic anhydrase (Schwimmer, 1969; Roth et al., 1997; Nicholson et al., 1998), monoamine oxidase (Warenycia et al., 1989a; Skrajny et al., 1992) and membrane lipids (Beck et al., 1983) as targets of H_2S toxicity. As well, it is reported that H_2S affects adenosine triphosphatase (ATPase) and alkaline phosphatase activity (Roth, 1999).

Research in the past century focused on the mechanism of action for high dose H_2S exposure. More recently, H_2S effects on the N-methyl-D-aspartate (NMDA) receptor have become the focus of research (Abe & Kimura, 1996; Kimura, 2000). It has been postulated that H_2S is present endogenously in the brain and may act as a neuromodulator (Abe & Kimura, 1996). Experiments suggest that physiological concentrations of H_2S may facilitate NMDAR-mediated effects that induce long-term potentiation (LTP) in the brain (Abe & Kimura, 1996

1.2.3   Properties of Hydrogen Sulphide

1.2.3.1 Physical Properties

H_2S is an extremely toxic pollutant (Smith, 1989; Roth, 1993; MPCS, 1999). It is a colourless, flammable gas that is heavier than air (density = 1.19, standard

temperature and pressure) and has a characteristic "rotten-egg" odour (Beauchamp et al., 1984; Roth, 1993). H_2S can undergo two different oxidative reactions (Beauchamp et al., 1984): it burns in air with a blue flame and forms sulphur dioxide (SO_2) and water in the presence of sufficient oxygen (O_2) (equation 1.1), or water and deposits of elemental sulphur in the deficiency of O_2 (equation 1.2). Explosive limits of H_2S range from 4.3 - 46% volume in air, and the autoignition temperature is $260^{\circ}C$ (Beauchamp et al, 1984; Roth, 1993).

$$2H_2S + 3O_2 \rightarrow 2H_2O + 2SO_2 \qquad \text{(eq. 1.1)}$$
$$2H_2S + O_2 \rightarrow 2H_2O + 2S \qquad \text{(eq. 1.2)}$$
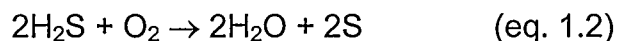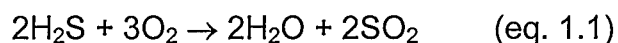
1.2.3.2   Chemical Properties

H_2S is soluble in aqueous and organic solutions (Roth, 1993). In aqueous solutions, H_2S dissociates into two ions: hydrosulphide (HS^-) and sulphide (S^{2-}) ions (Beauchamp et al., 1984; Reiffenstein et al., 1992; Roth, 1993) (equation 1.3). The pK_a values for the two different ions are 7.04 (HS^-) and 11.96 (S^{2-}). Therefore, at physiological pH (pH=7.4), two-thirds of the total sulphide will be present as HS^- and the remainder as the undissociated form (H_2S). The undissociated form of H_2S is lipid soluble and therefore readily crosses biological membranes (Beauchamp et al., 1984; Reiffenstein et al., 1992; Roth, 1993).

$$H_2S \leftrightarrow H^+ + HS^- \leftrightarrow H^+ + S^{2-} \qquad \text{(eq. 1.3)}$$
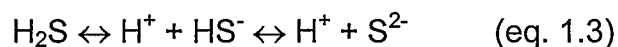
1.2.4 Sources of Hydrogen Sulphide

1.2.4.1 Natural Sources

H_2S is part of the natural cycle of sulphur in the environment (Roth, 1993; PCF, 2000). There are a variety of natural sources of H_2S including crude petroleum, natural gas (sour gas), volcanoes, sewers, sulphur deposits and springs, as well as decaying organic material (Beauchamp et al., 1984; PCF, 2000). These natural sources account for nearly 90% of the H_2S content in the atmosphere (Roth, 1993). It is estimated that 100 million tonnes of H_2S are released annually into the atmosphere from natural sources (Beauchamp et al., 1984).

1.2.4.2 Industrial or Other Anthropogenic Sources

H_2S is a by-product of numerous industrial processes including petroleum refineries, natural gas, coke oven and petrochemical plants, kraft paper mills, viscose rayon production, sulphur production for chemical use, iron smelters, food processing plants, tanneries and sewage treatment plants (Beauchamp et al., 1984; Roth, 1993; MPCS, 1999). Recovery of sulphur compounds is an important part of all these operations (Beauchamp et al., 1984). Industrial sources account for the other 10% of the H_2S released into the atmosphere. When considering all of these industrial sources of H_2S, many occupations as well as the general population are at risk for potential exposures. Communities that surround natural and industrial sources of H_2S are routinely exposed to ambient levels in the air that are far below occupational limits (White et al., 1999).

1.2.5   Classification as a Toxic Agent

1.2.5.1 Acute High Dose Exposure vs. Chronic Low Dose Exposure

All chemicals, including H_2S, are potentially toxic, depending on the duration and type of exposure, dose administered, organ(s) targeted and effects produced. Exposures to chemicals, such as hydrogen sulphide, are normally divided into four different categories: acute, subacute, subchronic and chronic (Casarett & Doull, 1996). An acute exposure typically lasts up to 24 hours, and generally refers to a single exposure to one or more chemicals; however, repeated exposure to a chemical(s) can occur during this period, with varying degrees of toxic effects depending on the chemical and dose received (Casarett & Doull, 1996). A sub-acute exposure lasts up to one month, and a subchronic exposure is from one to three months. Chronic exposure typically lasts longer than three months, with either continuous or repeated exposure to a chemical(s) over the extended period of time (Casarett & Doull, 1996).

There is a spectrum of toxic effects that can be produced by one or more chemicals, depending on the exposure scenario (duration and frequency of exposure) and the amount of exposure (high vs. low dose). Adverse effects of any given chemical can include: chemical allergic reactions, idiosyncratic reactions (genetic abnormal reactivity), immediate or delayed toxicity (up to 30 – 40 years), reversible or irreversible effects, and local or systemic toxicity (Casarett & Doull, 1996). Each of these effects has varying degrees of severity.

Acute exposures usually produce effects that are quite different from those associated with chronic exposures. Acute high dose exposures typically produce immediate severe toxic effects, but also may produce delayed toxicity that differs significantly from toxicity produced by chronic low dose exposures. Toxic effects attributed to high-level H_2S exposures have been studied at great length over the years. For example, acute high dose exposures to H_2S above 75 ppm are generally regarded to be toxic (Reiffenstein et al., 1992). Higher concentrations of H_2S are potentially fatal depending on the duration of exposure (Reiffenstein et al., 1992; Roth, 1999). High-level H_2S exposure is one of the leading causes of sudden accidental death in the workplace (Ellenhorn, 1997; NIOSH, 1977), with several deaths reported each year (Guidotti, 1994). In addition, high-level exposures that cause significant health effects, including brief periods of unconsciousness, are common but often go unreported in Alberta oil fields (Hessel & Melenka, 1999).

In contrast, chronic low dose exposures may produce immediate effects after each individual exposure, as well as long-lasting chronic effects. In the past, chronic low-level exposures to H_2S (< 10 ppm) have been considered to be innocuous, therefore the effects at these low levels have not been extensively studied (Kilburn, 1997), and most reports of adverse effects are unconfirmed and anecdotal (PCF, 2000). Consequently, very little is known about the health effects of chronic low-level exposure to H_2S (Hessel & Melenka, 1999), and the

general public's concerns regarding possible acute and chronic health effects as a result of ambient level exposure to H_2S persist (White et al., 1999).

"Cumulative" is a term often used in toxicology. It can refer to different exposure scenarios (EPA, 1999): repeated exposures to one chemical over time (as in a chronic exposure) or a single or repeated exposure to a number of chemicals at one time. Exposures in both instances can involve multiple sources of the chemical(s), as well as multiple pathways (e.g. air, water, and soil) and routes of exposures at one time (EPA, 1997). The latter scenario can lead to additive effects (addition of toxic effects from the individual chemicals), synergistic effects (toxic effects are greater than the addition of individual effects) or antagonistic effects (chemicals interfere with individual effects and resulting in overall reduced toxicity). The cumulative effects of a sour gas mixture have not been studied previously in the lab, and the only information of toxic effects has been derived from epidemiological studies.

1.2.5.2 Guidelines of Exposure

The maximum allowable concentration of ambient H_2S in the air is 10 parts per billion (ppb) (or 0.010 ppm) averaged over one hour to a total of 3 ppb over 24 hours, according to the Alberta Ambient Air Quality Guidelines (Alberta Environment, 2002). The maximum allowable exposure level in the workplace, averaged over eight hours, is considerably higher at 10 ppm (0.001%) (Alberta

Environment, 2002). This guideline is in place to protect workers from acute health effects (e.g. eye irritation). Evacuation of a work site occurs if H_2S concentrations exceed 20 ppm (ceiling limit). Alberta H_2S guidelines are equivalent, or stricter, than guidelines in other provinces or countries (PCF, 2000; Alberta Environment, 2002).

Threshold limit values (TLVs) are guidelines for airborne concentrations for a particular substance that correspond to conditions that any worker may be exposed to, day after day, without any adverse health effects (Bhambhani, 1999). There are three levels of TLVs established by the American Conference of Governmental Industrial Hygienists, 1993 (cited in Bhambhani, 1999). The first level is the TLV-Time Weighted Average (TLV-TWA), set to avoid acute ocular effects, to which workers may be exposed to for a normal 8-hour workday (cited in Bhambhani, 1999). The TLV-TWA for H_2S is 10 ppm. The second level is the TLV-Short Term Exposure Limit (TLV-STEL) to which workers may be exposed to for a short period of time without suffering from "irritation, chronic or irreversible tissue damage, or narcosis of sufficient degree to increase the likelihood of accidental injury, impair self-rescue or mutually reduce work efficiency" (cited in Bhambhani, 1999). The TLV-STEL is a 15-minute TWA exposure not to be exceeded during the workday, even if the 8-hour TWA is within the TWA limit (cited in Bhambhani, 1999). The TLV-STEL for H_2S is 15 ppm. The ACGIH (2001) has proposed that the TWA should be reduced to 5

ppm and the STEL be abolished. The third level is the TLV-Ceiling (TLV-C) that is not to be exceeded during any portion of a workday, or immediate evacuation of the workplace must follow (cited in Bhambhani, 1999). The TLV-C for H_2S is 20 ppm.

The Agency for Toxic Substances and Disease Registry (ATSDR) has established minimal risk levels for H_2S. The minimal risk level is an estimate of "the exposure level at which adverse effects are not expected to occur in humans". As with other toxicants, the levels for H_2S are based largely on studies in animals with limited evidence regarding human health effects (White et al., 1999). The minimal risk level for acute inhalation exposure is 0.07 ppm (70 ppb) and for intermediate inhalation exposure is 0.03 ppm (30 ppb) (White et al., 1999).

1.2.6   Toxicokinetics of Hydrogen Sulphide

Toxicokinetics refers to the absorption, distribution, biotransformation (metabolism) and excretion (elimination) of chemicals (Casarett and Doull, 1996). All of these factors contribute to the toxic effects of a chemical. The toxicokinetics of H_2S have been well studied in animals, but to a lesser degree in humans. In humans and animals, H_2S is quickly absorbed via inhalation through the lungs into the body (primary route of exposure), with minimal absorption through the skin in occupational settings (Beauchamp et al., 1984). Subsequent

to absorption, H_2S is distributed to the brain, liver, kidney, pancreas and small intestine (Roth, 1993).

There is no information available for metabolism of H_2S in humans, however results from animal studies have revealed three pathways of H_2S metabolism (Beauchamp et al., 1984; Bhambhani, 1999): (1) oxidation to sulphate or thiosulphate; (2) methylation; and (3) reaction with metallo- or disulphide-containing proteins. The first two pathways lead to detoxification of H_2S; the third reaction, combining H_2S with essential proteins, is responsible for the toxic effects of H_2S (Beauchamp et al., 1984). Some of these proteins include enzymes, e.g. cytochrome oxidase, and reactions with these enzymes may lead to inhibition of mitochondrial electron transport and subsequent interruption of cellular respiration, causing histotoxic hypoxia (Milby and Baselt, 1999a), leading to lethal effects (Beauchamp et al., 1984). Central neurotoxicity (hypoxic-ischemic encephalopathy) is the most significant, severe consequence of inhibition of cytochrome oxidase (Milby and Baselt, 1999a).

H_2S is detoxified quickly (half life of 60 minutes) by oxygen bound to hemoglobin in the blood (Bhambhani, 1999; Milby &Baselt, 1999a). This oxidation to sulphate is the primary metabolic pathway and subsequent excretion follows via the kidneys, with significant levels of sulphate detectable in the urine within less than 24 hours (Dziewiatkowski, 1945). Minor excretion also occurs via a similar

oxidation process in skeletal muscle by oxygen bound to myoglobin and also in the gastrointestinal tract (Bhambhani, 1999). The remaining unoxidised H_2S is eliminated by the lungs via normal exhalation (Bhambhani, 1999). The ability of the body to detoxify H_2S in the blood depends on the individual's cardiac output and thus the availability of oxygenated blood (Bhambhani, 1999). Therefore, in a working individual, the cardiac output increases, reducing the presence of oxygenated blood available to oxidise H_2S. Women are less able to detoxify H_2S due to their lower blood volume per kilogram of body weight, lowering the availability of oxygenated blood by 10% (Bhambhani, 1999).

Due to the rapid oxidation of H_2S in the blood (Bhambhani, 1999), H_2S is generally not considered to be a cumulative[1] toxicant (Beauchamp et al., 1984, Bhambhani, 1999). However, it has been suggested that H_2S may accumulate in certain regions of the body, including the central brain and therefore the amount of sulphide from one exposure may be added to the load already present from previous exposures (Guidotti, 1994). As well, H_2S is lipophilic suggesting that accumulation in biological tissues is possible. Accumulation and persistence of H_2S in the brain may be responsible for the long-lasting effects from H_2S exposures. Skrajny et al. (1996) documented cumulative effects on hippocampal function of repeated low-level exposure (25 – 100 ppm H_2S). Cumulative effects

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[1] "Cumulative" in this case refers to the summation of effects produced by the presence of a substance in the body over a period of time.

have also been reported in mice exposed repeatedly to 100 ppm H₂S (i.e. increased inhibition of cytochrome oxidase activity).

1.2.7   Toxic Effects of Hydrogen Sulphide Exposure

1.2.7.1 General Effects

H$_2$S is a broad-spectrum toxicant, due to its ability to affect a wide variety of organs (Roth, 1999). However, organs with exposed mucous membranes and/or high oxygen demand are most susceptible to H$_2$S toxicity (Roth, 1999). Each organ system exhibits different "threshold responsiveness" to H$_2$S exposure, with the degree of effect dependent on the organ targeted (Reiffenstein et al., 1992). Toxic effects resulting from H$_2$S exposure depend on the concentration, rate and duration of exposure.   In addition, the lungs are primarily responsible for absorption of H$_2$S and hence the ventilation rate of the exposed individual is a factor (Reiffenstein et al., 1992; Bhambhani, 1999). Since H$_2$S is rapidly detoxified by the body (Bhambhani, 1999), effects are more related to concentration rather than duration of exposure to H$_2$S (Milby & Baselt, 1999a), with severe consequences of H$_2$S exposure resulting from high level (> 250 ppm) concentrations. Fatalities have occurred in individuals exposed to 150 ppm H$_2$S for 6 hours or to 650 ppm H$_2$S for 8.5 minutes (Guidotti, 1994).   Originally H$_2$S was thought to affect the respiratory system (Smith, 1989). The brain is now considered to be the primary target of H$_2$S, although olfactory, ocular, respiratory and cardiovascular systems are also appreciably affected (Roth, 1993).

1.2.7.1.1   Olfactory Effects

Humans are able to detect H_2S odour at very low concentrations (5 ppb) (Au and Legator, 1999).   The characteristic "rotten-egg" odour at these ambient concentrations is the source of numerous complaints (e.g. headaches and nausea) and concerns from the general public (Haahtela et al., 1992). The odour affects specific areas of the brain (cortex, subcortex, cranial nerves), however the health effects due to H_2S odour are not well understood (Roth, 1999) nor accepted. Often if a person smells an odour and then experiences adverse health effects, he associates the undesirable symptoms experienced with the odour (White et al., 1999). However, an individual's response to an odour and presumed associated health effects are often influenced by preconceived beliefs about the odour rather than odour itself (Dorman et al., 1999). Malodourous chemicals (e.g. H_2S) can elicit pronounced psychological reactions regardless of the degree of toxicity (Knasko, 1993), leading some to believe that the line between "nuisance odour" and "adverse health effect" has become blurred for the general public (Shusterman, 1992; Granville, 1999). At the very least, however, the presence of the H_2S odour alone could have a negative impact on an individual's quality of life (White et al., 1999), suggesting that the effects of low-level H_2S odour should be a focus of future research.

At higher concentrations (30 – 100 ppm), H_2S odour becomes "sickeningly sweet" (Glass, 1990). Progressing to even higher concentrations, anosmia or

olfactory paralysis is reported as a result of olfactory fatigue due to exposures considered to be toxic and potentially fatal (> 100 ppm) (Glass, 1990; Roth, 1993), rendering odour as an ineffective warning device. The cause of the paralysis is not well understood and could even be a result of memory loss that occurs after acute high dose exposure.

1.2.7.1.2   Ocular Effects

As initially reported in 1713, the effects of H_2S exposure on mucous membranes are first observed in the form of eye irritation, or "sore-eyes" (5 – 10 ppm H_2S). "Kerato-conjunctivitis", or "gas-eye", occurs at slightly higher concentrations (10 – 50 ppm H_2S) as a result of inflammation of the conjunctiva and cornea (Beauchamp et al., 1984). This painful inflammation is consistent with the irritating effects of malodourous sulphur compounds (Jaakkola, 1990). Ocular effects are reported in individuals exposed to far lower concentrations (~30 ppb) of H_2S (Haahtela et al., 1992). Residents in Rotorua, New Zealand, where ambient H_2S concentrations were measured to be 14 ppb, experienced increases in the incidence of disorders of the eye and lacrimal glands, including cataracts, conjunctivitis and disorders of the orbit (Bates, 1997).

1.2.7.1.3   Respiratory Effects

In addition to irritation and inflammation of the eye tissue, low-level H_2S exposure (~50 ppm) can affect the respiratory tract, causing rhinitis, pharyngitis, laryngitis,

bronchitis and pneumonia (Milby, 1962; Beauchamp et al., 1984). Symptoms reported in individuals exposed to ambient levels (as low as ~10 ppb) include asthma-like symptoms (cough, sore throat, chest pain, increased nasal secretions and dyspnea) (Dales et al., 1989; Haahtela et al., 1992) that become more pronounced with prolonged (chronic) low-level exposures (Beauchamp et al., 1984). Current literature suggests that inhalation of 2 – 10 ppm H_2S for 15 – 30 minutes does not affect the respiratory function of healthy, exercising men and women (Bhambhani, 1999). Conversely, individuals exposed to higher levels (250 ppm H_2S) exhibit symptoms of bronchial hyperresponsiveness (Hessel et al., 1997) and pulmonary edema (Milby and Baselt, 1999b). At lethal concentrations (500 ppm H_2S), respiratory failure may occur as a result of paralysis of the respiratory centre in the brain (caused by histotoxic hypoxia), and this in turn can lead to subsequent asphyxia and cardiac failure (Beauchamp et al., 1984).

1.2.7.1.4 Cardiac Effects

Alterations in cardiac function are also associated with H_2S exposure. Acutely exposed workers exposed to >100 ppm H_2S exhibit changes in cardiac function (e.g. sinus tachycardia, hypotension and elevated cardiac enzymes) that are associated with myocardial infarction (Jappinen, 1987; Tvedt, 1991a). However, individuals exposed to ambient H_2S levels (~ 10 ppb) do not have an increased risk for cardiovascular disease (Jaakkola et al., 1990 ; Bates et al., 1997).

1.2.7.2 Neurotoxicology of Hydrogen Sulphide

1.2.7.2.1 Effects in Humans

Neurotoxic effects of high level H_2S exposure (> 250 ppm) are the most documented and dramatic of all organ system effects. Minor, transient symptoms occur first, including headache, vertigo, incoordination and intense fatigue and anxiety (Milby and Baselt, 1999a). If exposure continues, unconsciousness and death can occur from 500 ppm H_2S for 4 − 8 hours, 750 ppm H_2S for 5 minutes or a few breaths of 1000 ppm H_2S (Guidotti, 1996).

A well-known phenomenon, termed "knockdown", occurs in oil field workers exposed to extremely high concentrations (750 − 1000 ppm) of H_2S (Reiffenstein et al., 1992). Inhalation of these high levels for only a few minutes can lead to abrupt physical collapse, with a loss of consciousness (Roth, 1993). This effect is likely due to a direct toxic action of H_2S on the brain, i.e. the intracellular inhibition of cytochrome oxidase by the sulphide ion that prevents cellular utilisation of oxygen (Nicholls, 1975). When exposure is sustained, this sudden collapse can quickly lead to fatal respiratory failure (Guidotti, 1994).

In the case where acute high dose exposure is promptly terminated, rapid and full recovery is expected (Burnett et al., 1977; Arnold et al., 1985; Milby and Baselt, 1999b), although subsequent brain damage with serious neurological and psychiatric sequelae (e.g. amnesia, motor deficits, severe headaches,

hallucinations, etc.) has been reported (Tvedt et al., 1991b; Kilburn, 1993). Some researchers have reported that impairment of neurological function persists over time, with patients showing deficits in memory and motor functions up to five years post-exposure (Tvedt et al., 1991a). It has been suggested that these symptoms are a consequence of hypoxia secondary to respiratory insufficiency, rather than due to a direct toxic action on the brain (Milby & Baselt, 1999b). This respiratory insufficiency occurs as a result of apnea caused by either paralysis of the respiratory centre of the brain, or interference with airflow by airway obstruction or pulmonary edema (Milby & Baselt, 1999b). It has been suggested that any patient that survives "knockdown" by H_2S be followed for up to at least 5 years so that the understanding of subtle yet permanent alterations of central nervous system functions that follow high dose H_2S exposure can be improved (Snyder et al., 1995). Another significant health outcome of knockdown that is often under appreciated is trauma (Hessel & Melenka, 1999). Traumas including severe fractures, lacerations and abrasions are attributed to falls following loss of consciousness, and these can be serious and require long-term rehabilitation and occupational therapy (Hessel & Melenka, 1999).

The effects of prolonged or repeated exposure to low-levels of H_2S are not well understood (Hessel & Melenka, 1999; Partlo et al., 2001) and have traditionally been regarded as relatively innocuous (Kilburn, 1997). However, exposures to low, sub-lethal, ambient levels of H_2S have recently been associated with

neurophysiological and neuropsychological sequelae, including memory loss, psychiatric disturbances and motor problems (Kilburn, 1999), implicating the central nervous system as the primary target of low-level H_2S exposure (Beauchamp et al., 1984). In one study, individuals living downwind from oil wells chronically exposed (~10 years) to ambient levels of H_2S reported memory loss and difficulty concentrating, and subsequent neurobehavioural testing showed impaired balance, delayed reaction times, disrupted visual fields and impaired verbal recall (Kilburn, 1997). Similar reports of neurobehavioural dysfunction, including motor deficits and affective disturbances (Kilburn & Warshaw, 1995a; Haahtela et al., 1992) have been linked to chronic, ambient (as low as 30 ppb) H_2S exposure. Common complaints of residents living downwind from sour gas plants include frequent headaches, nausea, vomiting, dizziness, pain and numbness in the extremities, disorientation, depression, personality changes, nosebleeds and respiratory problems (Haahtela et al., 1992; Kilburn & Warshaw, 1995a; White et al., 1999).

Low-level exposures are now thought to be responsible for a variety of symptoms reported in individuals that affect quality of life, and this could potentially be a serious problem (Kilburn & Warshaw, 1995a,b; Kilburn, 1997; Kilburn, 1999). However, the results of these low-level exposure studies are regarded to be qualitative and are not widely accepted across the scientific community. It has been suggested that little "quantitative" evidence exists on the potential health

effects in individuals exposed to levels below the occupational limits (< 10 ppm H_2S) (Granville, 1999). Industry health professionals support the current guidelines for H_2S exposure, however they are in agreement that more research into low-level H_2S health effects is warranted (Granville, 1999).

1.2.7.2.2 Effects in Animals – *in vivo*

In 1966, investigators exposed rhesus monkeys to 500 ppm H_2S for less than 30 minutes, and observed necrosis of the cerebral cortex, Purkinje cell death and focal gliosis (Lund and Weilund, 1966). Since that study, numerous investigations using animal models have documented significant H_2S neurotoxicity, including morphological and neurophysiological (Hannah & Roth, 1991; Roth & Hannah, 1989), neurochemical (Hannah et al., 1989; Skrajny et al., 1992), behavioural (Dorman et al., 1999; Partlo et al., 2001) and electrophysiological (Skrajny et al., 1996) effects.

In morphological and neurophysiological studies, H_2S exposure (20-75 ppm) has been reported to affect developing rat cerebellar Purkinje cell density, dendritic architecture and overall growth processes, as well as developing and mature brain neurotransmitter levels (Roth & Hannah, 1989; Hannah & Roth, 1991). In neurochemical studies, H_2S (20 - 50 ppm) exposure also resulted in decreased levels of neurotransmitter content (aspartate, glutamate and γ-amino butyric acid [GABA]) in the developing rat brain (Hannah et al., 1989) that could reflect

changes in synthesis and/or release of neurotransmitter or neuron death (Reiffenstein et al., 1992). In similar studies, alterations in serotonin (5-hydrotryptamine [5-HT]) and norepinephrine (NE) levels were observed in cerebellum and frontal cortex of developing rats exposed to comparable concentrations (25 and 75 ppm) of H_2S (Skrajny et al., 1992).

Behavioural studies using animal models have only recently been added to the literature on H_2S research. In one study, repeated, sublethal (125 ppm) H_2S exposure experiments were conducted to investigate effects on neurobehavioural function in adult Sprague Dawley rats using a 16-arm radial arm maze (RAM) (Partlo et al., 2001). While repeated exposure to 125 ppm H_2S did not affect previously learned spatial tasks, it had a detrimental effect on the rat's ability to relearn complex RAM tasks (Partlo et al., 2001). As well, in an investigation conducted by the Chemical Industry Institute of Toxicology (CIIT), behavioural tests in rats revealed cognitive dysfunction when animals were exposed to $\geq$ 80 ppm H_2S, with significant attenuation of motor activity and water maze performance following exposure to $\geq$ 80 ppm H_2S (Dorman et al., 1999; Struve et al., 2001). The data provide some evidence of mild brain dysfunction following repeated, prolonged sublethal H_2S exposure.

Electrophysiological investigations have provided insight into the effects of H_2S on brain activity (Reiffenstein et al., 1992; Roth, 1999). In particular, one study,

using a whole-animal (*in vivo*) model, examined the effects of low-level H_2S exposure on the hippocampus and neocortex of freely moving rats by recording changes in electroencephalographic (EEG) activity (theta rhythm) as a measurement of neuronal function (Skrajny et al., 1996). Hippocampal theta rhythm has been associated with memory, attention, spatial learning and motor control, which can all be affected by H_2S exposure (Dorman et al., 1999). Rats exposed to varying concentrations of H_2S (25 - 100 ppm) demonstrated significant changes in hippocampal, but not neocortical, function, suggesting a selective site of action (and therefore toxicity) in the brain for H_2S exposure (Skrajny et al., 1996).

1.2.7.2.3 Effects in Animals - *in vitro*

In vitro neuronal preparations have been useful in attempts to elucidate a mechanism of action for H_2S neurotoxicity. The rat hippocampal slice preparation is often used due to interest to study neurological effects including memory loss and cognitive dysfunction. Intracellular recordings in current-clamp mode from hippocampal CA1 neurons demonstrated that $\geq$ 80 µM sodium sulphide (NaHS), a source of H_2S, caused a concentration-dependent membrane hyperpolarization and reduction in membrane resistance, with further hyperpolarization during washout periods (Baldelli et al., 1989). Extracellular recordings of field population spikes (PSs) and excitatory post-synaptic potentials (EPSPs), as well as intracellular EPSPs, were also conducted, revealing a

reversible depression of synaptic transmission due to $\geq$ 60 μM NaHS application (Baldelli et al., 1989). Similar results were observed in dorsal raphe serotonergic neurons exposed to 200 μM NaHS, with both depolarization and hyperpolarization recorded (Kombian et al., 1993). The potassium (K^+) channel has been suggested as a site of action of H_2S for hyperpolarization effects (Reiffenstein et al., 1992) and activation of Na^+-K^+/ATPase is thought to cause alterations in membrane resistance (Reiffenstein et al., 1992).

More recently, it has been suggested that H_2S is generated by enzymes and exists endogenously in relatively high concentrations (50 - 160 μM) in the brain (Abe & Kimura, 1996). The investigators suggest that H_2S may actually have a physiological function and refer to it as a neuromodulator (Abe & Kimura, 1996). At concentrations of 10-130 μM, NaHS was found to alter excitable properties of neurons (Abe & Kimura, 1996). Furthermore, these physiological concentrations have been found to enhance NMDA receptor-mediated responses in the CA1 region of the hippocampal slice, and induce hippocampal long-term potentiation (LTP) in the presence of electrical stimulation (Abe & Kimura, 1996).

1.3 Hippocampus

1.3.1 Anatomy

The hippocampal formation is part of the limbic system and one of the major components of the cerebral hemispheres. It is located in the temporal lobe and

thought to be involved in mood and learning and memory (Johnston & Amaral, 1998). In the 19th century, a German neuroanatomist, Alois Alzheimer, recognised changes in the region of the hippocampal formation associated with dementia (cited in Bears et al., 1996). Further insight into the function of the hippocampal formation was gained by observing a now famous patient, "H.M". After surgical removal of the region bilaterally, H.M. lost the ability to consolidate short-term memory (STM) into long-term memory (LTM), but retained memories of events that occurred prior to the lesion (cited in Johnston & Amaral, 1998). This loss of anterograde memory was attributed to the lesion of the hippocampal formation (cited in Johnston & Amaral, 1998). Similar loss of memory has been noted in patients whose medial-temporal lobe has been damaged or removed due to temporal lobe epilepsy (cited in Johnston & Amaral, 1998).

The hippocampal formation consists of distinct components: 1) subiculum; 2) presubiculum; 3) parasubiculum; 4) hippocampus proper (hippocampus); 5) dentate gyrus (DG); and 6) entorhinal cortex (Johnston & Amaral, 1998). The subiculum, hippocampus and DG all have a single cell layer (Bears et al., 1996), with acellular molecular and less cellular polymorphic layers located above and below it (Johnston & Amaral, 1998). In the subiculum and hippocampus, the single cell layer is made up of pyramidal cells, and in the dentate gyrus this layer is made up of granule cells (Johnston & Amaral, 1998). In the hippocampus, the regions above and below the pyramidal single cell layer are divided into strata:

stratum lucidum, stratum radiatum and stratum lacunosum moleculare (apical dendrites of pyramidal cells, superficial to the pyramidal cell layer) and stratum oriens (basal dendrites of pyramidal cells) (Johnston & Amaral, 1996).

Pyramidal cells are the principle neurons in the hippocampus and they are arranged in the pyramidal cell layer (stratum pyramidale), which is further divided into three regions, based on the size and appearance of neurons (Johnston & Amaral, 1998). These divisions are abbreviated CA (cornu Ammonis [latin for Ammon's Horn]), and numbered CA1, CA2 and CA3. In addition to the differences in size and appearance of neurons, CA3 also differs from CA1 due to the fact that CA3 receives mossy fibre input from the DG, unlike the CA1 region (Johnston & Amaral, 1998).

1.3.2   Electrophysiology of the Major Neuronal Circuits

The primary circuitry of the hippocampus is based on synapses that sequentially relay information in a specific direction (Stoltenburg-Didinger, 1994) (Figure 1). The major input into the hippocampal formation is the entorhinal cortex that sends information via a bundle of axons termed the perforant path, through the subiculum, and then synapses with the neurons in the DG (Bears et al., 1996; Johnston & Amaral, 1998). DG neurons have axons (mossy fibres) that synapse on the proximal dendrites of CA3 pyramidal cells (Bears et al., 1996; Johnston & Amaral, 1998). The CA3 cell axons have two different destinations: one branch
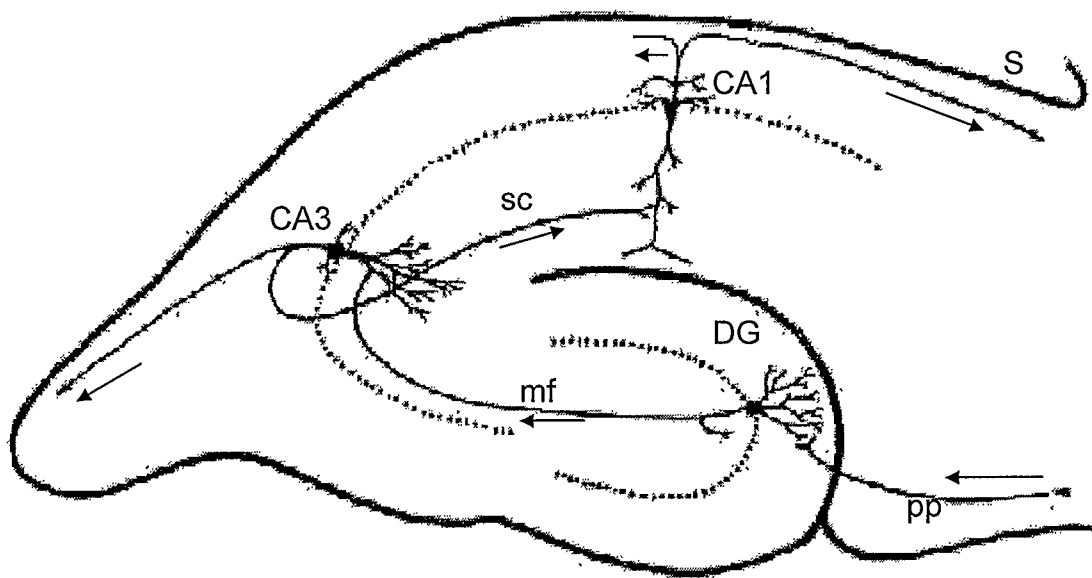
Figure 1. A drawing of a transverse slice showing several components of the hippocampal formation and some of the major circuitry. The perforant path fibers extend from the entorhinal cortex to the dentate gyrus (DG). The mossy fibres (mf) project to the CA3 region of the hippocampus. The Schaffer collateral fibres (sc) project from the CA3 to the CA1 region of the hippocampus. (Adapted from MacIver & Roth, 1987.)

leaves the hippocampus via the fornix, and the other branch, the Schaffer collateral projection, synapses with neurons in the CA1 region of the hippocampus (Johnston & Amaral, 1998). CA1 neurons in turn send their fibres to the subiculum and to deep layers of the entorhinal complex (Stoltenburg-Didinger, 1994; Johnston & Amaral, 1998). The entorhinal cortex then sends information back to many of the same cortical areas that originally relayed information to the entorhinal cortex. This allows information that enters the entorhinal cortex to traverse the entire hippocampus and then be returned to the original cortical area, allowing information to be stored as long-term memories (Johnston & Amaral, 1998).

The pathway of interest for this thesis is the projection from the Schaffer collateral fibres in the stratum radiatum of CA3 pyramidal cells to the pyramidal cell body region of CA1. Each CA3 neuron synapses with the dendrites of many CA1 pyramidal cells (e.g. a single CA1 neuron can be innervated by 5000 or more CA3 cells) (Johnston & Amaral, 1998).

The circuits of the hippocampus use the amino acid glutamate as a major excitatory neurotransmitter. Glutamate released from the Schaffer collateral fibres acts on metabotropic and ionotropic receptors, including AMPA ([RS]-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), kainate and NMDA receptors. The NMDA receptor plays a role in the induction of long-term

potentiation (LTP) (Bliss & Collingridge, 1993; Malenka & Nicoll, 1993). Inhibitory interneurons use GABA as a neurotransmitter to regulate synaptic transmission (Stoltenburg-Didinger, 1994) that acts on $GABA_A$ and $GABA_B$ receptors (Johnston & Amaral, 1998).

1.4 Hippocampal Slice Preparation as a Model to Study H₂S and Sour Gas Neurotoxicity

H_2S exposure (high or low-level) is reported to have cognitive effects (Tvedt et al, 1991a,b; Kilburn & Warshaw, 1995a,b; Kilburn, 1997; Kilburn, 1999), and therefore may affect hippocampal function (Skrajny et al., 1996). The hippocampal region of the brain has been well studied and is involved in cognitive processes such as memory and learning (Fountain et al., 1992). The *in vitro* hippocampal slice preparation is a well-utilised model system for neurotoxicity testing (Fountain et al., 1992) because it has been shown to be susceptible to a wide variety of toxic insults (Stoltenburg-Didinger, 1994), including H_2S (Skrajny et al., 1996). Studies using the hippocampal slice do not provide information on how *in vivo* functions are affected (since external inputs have been eliminated) but the slice does reflect the complexity of the *in vivo* nervous system (Fountain et al., 1992).

One major advantage of using an *in vitro* hippocampal slice preparation is that the classic, synaptic neuronal circuitry is preserved in each slice and this is very

useful for studying discrete synaptic pathways and their excitatory and inhibitory properties (Fountain et al, 1992). Other advantages compared to *in vivo* testing include control over the physical and chemical environment, freedom from *in vivo* anaesthetic and paralytic drug effects, direct visual control over electrode placement, lack of *in vivo* systemic effects and control over toxic exposures where a variety of concentrations can be studied (Costa, 1998). Disadvantages also exist, in that the hippocampal slice is deprived of a blood brain barrier as well as afferent inputs and efferent connections to other structures in the brain (Costa, 1998).

1.5 Paired Pulse Extracellular Recording

The Paired Pulse (PP) method of extracellular recording is a common approach to assessing the status of hippocampal inhibitory and excitatory systems (Fountain et al., 1992). It is the simplest form of synaptic plasticity (Archer et al., 2001). It involves stimulating the same pathway (i.e. the projection from CA3 to CA1 for the purpose of this thesis) twice using a stimulating electrode on the Schaffer collateral fibres in the stratum radiatum of CA3 (Fountain et al., 1992). Stimulation first produces a brief, negative-deflected transient presynaptic fibre volley, caused by the volley of action potentials in the presynpatic fibres, followed by slow, positive-deflected population[2] excitatory post synaptic potentials (EPSP)

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[2] The term "population" signifies that the measured potential is actually a summation of activity from the entire population of cells (Johnston & Amaral, 1998).

in the stratum radiatum of CA1 region (Johnston & Amaral, 1998).The EPSP increases in amplitude with increasing stimulus intensity, until triggering a fast, negative-deflected population spike (PS) that is produced by the inward current during the postsynaptic action potentials and recorded in the stratum pyramidale of CA1 (Johnston & Amaral, 1998) (Figure 2).

When the CA3 to CA1 hippocampal pathway is stimulated with a paired-pulse, there is an increase in response to the second pulse due to activation by the first conditioning pulse when the appropriate interstimulus interval is used (Kuhnt & Voronin, 1994; Hirota & Roth, 1997; Archer et al., 2001). The activation of presynaptic GABA$_B$ autoreceptors via GABA released by the first stimulus produces a feedback inhibition of GABA release from the presynaptic terminal (Hirota & Roth, 1997). Therefore, the evoked neuronal response to the second stimulus becomes greater than the first response, leading to a second evoked population spike (PS2) that is larger and generally more stable than the first (PS1), a form of short-term synaptic plasticity known as paired-pulse facilitation (PPF) (Hirota & Roth, 1997; Archer et al., 2001). Consequently, PS1 is not consistently recorded and results are taken from the second evoked response (Archer et al., 2001) (see Figure 2). Measurements of the amplitude of the second PS represent the number of neurons that reach threshold as a result of stimulation (Johnston & Amaral, 1998). Increases in PS2 amplitudes relate to
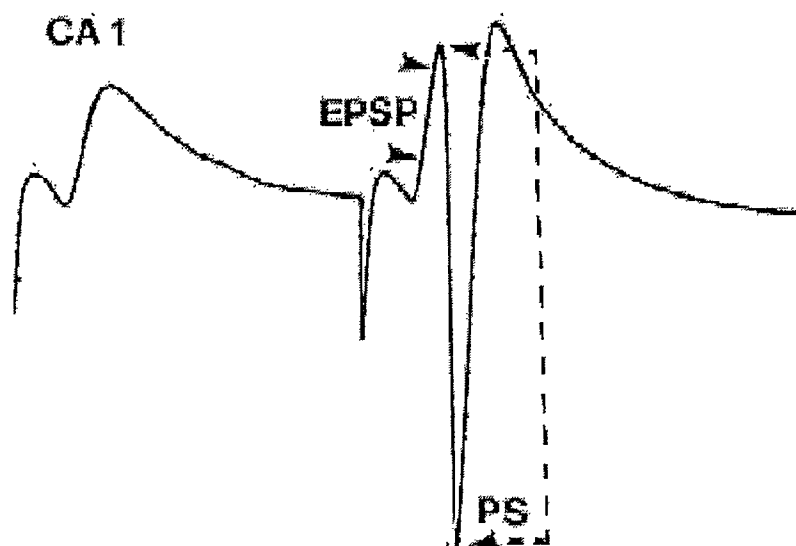
Figure 2. A sample of the paired pulse recording in the CA1 region of the hippocampus. Paired pulse stimulation produces a potentiation of the second recording, known as paired-pulse facilitation (PPF), and measurements are made on the second response. The recording indicates the excitatory post synaptic potential (EPSP) and the population spike (PS). The arrows indicate where the PS amplitude is measured. (Adapted from MacIver and Roth, 1987.)

enhancement of synaptic transmission in the CA3 to CA1 pathway, decreases relate to depression of synaptic transmission (Archer et al., 2001).

1.6 Hypothesis

Sour gas contains H_2S as well as many other components that may interact with H_2S, thus it is important to study the effects of sour gas exposure and compare them to the effects of exposure to H_2S alone. The overall hypothesis is that the neurotoxic effects of sour gas exposure are largely dependent on the presence of H_2S, the major toxic component. It is expected that the effects of sour gas and H_2S will be similar, and any differences that exist will be statistically insignificant.

1.7 Objectives

The objectives of this thesis were:

1) to conduct concentration-response experiments for low-level sour gas over a specific concentration range (sour gas containing 1, 5, 10, 25 ppm H_2S);

2) to conduct concentration-response experiments for low-level H_2S over an identical concentration range (1, 5, 10, 25 ppm);

3) to compare the effects of the sour gas mixture and H_2S alone;

4) to conduct experiments to explore a possible NMDA receptor-dependent mechanism of action for H_2S activity using a pharmacological antagonist of the NMDA receptor [2-Amino-5-phosphonopentanoic Acid (AP5)];

5) to conduct control experiments to test whether the major components of the gas cylinders [methane (largest component of sour gas ~85%), nitrogen (largest component of the H_2S tank ~99.8%)] can cause effects alone, and also to ensure stability of the slice preparation (time control).

1.8   Rationale

While the effects of high-level H_2S have been studied extensively and are fairly well documented, research into the health consequences of low, ambient level H_2S exposure has been deficient. Public concerns have increased the need for research and knowledge in this area. There is no evidence for a direct causal relationship between low-level exposure to H_2S and the symptoms associated with it. It is also not definitively known if the effects of sour gas are caused only by the presence of H_2S alone. Previous research has been conducted specifically on H_2S alone while the effects of "cumulative" exposure to mixtures (i.e. sour gas) have not been studied. However, research into low, ambient-level sour gas (mixture) exposures would better represent the actual exposure scenario for residents in the proximity of, or individuals that work on, sour gas operations. Therefore, there was a need to study the effects of exposure to sour gas containing low-levels of H_2S and compare them to the effects of exposure to low-levels of H_2S alone. As well, there is increased public interest in health, safety and environmental issues relating to sour gas exposure, especially in Alberta.

2.0   Methods

2.1   Animals

Male Sprague-Dawley rats, aged 20 – 30 days, were purchased from the University of Calgary Biosciences Vivarium. The animals were housed in plastic cages on a 12-hour light-dark cycle and supplied with food and water *ad libitum*.

2.2   Artificial Cerebrospinal Fluid Preparation

Artificial cerebrospinal fluid (ACSF) was prepared following previously described methods (MacIver & Roth, 1987; Hirota & Roth, 1997; Archer et al., 2001). High purity water (~18 MΩ) (filtered with a Millipore Super Q water system) and cell culture tested chemicals (Sigma Aldrich, Calgary) were used. The composition of ACSF was (mM): NaCl – 124, $NaHCO_3$ – 26, Glucose – 10, KCl – 5, NaH_2PO_4 · H_2O 1.25, $MgSO_4$ · H_2O – 2, $CaCl_2$ – 2 (pH 7.3 – 7.4). The ACSF (pH 7.4) was cooled on ice (4° – $8^\circ C$) and kept saturated with a 95% O_2 / 5% CO_2 (carbogen) gas mixture (Praxair, Calgary) for at least one hour prior to use.

2.3   Dissection and Slice Preparation

The technique for the preparation of hippocampal slices was approved by the Animal Care Committee at the University of Calgary, in accordance with the Canadian Council on Animal Care standards set out in the *Guide to the Care and Use of Experimental Animals*. The methods for preparing and maintaining rat

hippocampal slices were adopted from previously documented methods (MacIver and Roth, 1987; Hirota and Roth, 1997; Archer et al., 2001). For all experiments, rats were first anaesthetised with ether (VWR Canlab, Calgary). Upon complete cessation of breathing and reduced cardiac function, the brain was then rapidly removed and placed in precooled, oxygenated ACSF. The hippocampus was dissected from the right hemisphere and transverse slices 400 μm thick were cut using a Stoelting tissue chopper (Figure 3). Slices were placed on a nylon mesh screen at the gas-liquid interface in a tissue chamber. Humidified carbogen circulated over the tissues (1.0 L/min) and ACSF solution was perfused through the chamber, passing over the slices at a rate of 1.0 mL/min. Slices were incubated and warmed to 35°C for ~90 minutes without electrical stimulation.

2.4 Stimulating and Recording Electrodes

For each experiment, a bipolar tungsten stimulating electrode, coated with Kynar, a vinylidene fluoride resin (Pennwalt Corp., Philadelphia, PA), was placed on the Schaffer collateral fibres in the stratum radiatum of CA3 of the hippocampal slice in order to activate excitatory synaptic inputs to CA1 pyramidal neurons. An extracellular glass microelectrode (resistance of 3-5 MΩ) filled with ACSF, buffered with HEPES (N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid) (Sigma Aldrich, Calgary), was placed in the CA1 cell body region in order to record synaptically evoked field potentials in response to the orthodromic stimulation (Figure 4).
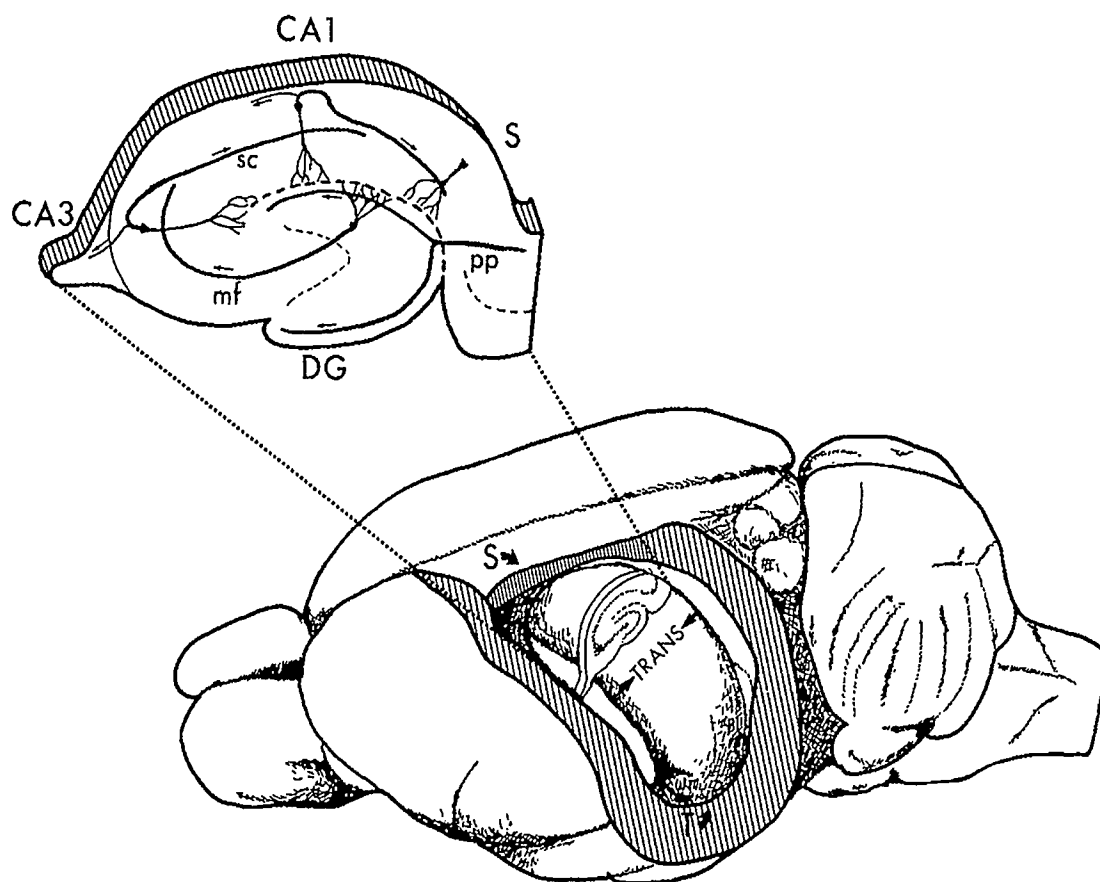
Figure 3. A drawing of the rat brain showing the location of the hippocampal formation. (Rostral, right; caudal, left.) The hippocampus is dissected out, and a transverse section is made that is useful for *in vitro* extracellular recordings. "Reprinted from *Neuroscience,* 31(3): 571-591, Amaral & Witter: The Three-Dimensional Organization of the Hippocampal Formation: A Review of the Anatomical Data, p. 573, Copyright (1989), with permission from Elsevier Science".
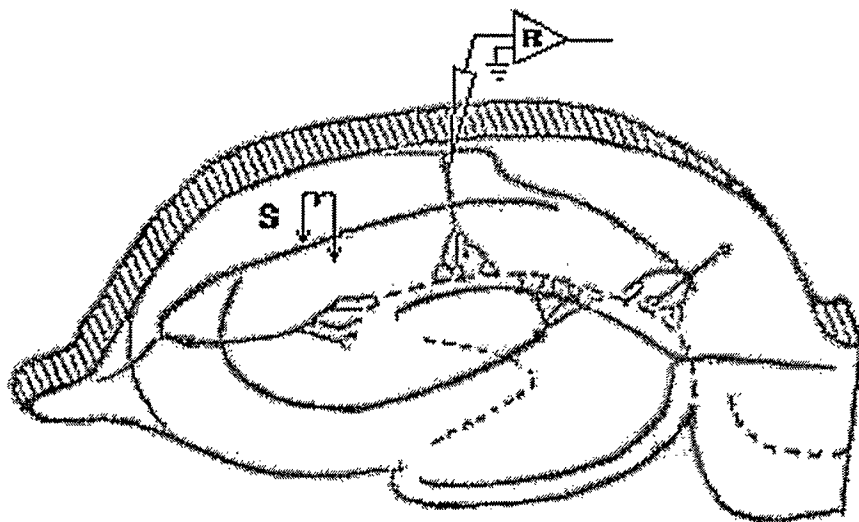
Figure 4. A drawing of the hippocampal slice preparation showing the position of electrodes. The stimulating electrode (S) is placed on the Schaffer collateral fibres (sc) of CA3, and the recording electrode (R) is situated in the cell body region of CA1. (Adapted from Amaral & Witter, 1989.)

Paired stimulus pulses of 0.1 ms duration were delivered to the slice every 100s (0.01 Hz), with a 40 ms delay between pulses (interstimulus interval) set to achieve maximal PPF, using a Grass S88 stimulator and a Grass SIU5 stimulus isolation unit (Grass Corp., Quincy, MA). For the purpose of these experiments, once the maximal population spike (PS2) amplitude was determined, the stimulation voltage was decreased in order to attain half-maximal PS2 amplitude. All experiments were conducted at half-maximum to permit observation of synaptic depression and enhancement (Figure 5).

2.5   Paired Pulse Analysis

Field potentials produced by paired pulse stimulation were amplified (x1000) using a Grass P15 amplifier (Grass Corp., Quincy, MA), with low- and high frequency filters set at 1 and 50 kHz respectively. The recorded signals were collected and stored on computer using a Neurodata converter (NeuroData Instruments Corp., New York, NY). Analysis was conducted using custom Labview®-based software (Advanced Measurements, Calgary, AB). Measurements were made on the amplitude of PS2 of the evoked field potential.

2.6   Administration of Gases

Control gases (carbogen, methane or nitrogen) were obtained from Praxair, Calgary. The H_2S gas mixture (0.2% H_2S, balance N_2) was obtained from Praxair, Calgary, with the analysis provided by Praxair, Calgary. Shell Canada
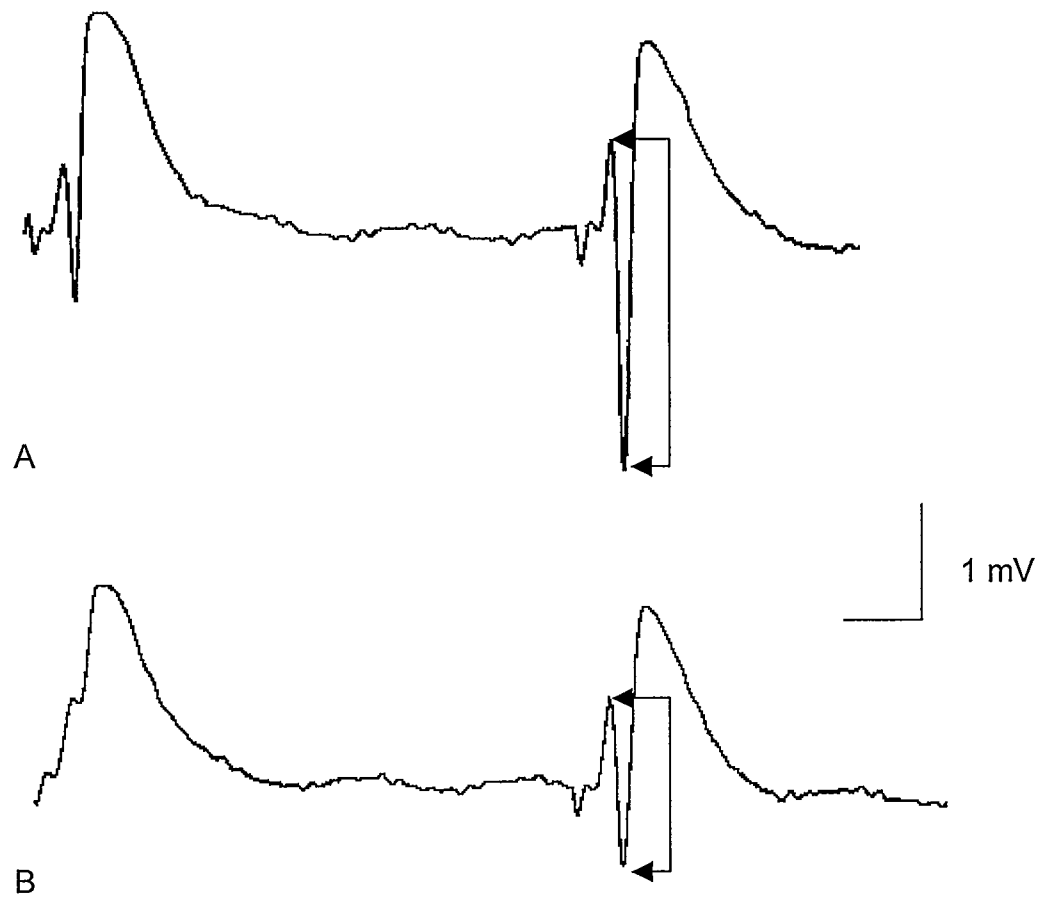
Figure 5. Sample recordings of the response in CA1 to paired-pulse stimulation of CA3 of the hippocampus. The maximal response of the slice (A) is first determined, and following this the stimulation voltage is decreased so the only (B) half the maximal PS amplitude (PS ½ max) is achieved. This amplitude is used for the pre-exposure level.

(Bob Wrublewski, Calgary) donated the sour gas sample and Maxxam Analytical (Bernie Brassard, Edmonton) provided the sour gas collection and analysis, as well as the rental of the cylinder.

At the beginning of each experiment there was a 20-minute "pre-exposure" period where only carbogen was supplied to the slice, and PS2 amplitudes were recorded every 100 seconds. Following the pre-exposure period, a control gas (methane, nitrogen or carbogen) or test gas (e.g. sour gas or H_2S) was administered to the slices via the warmed, humidified air (O_2/CO_2) that circulated over the slices (1.0 L/min) using calibrated, commercial flow meters (Gilmont Instruments Inc.). Appropriate flow rates were verified by gas chromatographic analysis. Gas exposure periods were 30 minutes in duration, with a period of at least one hour following exposure to allow for "wash-out" of test or control gas. For the time control experiments, carbogen was supplied continuously to the slices for 110 minutes. The 30 minute and 60 minute periods following the first 20 minutes were referred to as the "gas exposure" and "washout" periods respectively for the purpose of comparing effects during similar time periods for other experiments when various control and test gases were supplied to the slices.

Sour gas containing a range of H_2S concentrations (1, 5, 10 and 25 ppm) was tested in a series of separate experiments. The equivalent concentrations of H_2S

alone were also studied. The effects of methane were studied at concentrations equivalent to the amount present in sour gas containing 25 ppm H_2S. The effects of nitrogen were studied at concentrations equivalent to that present in a sample of 25 ppm H_2S. Time control experiments were conducted whereby only carbogen was supplied to the slices for the entire experiment.

2.7   Use of 2-Amino-5-phosphonopentanoic Acid (AP5)

AP5 (Sigma Aldrich, Calgary), a pharmacological NMDA receptor antagonist, was used in a series of experiments conducted at 25 ppm H_2S to determine whether H_2S affects NMDA receptor activity. Some preliminary experiments with AP5 were also conducted at 1 ppm H_2S. For the purpose of these experiments, 50 µM AP5 (Abe & Kimura, 1996; Archer et al., 2001) was added to the ACSF that bathed the slices for the course of the entire experiment.

2.8   Absence of Electrical Stimulation Experiments

To test whether the effects of H_2S on synaptic transmission were dependent on the presence of electrical stimulation, a series of experiments were conducted that involved a "lack of stimulation" period. Recordings during the pre-exposure period in response to electrical stimulation were obtained. Subsequent to this, 25 ppm H_2S was applied to the slice for 30 minutes, followed by a 60-minute washout period. During the total 90-minute period, no electrical stimulation was supplied to the slice. After the absence of electrical stimulation for 90 minutes,

the stimulator was turned back on and recordings were obtained for another 45 minute extended washout period.

2.9   Hydrogen Sulphide Monitoring

Continuous sampling and monitoring of the air above the slices was conducted during the entire experiment in order to verify the concentrations of H_2S (alone or as a component of sour gas) during periods of control, gas application and washout.   Detection was possible using a CiTicel 4-20MA H_2S-T3HH electrochemical H_2S sensor (McNeill International Inc., Mentor, OH, USA) and monitoring was performed using a Gastech Model Safe-T-Net 2000 H_2S monitor (Gastech Instruments Canada Ltd.).   Samples of air were removed from the chamber using a vacuum drawing at 300 – 500 mL/min and delivered to the sensor.   Levels of H_2S (displayed in ppm) were continuously displayed on the monitor in order to regulate the desired concentration of H_2S throughout the course of the exposure period.

2.10   Gas Chromatographic Analysis

Gas chromatographic (GC) analysis of the air above the slices during gas exposure periods was conducted in order to confirm H_2S concentrations measured with the H_2S electrochemical sensor and monitor.   The sample was drawn through a gas sampling valve at 6 mL/min or less from the slice bath chamber into a HP 5890A Gas Chromatograph.   The gas sampling valve injected

a 150 μL sample onto a DB1 30 M x 0.530 mm megabore column (J&W Scientific) through a split/splitless injector (1:1). The carrier gas (Helium) flow rate through the column was 15 mL/min. The oven temperature was kept constant at 40°C and the Flame Photometric Detector (FPD) was kept at 250°C. The resulting peaks were analysed by comparing them to a calibration curve that was generated with certified calibration gases (Praxair) under identical chromatographic parameters. The results from the GC analysis were used to set the flow rates for test gases into the chamber.

2.11   Composition of Sour Gas Sample

The sample used to conduct the research for this thesis was taken from the Hope Creek Gas Plant (Shell Canada) and has methane as the major component (85%) and H_2S present as 4% (40,000 ppm) of the gas mixture. The complete analysis of the sour gas sample is found in Appendix A.

2.12   Statistical Analysis

Each experiment was conducted on at least five slices obtained from different rats. For each slice, the last ten raw data points for the pre-exposure (baseline) period and the last five data points for the gas application and washout periods were averaged, yielding three data points for each slice. Statistical analysis was then performed, using two-way repeated measures analysis of variance (ANOVA) tests, for slices exposed to each of the H_2S concentrations tested (1, 5,

10 and 25 ppm) in sour gas and as H_2S alone, as well as control gases (Carbogen and Methane). In a two-way repeated measures ANOVA test, there are two experimental factors that may affect the experimental treatment; in this case the two factors are concentration of gas administered and the time after initial treatment (application of gas). The two-way design tested for differences between the concentrations of gases, between the two test gases, and also for differences occurring during the experiment. The analysis was performed using Sigmastat® software and graphs were plotted using Sigmaplot® software (SPSS, Chicago, IL). Statistical significance was inferred when $p < 0.05$.

3.0   Results

3.1   Controls

3.1.1   Time

Control experiments were conducted in order to ascertain that slice responsiveness in the absence of any intervention remained stable over time. Recordings of paired-pulse field potentials from the CA1 cell body region were recorded and stored every 100 seconds for 110 minutes.

In the presence of carbogen alone, no significant changes in synaptic transmission in the CA3/CA1 pathway were observed during the 110-minute recording period. An example of one representative slice is shown in Figure 6. There were no significant changes in the PS2 amplitude during the first 20 minutes (1.27 mV ± 0.23 mV) and the PS2 was stable for the remaining 90 minutes of the experiment (1.25 mV ± 0.26 mV) (Figure 6). There was variation in the PS2 amplitude over the 110-minute period (Figure 6) and the PS2 amplitude values (including the half-maximal PS2) were different when comparing slices. Therefore, data were normalised in order to compare effects between different slices (Figure 7). There was no change in the PS2 amplitude during the "gas exposure" (99.7% ± 11.2%) and the "washout" (96.8% ± 26.4%) periods in comparison to the initial levels in the first 20 minutes. As well, no statistically significant differences were found when the average raw data points
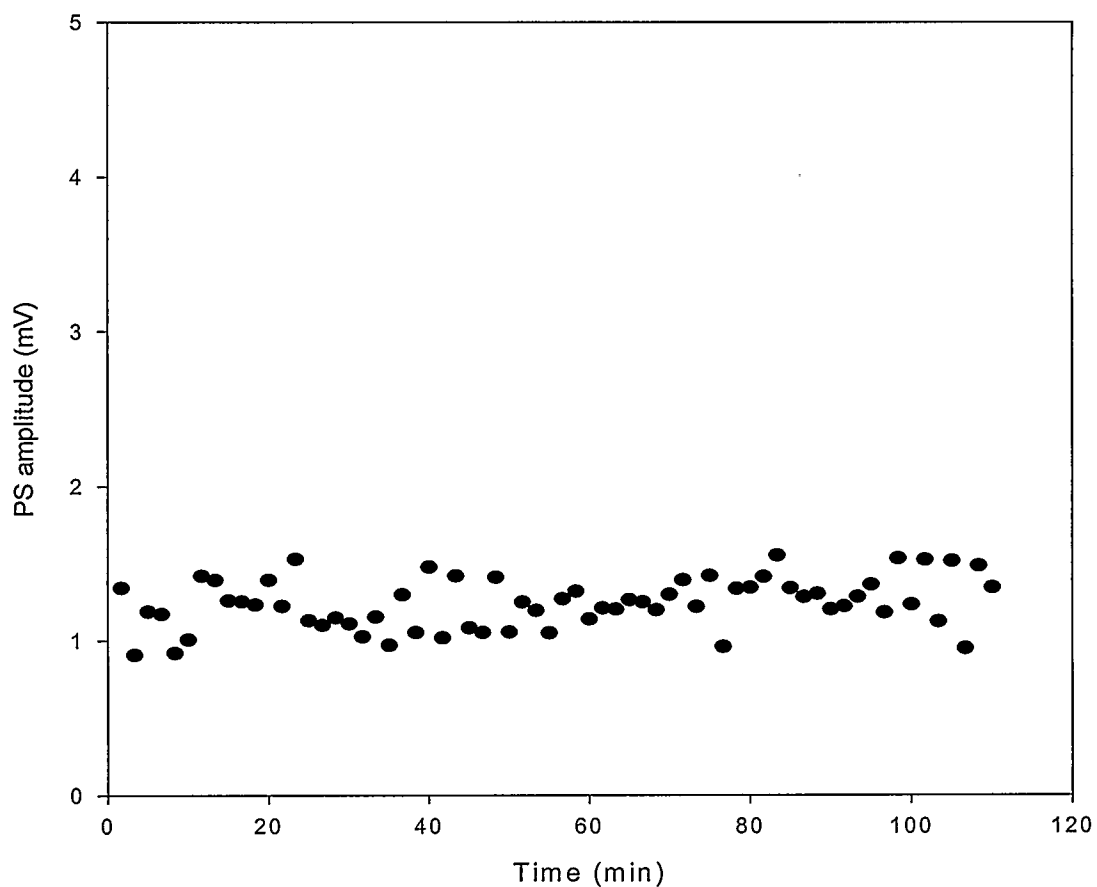
Figure 6. Results from a representative single time control experiment. No significant changes in PS2 amplitude (mV) for the duration of the experiment were seen. For all time-dependent plots, each point (•) represents one PS2 amplitude measured from one complex as a result of paired-pulse stimulation of the CA3-CA1 pathway in the hippocampus every 100 seconds.
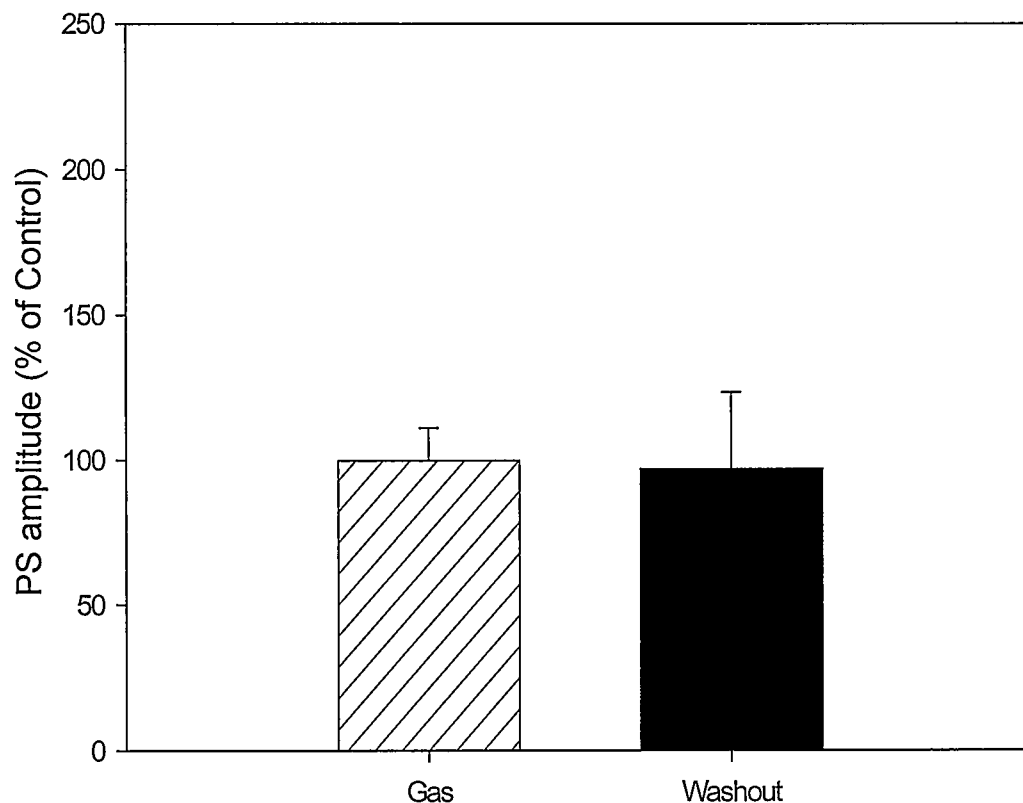
Figure 7. The PS2 amplitude was unchanged for the duration of the time control experiment in hippocampal slices exposed to carbogen alone. For each slice, the averages of the last five complexes for the "gas exposure" and "washout" periods are normalised with respect to the baseline value (average of 10 complexes). The "gas exposure" and "washout" values from each slice are then averaged. Hatched and black bars represent mean responses (n=5 slices) for the "gas" and "washout" periods respectively. Error bars represent standard deviation.

from each slice were analysed (one-way repeated measures ANOVA test: see Methods 2.12).

3.1.2 Methane

Methane was the most abundant component (85%) of the sour gas sample used for all sour gas experiments. H_2S was present in the next highest concentration (4%). Therefore, in order to ensure that methane alone did not cause effects on synaptic transmission, methane exposure experiments were conducted. Following the 20-minute pre-exposure period methane (1060 ppm) was delivered for 30 minutes (gas application period). The flow rate (1.25 mL/min) and concentration of methane were identical to those maintained when administering sour gas containing the equivalent of 25 ppm H_2S (the highest concentration tested) to the slice.

Exposure to methane did not result in any significant changes in synaptic transmission over 110 minutes. In one slice, the average PS2 amplitude during the pre-exposure period was 1.53 mV ± 0.10 mV (Figure 8). The PS2 amplitude remained stable within this range throughout the gas application (1.50 mV ± 0.09 mV) and washout (1.54 mV ± 0.07 mV) periods (Figure 8). Normalised data from six slices were averaged (Figure 9) and no changes in PS2 amplitude were observed when comparing the methane application (101.3% ± 11.0%) and the washout (94.4% ± 16.9%) periods to the pre-exposure period. Statistical
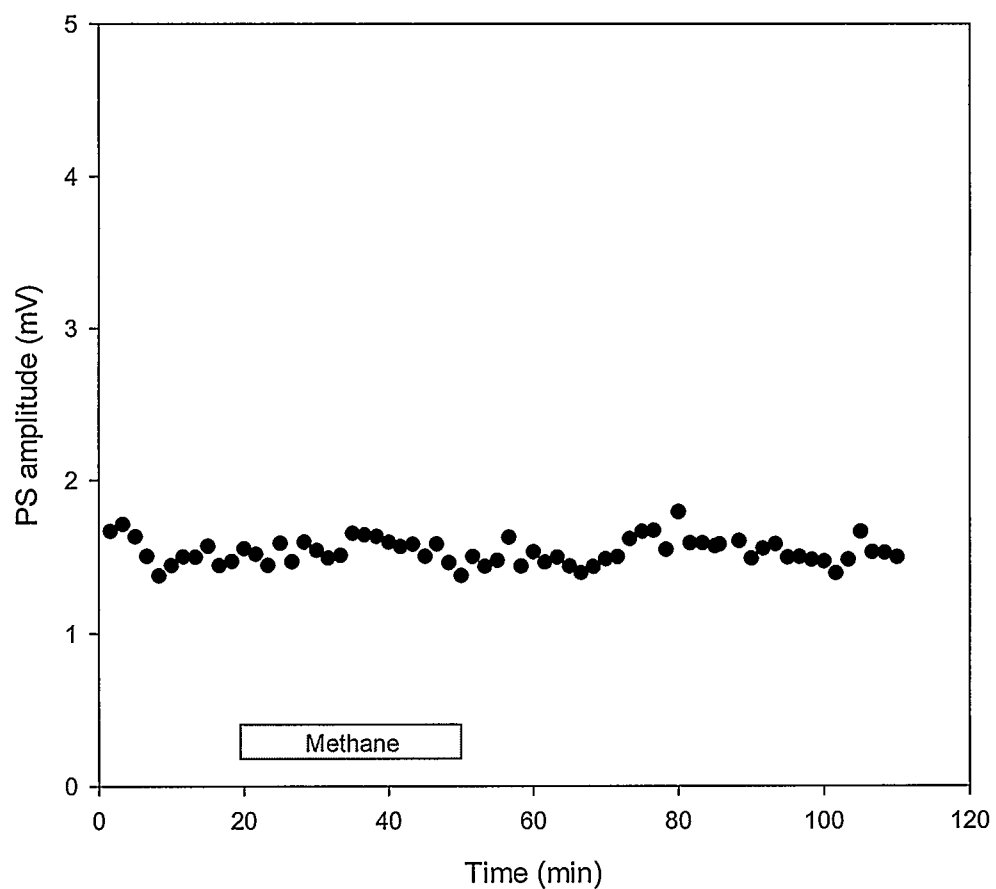
Figure 8. Results from a representative methane control experiment. There were no changes in PS2 amplitude (mV) during and after application of methane (indicated by shaded bar) when compared to the 20-minute pre-exposure period.
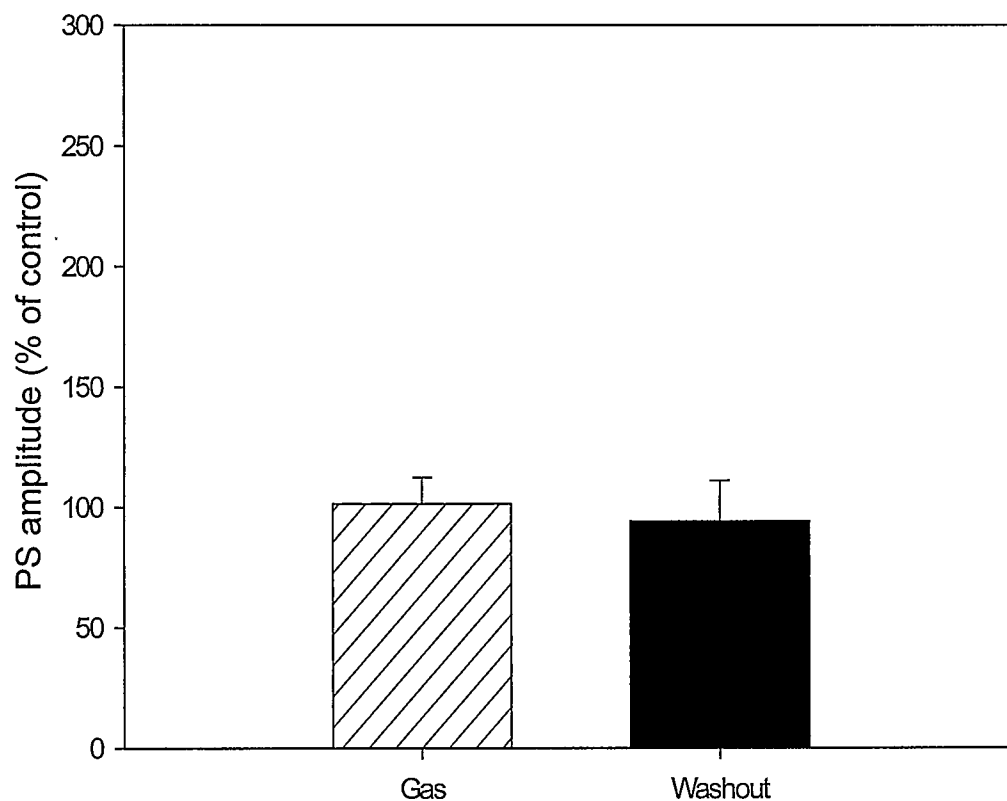
Figure 9. The PS2 amplitude did not change significantly during gas application and washout periods for slices exposed to methane (n=6 slices). For each slice, the averages of the last five complexes for the 30-minute gas (methane) application and 60-minute washout periods were normalised with respect to the 20 minute ACSF perfusion (pre-exposure) value (average of 10 complexes). The gas and washout values from each slice were then averaged and presented as mean ±SD (n=6).

analysis did not reveal any significant differences during methane exposure and washout compared to the pre-exposure period.

3.1.3   Nitrogen

The H_2S cylinder used for all H_2S experiments contained primarily nitrogen (~99.8%) with a balance of H_2S (~0.2% or 2010 ppm). Therefore, exposures to nitrogen alone were conducted to determine whether it was capable of producing effects on synaptic transmission and consequently changes in PS2 amplitude. Following the 20-minute pre-exposure period, nitrogen (12,500 ppm) was delivered for 30 minutes (gas application period) to the hippocampal slice. The flow rate (12.5 mL/min) and concentration of nitrogen was identical to that maintained when administering 25 ppm H_2S (the highest concentration tested).

As observed in slices exposed to methane and carbogen, exposure to nitrogen did not produce any significant changes in the PS2 amplitude. In one slice, the average PS2 amplitude during the pre-exposure period was 1.52 mV ± 0.35 mV, and the PS2 amplitude did not significantly change by the end of the nitrogen application (1.58 mV ± 0.16 mV) and washout (1.71 mV ± 0.15 mV) periods (Figure 10). When the normalised data from six slices were averaged and statistically analysed, no significant changes in the PS2 amplitude during the nitrogen exposure period (109% ± 8.9%) and the washout period (109.1% ± 25.5%) were observed when compared to the pre-exposure period (Figure 11).
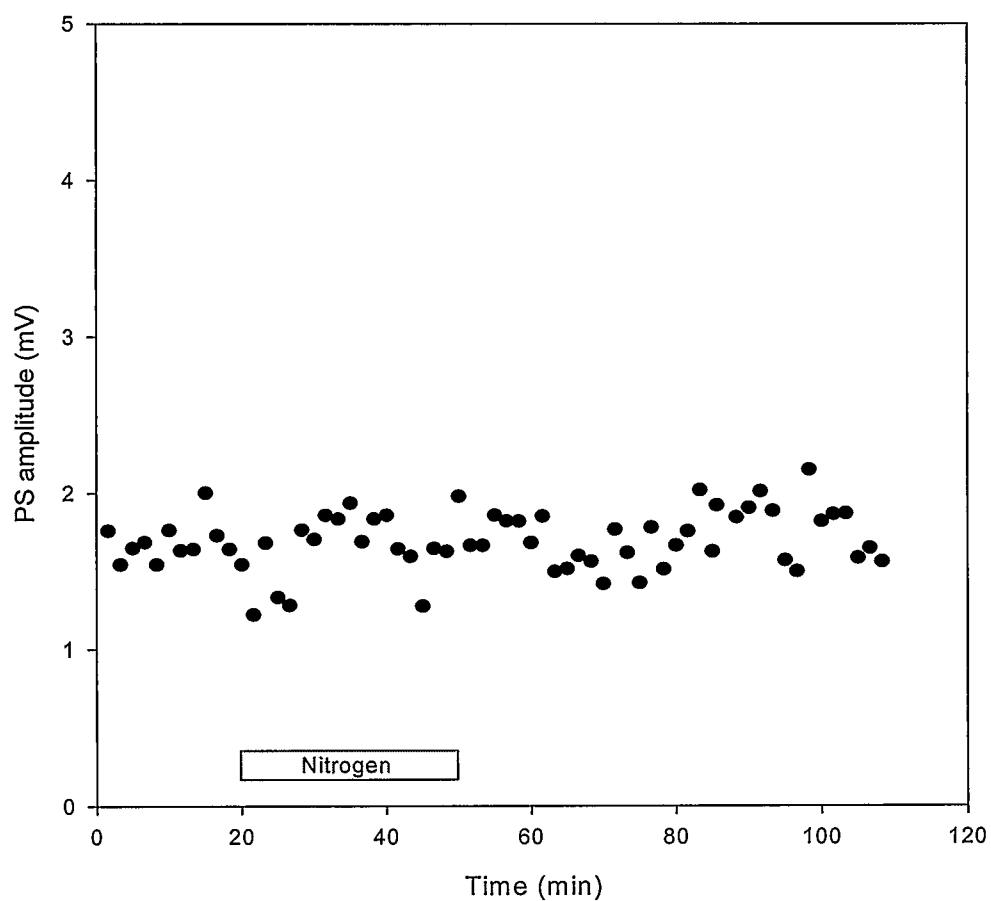
Figure 10. Results from a representative nitrogen control experiment. There were no changes in PS2 amplitude (mV) during and after application of nitrogen (indicated by shaded bar) when compared to the 20-minute pre-exposure period.
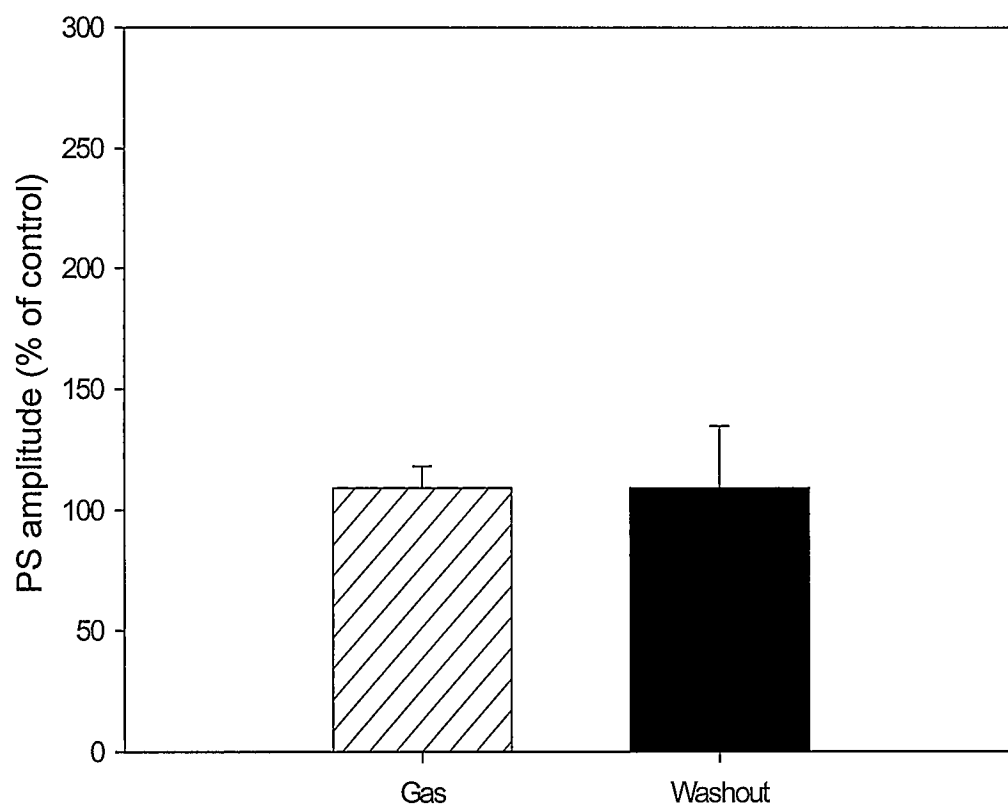
Figure 11. There were no significant changes in PS2 amplitude during gas application and washout periods for slices exposed to nitrogen (n=6 slices). For each slice, the averages of the last five complexes for the nitrogen application and washout periods were normalised with respect to the 20-minute ACSF perfusion (pre-exposure) value (average of 10 complexes). The gas and washout values from each slice were then averaged and presented as mean ±SD (n=6).

3.2   Effects of Sour Gas

3.2.1   Sour Gas Containing 1 ppm H_2S

In contrast to the effects observed with control gases, all concentrations of sour gas tested produced significant effects on synaptic transmission in the CA3 to CA1 pathway of exposed slices. In one representative slice exposed to sour gas containing 1 ppm H_2S, the average PS2 amplitude during the pre-exposure period was 1.83 ± 0.16 mV; by the end of the sour gas exposure period, the PS2 amplitude increased to 2.33 mV ± 0.12 mV and continued to increase to 3.69 ± 0.07 mV by the end of the one-hour washout period (Figure 12). When the normalised data from five slices were averaged, there was an increase in the PS2 amplitude (116.5% ± 20.8%) during the gas application period (Figure 13), however this increase was not significant when compared with the pre-exposure period. During the washout period, there was a further increase in PS2 amplitude (190.1% ± 41.7%) that was significantly different from the pre-exposure and gas application periods. To determine whether the effects of sour gas on synaptic transmission were dose dependent, subsequent experiments were conducted on slices exposed to sour gas containing 5, 10 and 25 ppm H_2S.

3.2.2   Sour Gas Containing 5 ppm H_2S

In a single slice exposed to sour gas containing 5 ppm H_2S, the average PS2 amplitude during the pre-exposure period was 2.11 mV ± 0.11 mV, and application of sour gas caused a gradual increase in the PS2 amplitude to 2.36

Figure 12. Results from a single representative sour gas (1 ppm H_2S) experiment. There was a gradual increase in PS2 amplitude (mV) after the pre-exposure period. At the end of the application of sour gas (indicated by the shaded bar) there was a slight increase in PS2 amplitude. During the one-hour washout period, there was a steady increase in PS2 amplitude, with the greatest increase present at the end of the experiment,

Figure 13. There was a gradual increase in PS2 amplitude during application of sour gas containing 1 ppm H_2S, and a further significant increase during the washout period (n=5 slices). For each slice, the last five complexes from the sour gas application and washout periods were averaged and normalised with respect to the pre-exposure value (average of 10 complexes). The gas and washout values from each slice were then averaged and presented as mean ± SD; * indicates significance (p<0.05) with respect to pre-exposure and gas application values.

mV ± 0.14 mV (Figure 14). This was followed by a maximum increase to 2.85 mV ± 0.25 mV) at the end of the washout period (Figure 14). When data were normalised and six slices were averaged (Figure 15), the result was a slight increase in PS2 amplitude during the gas application period (120.7% ± 18.5%) when compared with the pre-exposure period, but this increase was not statistically significant. However, there was a significant increase in the PS2 amplitude during the washout period (145.9% ± 32.0%) compared to the pre-exposure period (Figure 15). These effects were similar to those observed in slices exposed to sour gas containing 1 ppm H_2S (see Results Section 3.2.1).

3.2.3   Sour Gas Containing 10 ppm H_2S

In one representative slice exposed to sour gas containing 10 ppm H_2S (Figure 16), the average PS2 amplitude during the pre-exposure period was 1.69 mV ± 0.09 mV. There was a gradual increase in PS2 amplitude up to 2.32 mV ± 0.37 mV at the end of the sour gas application. This was followed by a further increase to a maximum of 3.96 mV ± 0.22 mV at the end of the washout period (Figure 16). When normalised data from six slices were averaged (Figure 17), a slight increase in PS2 amplitude was seen during the gas application period (138.7% ± 13.5%) when compared with the pre-exposure period (not statistically significant). Conversely, the increase in PS2 amplitude during the washout period (206.6% ± 36.6%) when compared to both the pre-exposure and gas application periods (Figure 18) was significant. The enhancement effects on
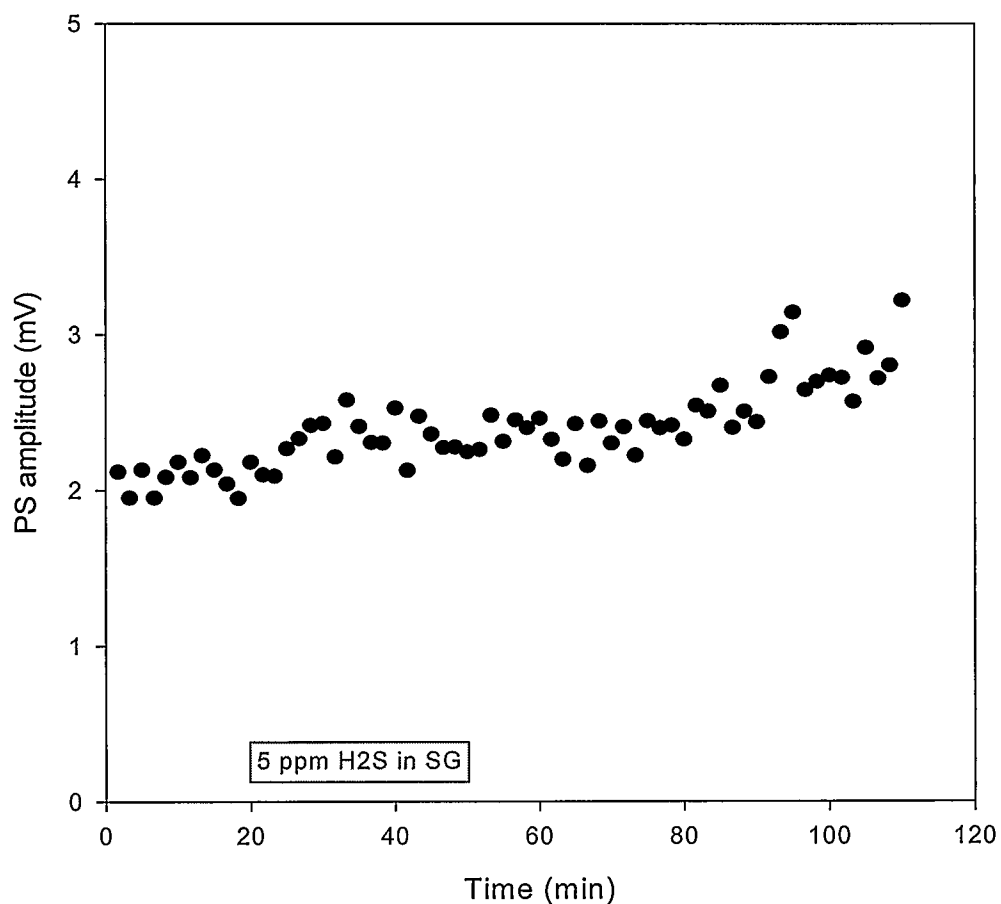
Figure 14. Results from a single sour gas (5 ppm H_2S) experiment. There was a gradual increase in PS2 amplitude (mV) after the pre-exposure period. During the application of sour gas (indicated by the shaded bar) there was a slight increase in PS2 amplitude. During the one-hour washout period, there was a further gradual increase in PS2 amplitude, with the greatest increase occurring at the end of the experiment.
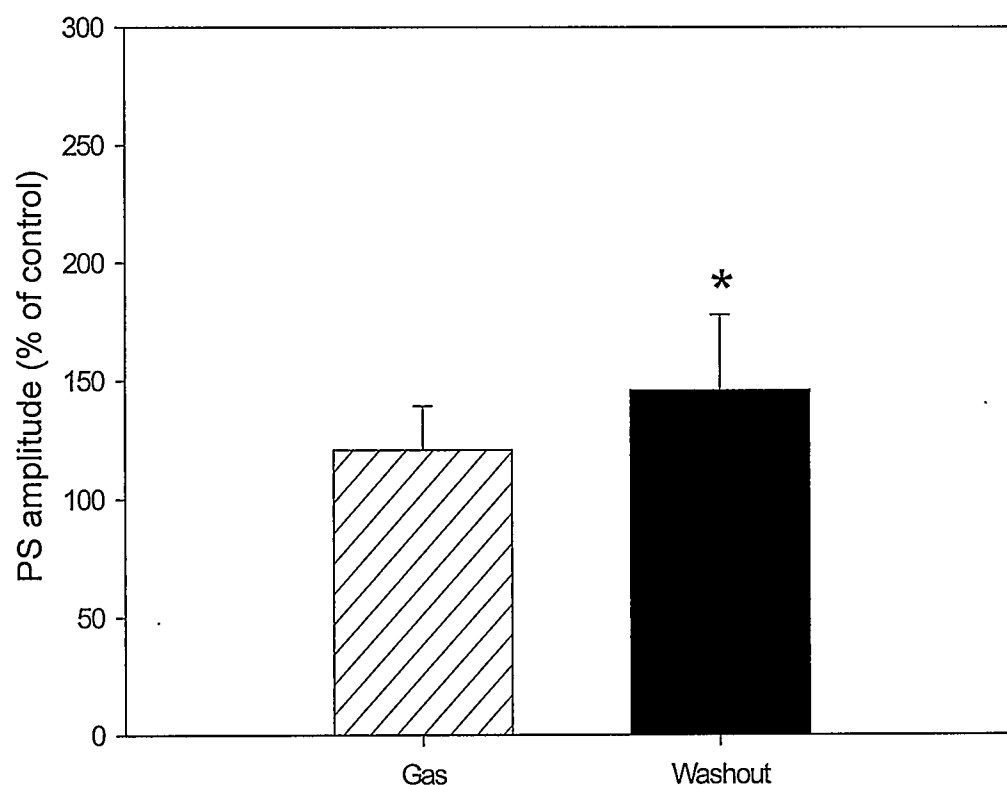
Figure 15. Exposure to sour gas containing 5 ppm H_2S produced a significant increase of PS2 amplitude during the washout period, with a gradual increase occurring during the gas application period (n=6 slices). For each slice, the last five complexes from the sour gas application and washout periods were averaged and normalised with respect to the pre-exposure value. The gas and washout values from each slice were then averaged and presented as mean ± SD; * indicates significance (p<0.05) with respect to pre-exposure and gas application values.
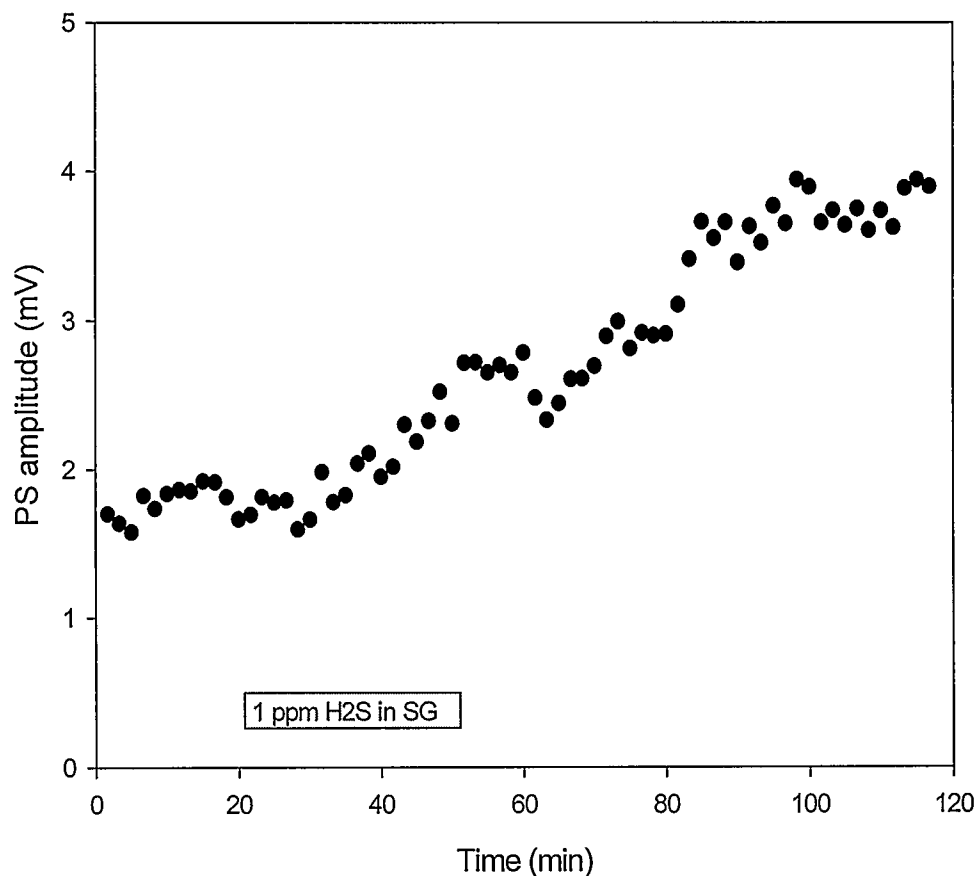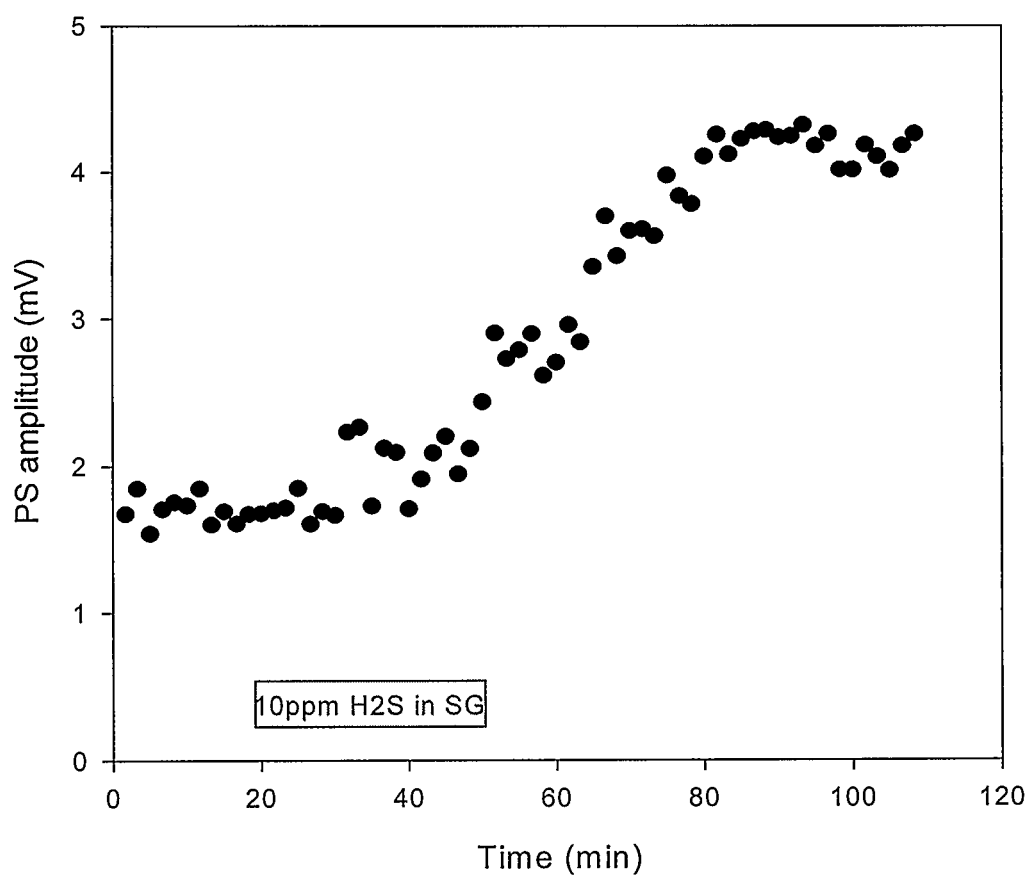
Figure 16. Results from a single representative sour gas (10 ppm H_2S) experiment. There was a gradual increase in PS2 amplitude (mV) after the pre-exposure period. At the end of the application of sour gas (indicated by the shaded bar) there was a slight increase in PS2 amplitude. During the one-hour washout period, there was a steady increase in PS2 amplitude, with the greatest increase occurring at the end of the experiment.
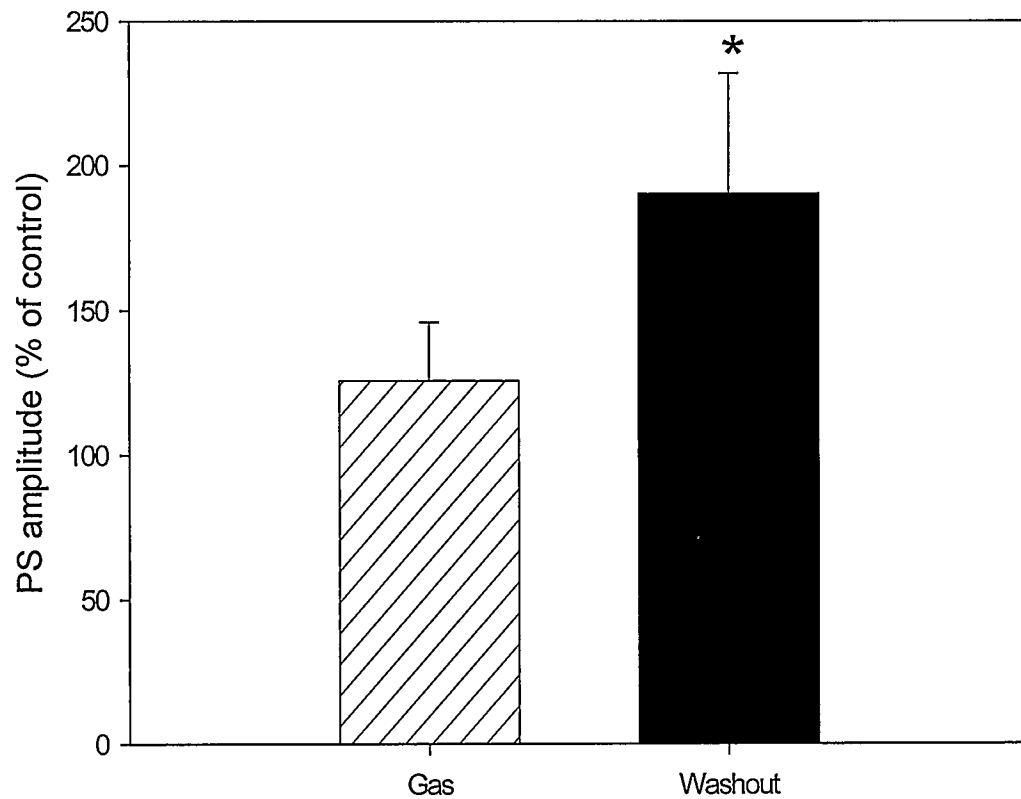
Figure 17. Exposure to sour gas containing 10 ppm H_2S had a significant effect on PS2 amplitude during the washout period (n=6 slices). For each slice, the last five complexes of the sour gas application and washout periods were averaged and normalised with respect to the pre-exposure value. The gas and washout values from each slice were then averaged and presented as mean ± SD; * indicates significance (p<0.05) with respect to pre-exposure and gas application values.

synaptic transmission observed were similar to those observed in slices exposed to sour gas containing either 1 ppm H_2S (see Results Section 3.2.1) or 5 ppm H_2S (see Results Section 3.2.2).

3.2.4   Sour Gas Containing 25 ppm H_2S

Exposure to sour gas containing 25 ppm H_2S produced very different effects on synaptic transmission in comparison to sour gas containing 1, 5 and 10 ppm H_2S. There was a biphasic response that involved an initial depression of PS2 amplitude during the gas application period, followed by a return to pre-exposure values and subsequent enhancement of PS2 amplitudes that extended throughout the entire washout period. For example, the PS2 amplitude in one slice during the pre-exposure period was 2.46 mV ± 0.15 mV (Figure 18). During the sour gas exposure, the PS2 amplitude decreased to 1.65 mV ± 0.26 mV (Figure 18). During the one-hour washout period, the PS2 amplitude quickly returned to pre-exposure levels (within five minutes) and then continued to increase with maximum enhancement occurring at the end of the experiment (5.60 mV ± 0.14 mV) (Figure 18). Normalised data from an average of five slices are presented in Figure 19. There was a decrease in the PS2 amplitude during the gas application period (67.5% ± 7.1%) that was not significant (p=0.05) when analysed using the two-way repeated measures ANOVA test. However, further statistical analyses using the t-test showed significant depression (p<0.05) when normalised PS2 amplitudes of pre-exposure and gas application periods
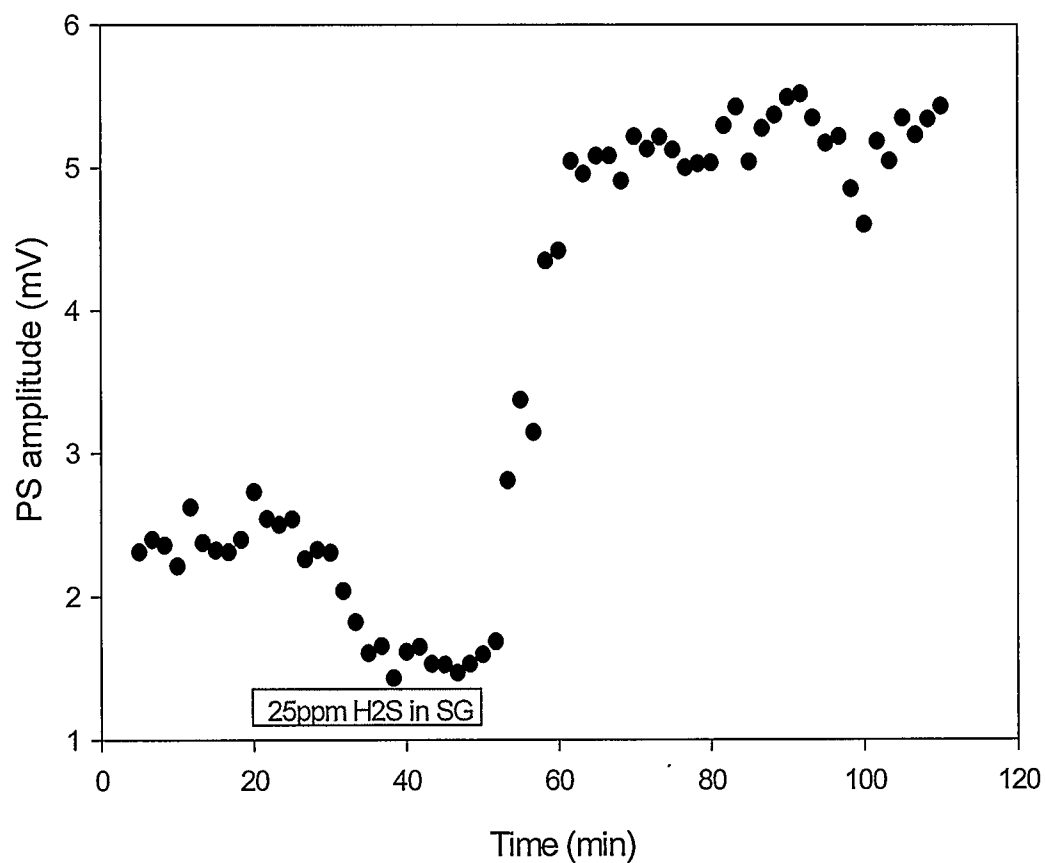
Figure 18.   Results from a representative single sour gas (25 ppm H_2S) experiment.   There was a unique biphasic effect characterised by a progressive decrease in PS2 amplitude (mV) during the sour gas application (indicated by the shaded bar).   Following this depression of synaptic transmission, the PS2 amplitude returned to pre-exposure values and then steadily increased to a maximum level by the end of the washout phase.
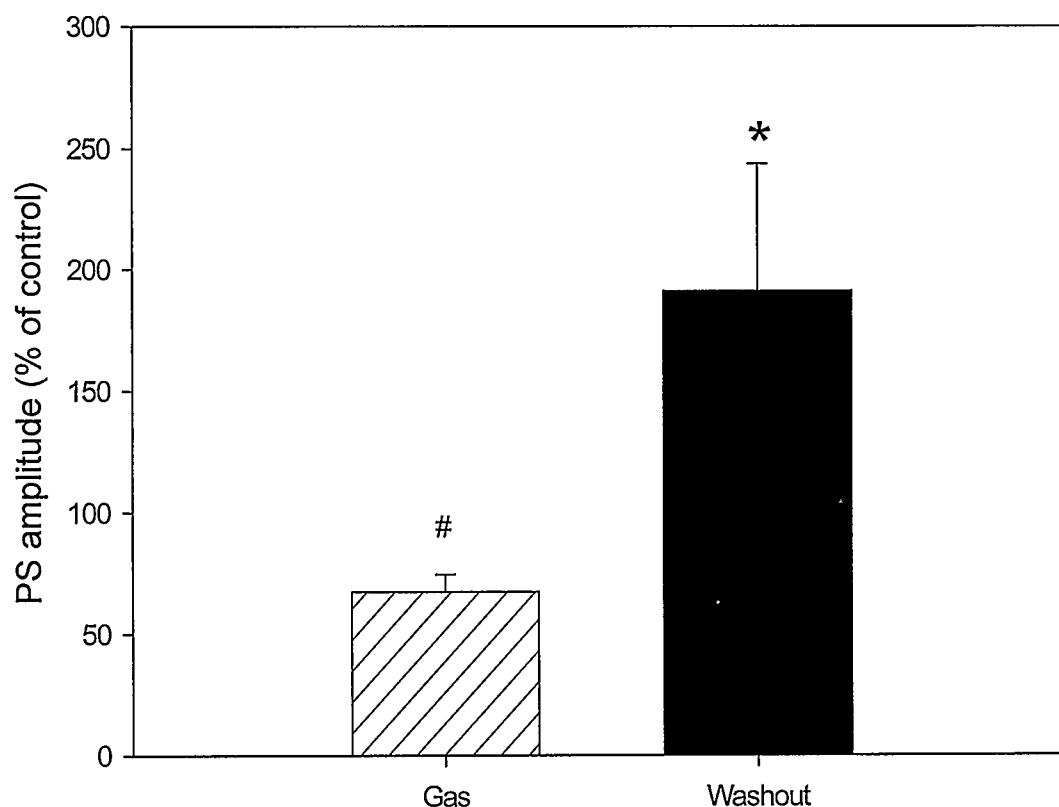
Figure 19. Sour gas containing 25 ppm H_2S had a biphasic effect on synaptic transmission. There was depression of the PS2 amplitude during the gas application period and enhancement of the PS2 amplitude during the washout period (n=5 slices). For each slice, the last five complexes of the gas application and washout periods were averaged and normalised with respect to the pre-exposure value. The gas and washout values from each slice were then averaged and presented as mean ± SD; * indicates significance (p<0.05) with respect to pre-exposure and gas application values; # indicates significant depression by t-test (p<0.05) with respect to the pre-exposure period.

averaged from five slices were compared (Figure 19). The increase in PS2 amplitude during the washout period (191.0% ± 52.6%) was significant ($p<0.05$) when compared to both the pre-exposure and gas application periods using the two-way repeated measures ANOVA test (Figure 19).

3.3   Effects of Hydrogen Sulphide

3.3.1   1 ppm H_2S

Application of 1 ppm H_2S produced enhancement effects on synaptic transmission that were similar to those observed with sour gas containing equivalent levels of H_2S (see Results Section 3.2.1). In one representative slice (Figure 20), the PS2 amplitude for the 20-minute pre-exposure period was 1.40 mV ± 0.22 mV. There was an increase in the average PS2 amplitude during the 30-minute gas application to 1.94 mV ± 0.15 mV in comparison to the average PS2 amplitude for the pre-exposure period, and the PS2 amplitude continued to increase during the washout period to a maximum 2.88 mV ± 0.14 mV (Figure 20). When normalised data from five slices were averaged, the result was an increase in the PS2 amplitude at the end of the gas application period (163% ± 68.8%), however this increase was not statistically significant when compared to the pre-exposure period (Figure 21). The greatest increase in PS2 amplitude was observed at the end of the washout period (286.7% ± 108.2%). This enhancement effect was significant ($p<0.05$) when compared to the pre-exposure and gas application periods.

Figure 20. Results from a representative 1 ppm H2S experiment. There was a gradual increase in PS2 amplitude (mV) after the pre-exposure period. At the end of the application of H2S (indicated by the shaded bar) there was a slight increase in PS2 amplitude. During the one-hour washout period, there was a steady increase in PS2 amplitude, with the greatest increase present at the end of the experiment.
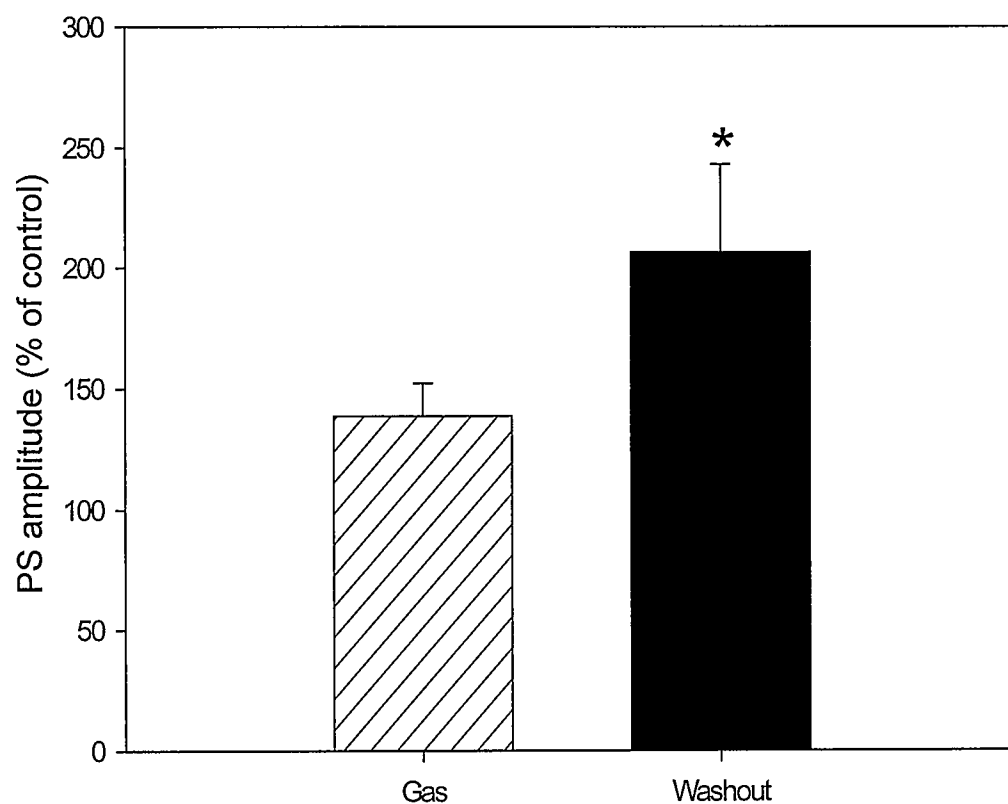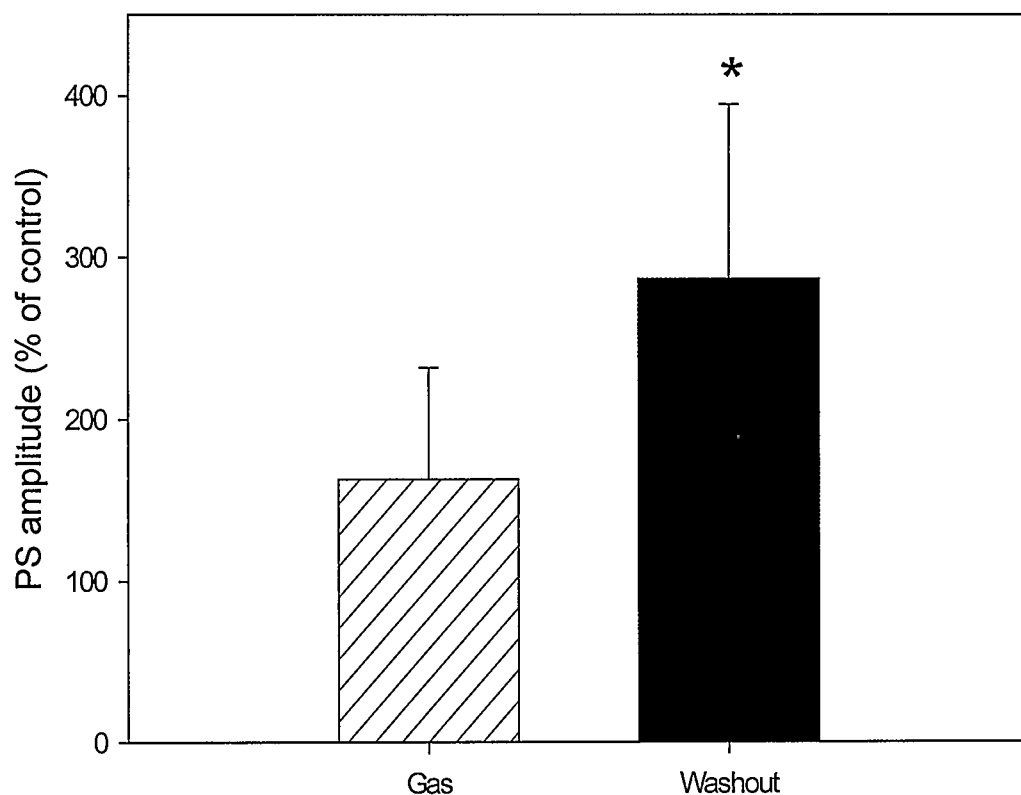
Figure 21. 1 ppm H₂S exposure had a significant effect on PS2 amplitude during the washout period, with a gradual increase occurring during the gas application period (n=5 slices). For each slice, the last five complexes from the gas application and washout periods were averaged and normalised with respect to the pre-exposure value. The gas and washout values from each slice were then averaged and presented as mean ± SD; * indicates significance (p<0.05) with respect to pre-exposure and gas application values.

3.3.2   5 ppm H$_2$S

Application of 5 ppm H$_2$S had effects on synaptic transmission that were similar to those observed with sour gas containing equivalent levels of H$_2$S (see Results Section 3.2.2). Compared to the pre-exposure period (1.30 mV ± 0.10 mV), there was no significant effect on the PS2 amplitude during the gas application period (1.40 mV ± 0.14 mV) (Figure 22). However there was a gradual increase during the one-hour washout period to 2.02 mV ± 0.23 mV (Figure 22). When normalised data averaged from five slices were analysed, there was an increase of PS2 amplitude that was not significant during the gas application period (116.9% ± 12.7%) when compared with the pre-exposure period (Figure 23). However, there was a significant increase in PS2 amplitude during the washout period (175.4% ± 65.4%) that was significant (p<0.05) in comparison to the pre-exposure and gas application periods (Figure 23). The effects on PS2 amplitude caused by exposure to 5 ppm H$_2$S were similar to those observed in slices exposed to 1 ppm H$_2$S (see Results Section 3.3.1).

3.3.3   10 ppm H$_2$S

As observed with 1 and 5 ppm H$_2$S (see Results Sections 3.3.1 and 3.3.2), 10 ppm H$_2$S produced effects on synaptic transmission that were similar to those observed with sour gas containing identical levels of H$_2$S (see Results Section 3.2.3). In one representative slice, the PS2 amplitude during the pre-exposure period was 1.31 mV ± 0.15 mV, and the amplitude did not significantly change by
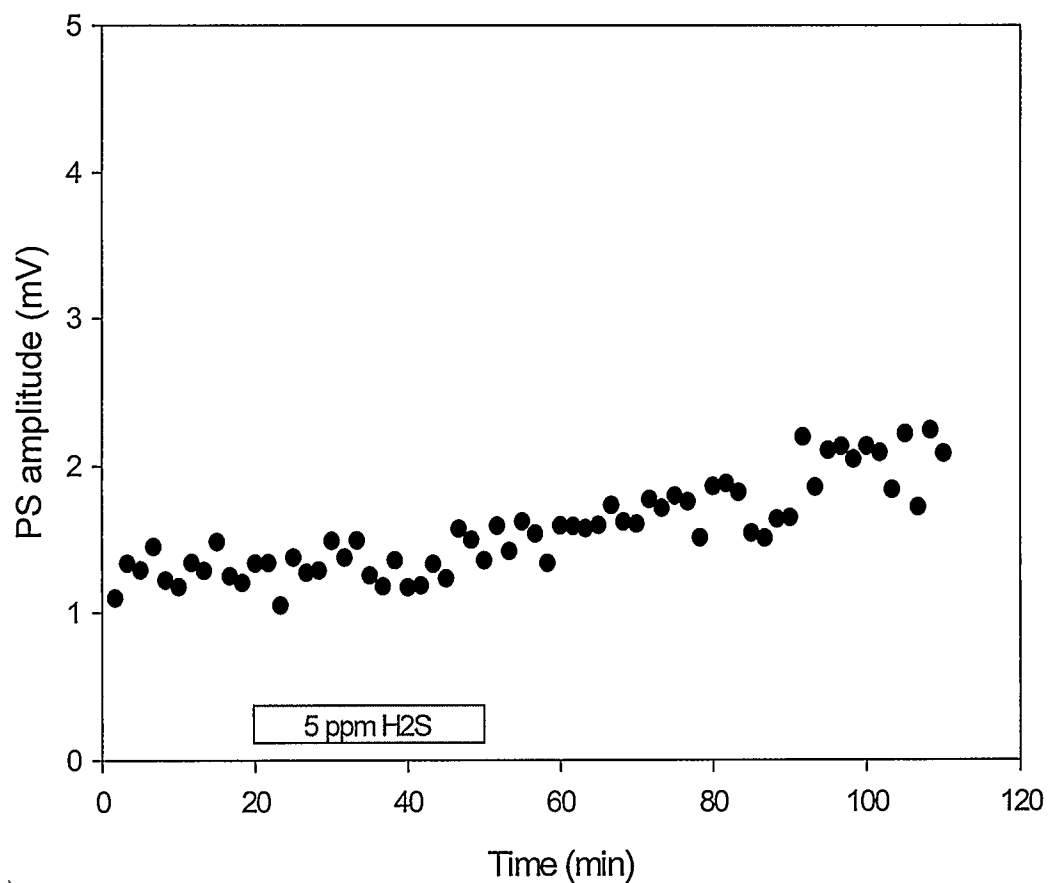
Figure 22. Results from a single 5 ppm H₂S experiment. There was a gradual increase in PS2 amplitude (mV) after the application of H₂S (indicated by the shaded bar). During the one-hour washout period, there was a steady increase in PS2 amplitude, with the greatest increase present at the end of the experiment.
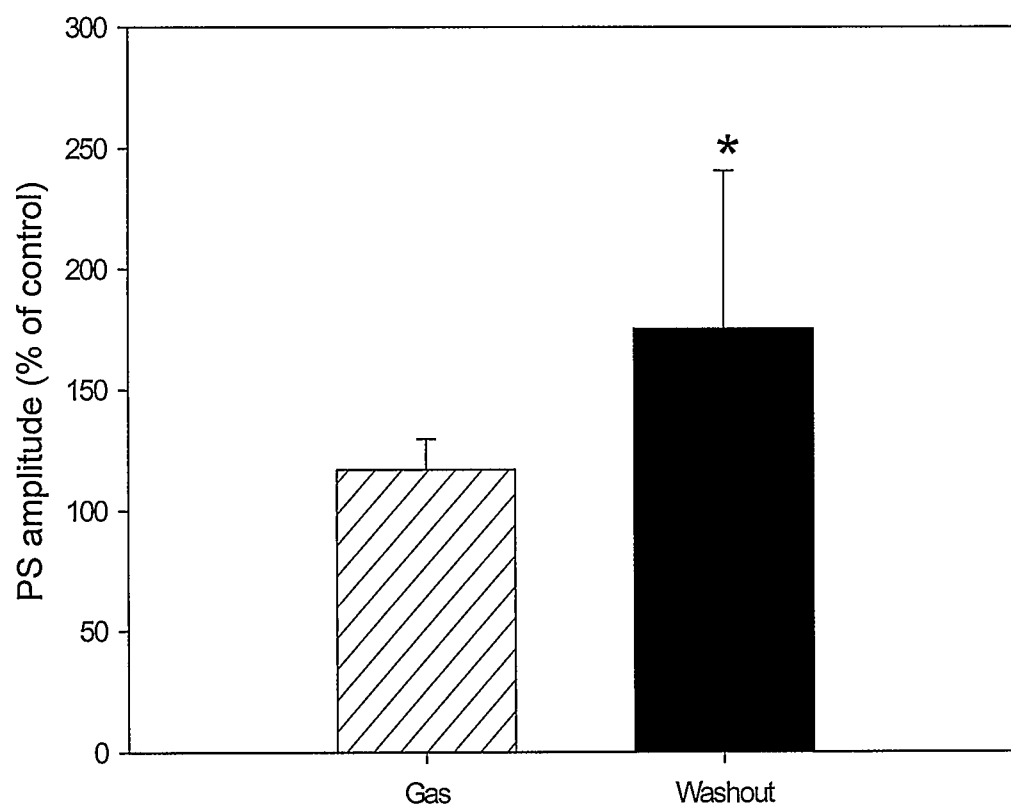
Figure 23. 5 ppm H₂S exposure caused a significant increase in PS2 amplitude during the washout period. There was no significant change occurring during the gas application period (n=5 slices). For each slice, the last five complexes from the gas application and washout periods were averaged and normalised with respect to the pre-exposure value. The gas and washout values from each slice were then averaged and presented as mean ± SD; * indicates significance (p<0.05) with respect to pre-exposure and gas application values.

the end of the gas application period (1.44 mV ± 0.37 mV) (Figure 24). However there was a progressive increase in PS2 amplitude during the washout period to a maximum 3.30 mV ± 0.60 mV by the end of the experiment (Figure 24). Normalised data from six slices were averaged (Figure 25) and analysis revealed a subtle increase in PS2 amplitude during the gas application period (122.6% ± 16.6%) that was not significant when compared with the pre-exposure period (Figure 25). However, there was a significant increase in the PS2 amplitude during the washout period (231.1% ± 24.4%) in comparison to both the pre-exposure and gas application periods (Figure 25).

3.3.4   25 ppm H_2S

Exposure to 25 ppm H_2S produced very different effects on synaptic transmission in comparison to 1, 5 and 10 ppm H_2S. Application of 25 ppm H_2S produced a biphasic effect on synaptic transmission similar to that reported with the equivalent level of H_2S in sour gas (see Results Section 3.2.4). In one representative slice during the pre-exposure period, the PS2 amplitude was 1.32 mV ± 0.13 mV, however the amplitude steadily decreased during the gas application, with the most significant decrease at the end of the gas application period (0.23 mV ± 0.03 mV) (Figure 26). During the one-hour washout period, PS2 amplitude returned to pre-exposure levels within three minutes and then continued to increase up to the end of the experiment (2.30 mV ± 0.13 mV) (Figure 26). When normalised data from six slices were averaged (Figure 27),
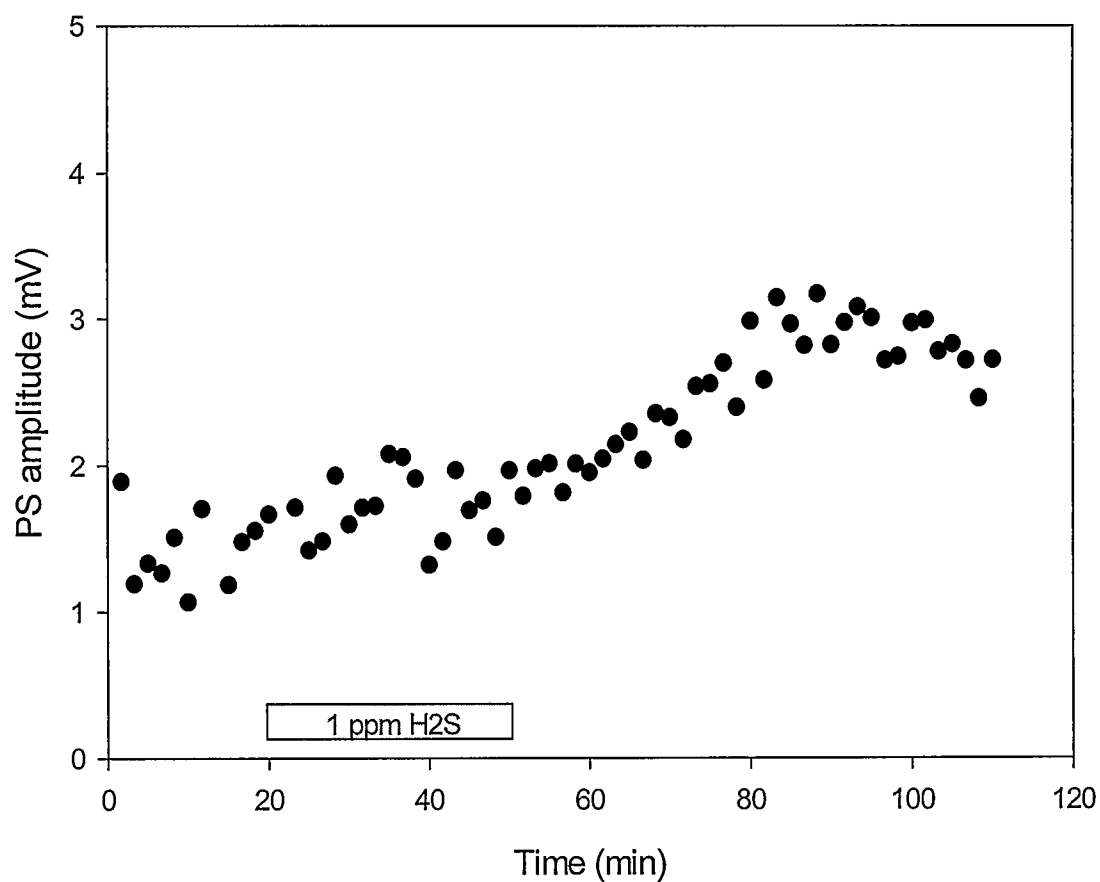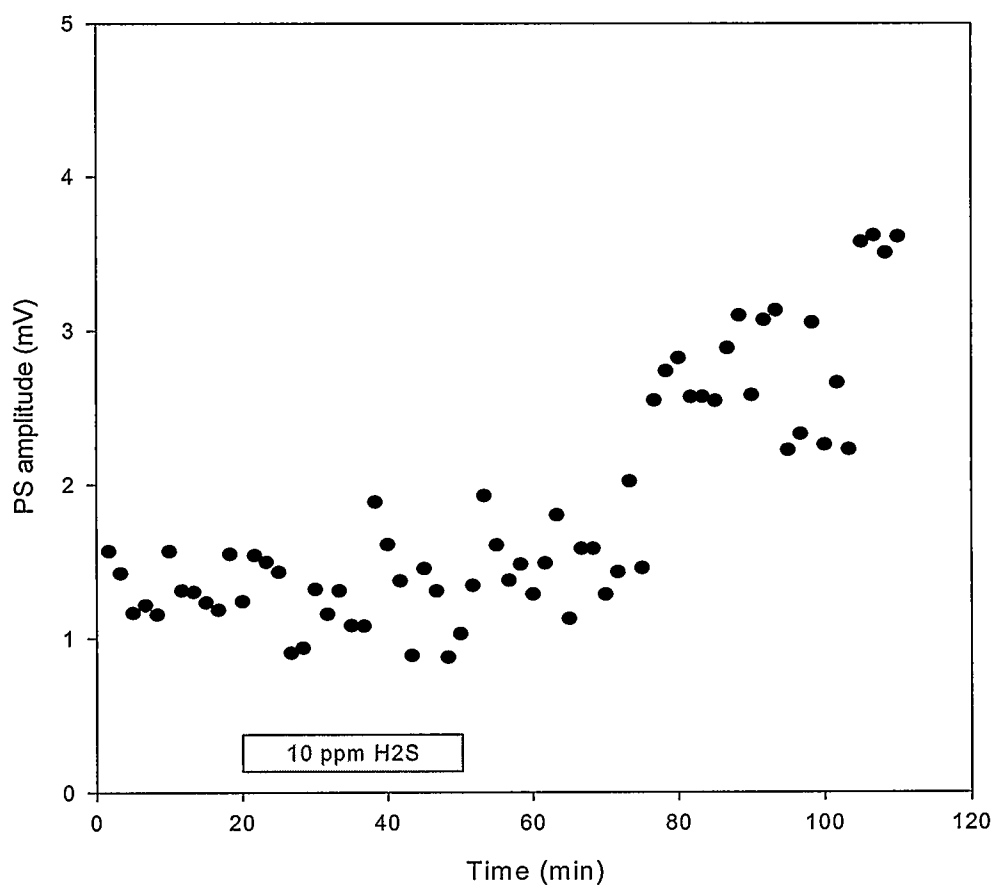
Figure 24. Results from a representative 10 ppm H₂S experiment. There was a gradual increase in PS2 amplitude (mV) after application of H₂S (indicated by the shaded bar). During the one-hour washout period, there was a gradual increase in PS2 amplitude, with the greatest increase present at the end of the experiment.
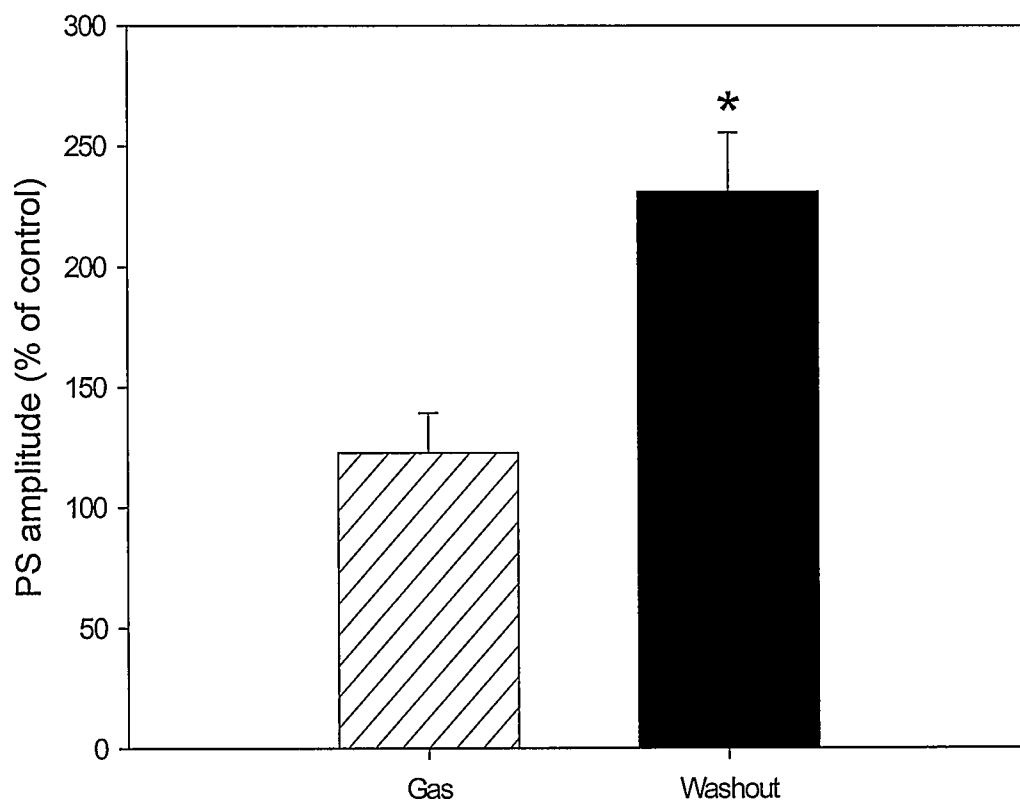
Figure 25. 10 ppm H_2S exposure did not produce any enhancement of PS2 amplitude during the gas application period but produced significant enhancement during the washout period (n=6 slices). For each slice, the last five complexes from the gas application and washout periods were averaged and normalised with respect to the pre-exposure. The gas and washout values from each slice were then averaged and presented as mean ± SD; * indicates significance ($p<0.05$) with respect to pre-exposure and gas application values.
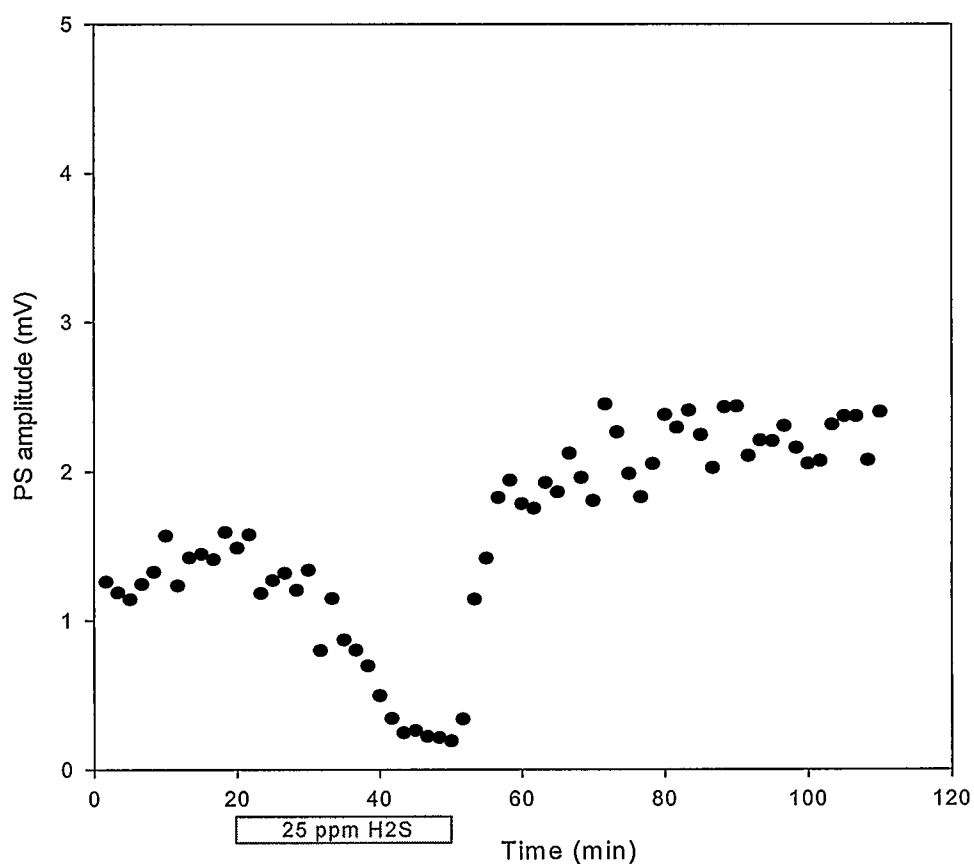
Figure 26. Results from a single 25 ppm H₂S experiment. There was a unique biphasic effect in response to application of H₂S. There was a progressive decrease in PS2 amplitude (mV) during the application of 25 ppm H₂S (indicated by the shaded bar). Following the depression of synaptic transmission, the PS2 amplitude returned to pre-exposure values quickly, and then steadily increased to a maximum level by the end of the washout phase.
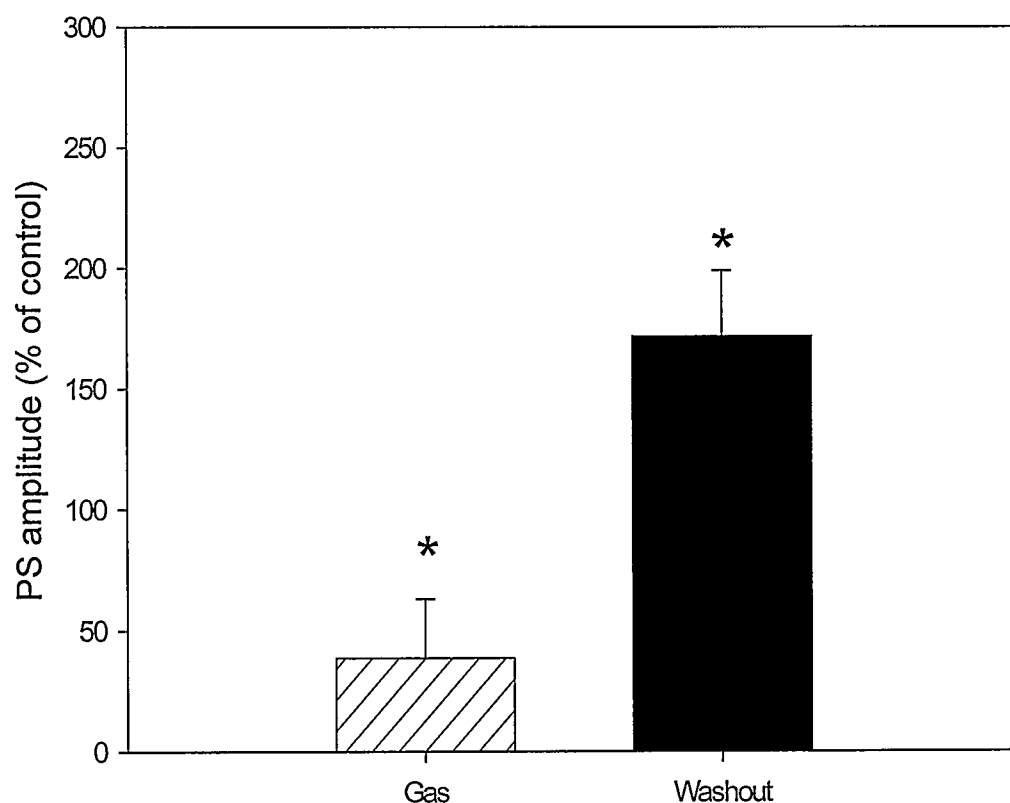
Figure 27. 25 ppm H$_2$S produced a biphasic effect on PS2 amplitude. There was depression of the PS2 amplitude during the gas application period and enhancement of the PS2 amplitude during the washout period (n=5 slices). For each slice, the last five complexes of the gas application and washout periods were averaged and normalised with respect to the pre-exposure value. The gas and washout values from each slice were then averaged and presented as mean ± SD; * indicates significance (depression and enhancement; p<0.05) of PS2 amplitudes with respect to pre-exposure and gas application values.

the result was a significant decrease in PS2 amplitude during the gas application period (38.6% ± 24.3%) when compared with the pre-exposure period (Figure 27). During the washout period, there was an increase in PS2 amplitude (171.6% ± 27.3%) that was significant when compared to the pre-exposure and gas application periods (Figure 27).

3.4   Mechanisms of Action for Hydrogen Sulphide

3.4.1   2-Amino-5-Phosphonopentanoic Acid (AP5)

3.4.1.1 Rationale

Previous studies by Abe and Kimura (1996) suggest a possible postsynaptic mechanism of action for H_2S effects on synaptic transmission in the hippocampal slice. They postulate that the NMDA receptor is involved in enhancement of synaptic transmission in the presence of H_2S (Abe & Kimura, 1996; Kimura, 2000). Therefore, experiments involving the application of AP5, a pharmacological antagonist of the NMDA receptor, to the slice concurrently with H_2S were conducted to determine the involvement of the NMDA receptor in PS2 amplitude enhancement. A return to pre-exposure PS2 amplitude levels during the washout period (with no subsequent enhancement) would implicate involvement of the NMDA receptor in enhancement of synaptic transmission caused by H_2S exposures observed during washout (see Results Section 3.3). Experiments were conducted at 25 ppm H_2S because of the unique biphasic

response observed at this concentration (significant depression followed by significant enhancement of PS2 amplitude; see Results Section 3.3.4).

3.4.1.2 AP5 Control

For the AP5 experiments, 50 µM AP5 was added to the ACSF at the beginning of the incubation period so that slices were exposed to AP5 for the duration of the experiment (110 minutes). In order to exclude the possibility that AP5 caused any effects on the PS2 amplitude over time, control experiments were conducted in the absence of any test gas, and measurements of PS2 amplitude were taken every 100 seconds for 110 minutes. The addition of AP5 alone did not result in any changes in synaptic transmission in the CA3 to CA1 pathway in the hippocampal slice, as indicated by constant PS2 amplitude measurements for the duration of the experiment (Figure 28).

3.4.1.3 AP5 + 25 ppm Hydrogen Sulphide

In a separate set of experiments, 25 ppm H_2S was administered to the slice in the presence of AP5. During the pre-exposure period, the PS2 amplitude in one slice was 1.56 mV ± 0.11 mV, and decreased to 0.60 mV ± 0.06 during the gas exposure period (Figure 29). This depressant effect was observed in slices exposed to 25 ppm H_2S (see Results Section 3.3.4). However, in the presence of AP5, the PS2 amplitude returned to pre-exposure levels within the first 15 minutes of the washout period and remained stable (1.42 mV ± 0.14 mV) for the
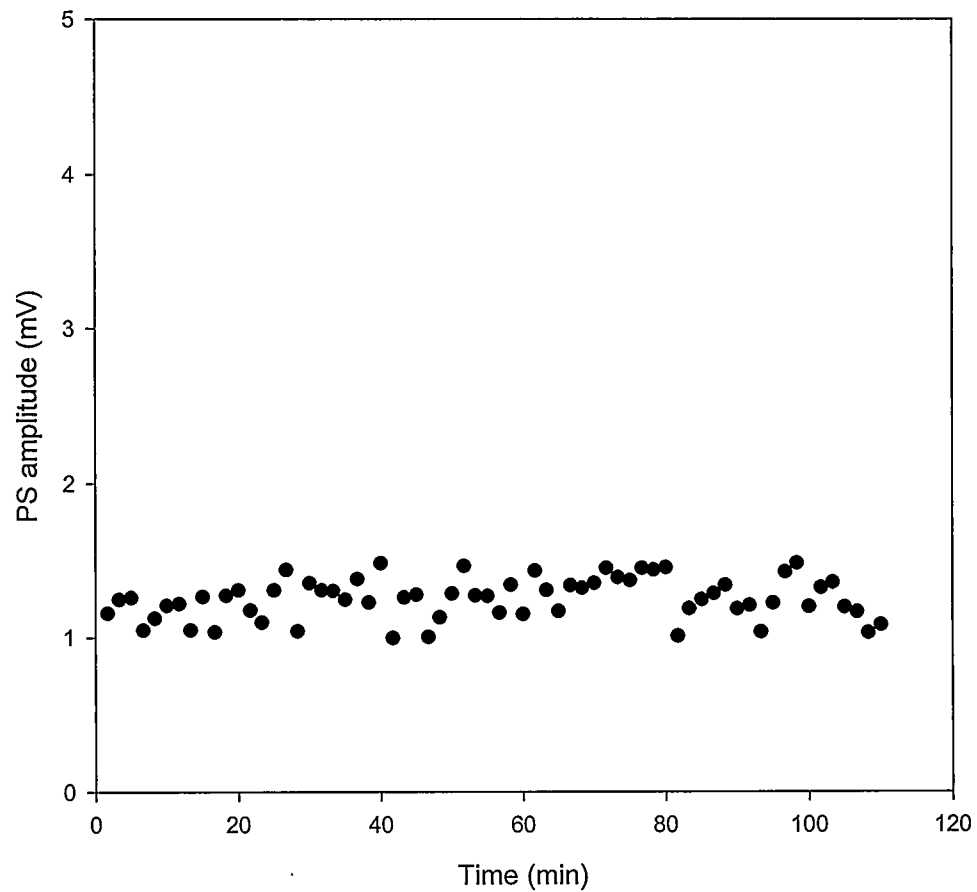
Figure 28. Results from a representative AP5 control experiment. There was no significant change in PS2 amplitude (mV) for the duration of the experiment. Each point (•) represents one PS2 amplitude measured from one complex as a result of paired-pulse stimulation every 100 seconds.
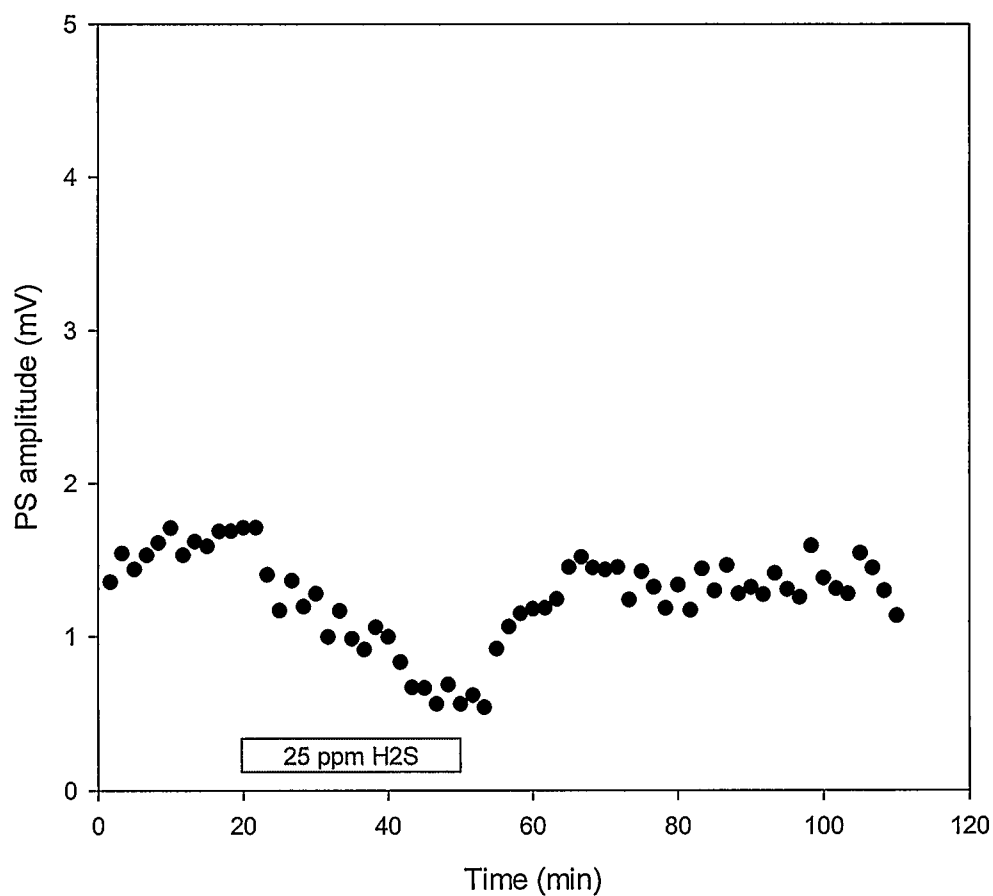
Figure 29. Results from a representative 25 ppm H₂S experiment in the presence of AP5. There was a progressive decrease in PS2 amplitude (mV) during the application of 25 ppm H₂S (indicated by the shaded bar) in the presence of AP5. Following the depression of synaptic transmission, the PS2 amplitude returned to pre-exposure values quickly, and then remained stable at these levels, with no enhancement of PS2 amplitude as a result of AP5 and 25 ppm H₂S exposure.

duration of the experiment (Figure 29). Normalised data from an average of six slices are presented in Figure 30. There was an overall depression of synaptic transmission during the gas application period (32.0% ± 24.0%) that was significant when compared with pre-exposure and washout periods (Figure 30). There was no significant enhancement of PS2 amplitude during the washout period (96.8% ± 7.2%) when compared with the pre-exposure period (Figure 30).

3.4.2   Absence of Electrical Stimulation + 25 ppm Hydrogen Sulphide

In order to establish whether the enhancement of synaptic transmission in the CA3/CA1 pathway (defined by a significant increase in PS2 amplitude) was dependent on electrical stimulation, absence of electrical stimulation experiments were conducted in the presence of 25 ppm H_2S. Following the pre-exposure period, stimulation was discontinued during the gas application and initial washout periods. Stimulation was then resumed after 110 minutes and recordings were made for a further 45 minutes of extended washout.

As mentioned above, PS2 amplitudes were measured from 110 – 155 minutes for the absence of electrical stimulation experiments. Therefore in order to compare effects in slices in the presence of electrical stimulation over the identical time frame, additional 25 ppm H_2S exposure experiments were conducted with extended washout periods (additional 45 minutes following the
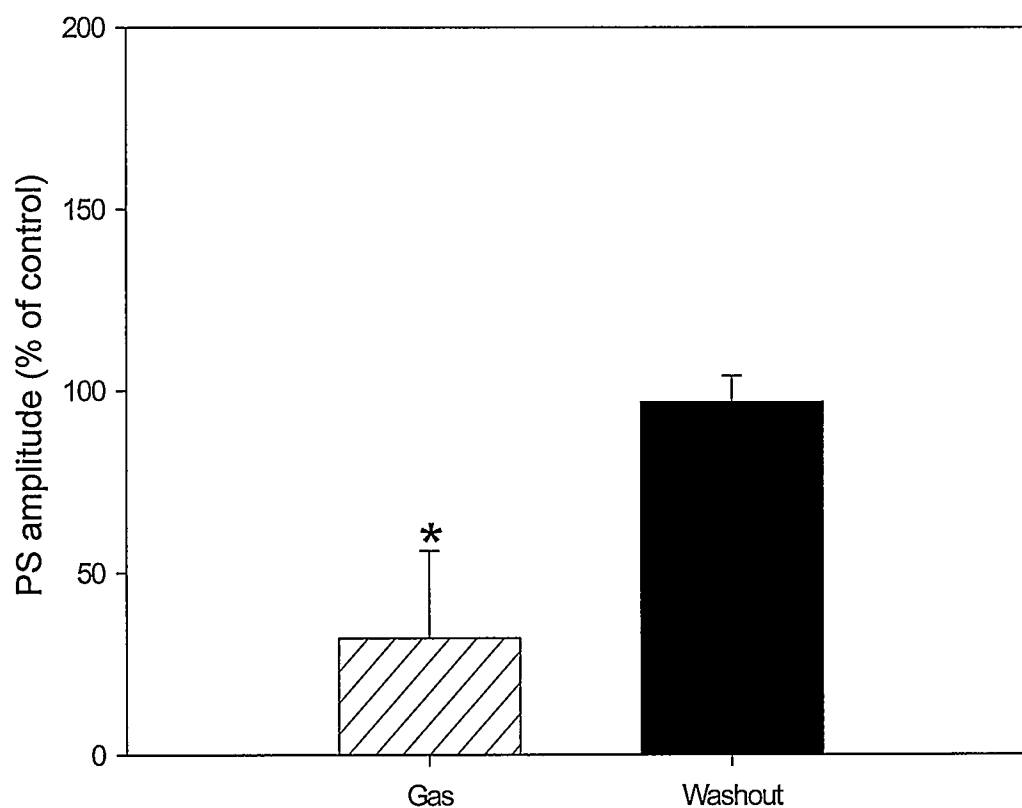
Figure 30. In the presence of AP5, 25 ppm H₂S did not cause a biphasic effect on PS2 amplitude (n=6 slices). There was a depression of the PS2 amplitude during the gas application period, but PS2 amplitude returned merely to pre-exposure values during the washout period with no further enhancement. For each slice, the last 5 complexes of the gas application and washout periods were averaged and normalised with respect to the pre-exposure value. The gas and washout values from each slice were then averaged and presented as mean ± SD; * indicates significant depression (p<0.05) of PS2 amplitudes with respect to pre-exposure and gas application values.

110 minute period) in the presence of electrical stimulation (Figure 31). In one representative experiment, the PS2 amplitude for the pre-exposure period was 1.39 mV ± 0.15 mV, and it decreased to 0.23 mV ± 0.03 mV by the end of the gas application. The PS2 amplitude then returned to pre-exposure values within three minutes and gradually increased throughout the entire washout period to 2.40 mV ± 0.22 mV (Figure 31).

The increased synaptic response that occurred in the presence of electrical stimulation following exposure to 25 ppm H_2S (see Figure 31) did not occur at the end of the experiment (155 minutes) when paired-pulse stimulation of the CA3/CA1 pathway was arrested (Figure 32). When stimulation of this pathway was resumed after the 90-minute (gas application and initial washout) period, the PS2 amplitude was elevated initially compared to pre-exposure levels (2.25 mV ± 0.07 mV) for the first 10 minutes (2.75 mV ± 0.02 mV or 122.4% ± 1.2%); however the PS2 amplitude then decreased and returned to pre-exposure levels (2.54 mV ± 0.04 mV or 113.2% ± 1.6%) by the end of the experiment (Figure 32). Normalised data averaged from five slices showed no significant enhancement of PS2 amplitude after the extended washout period (107.6% ± 12.6%) when compared with the pre-exposure values (Figure 33). However, there was a significant difference in the PS2 amplitude after the extended washout period when comparing effects in the presence (187.5% ± 29.7%) and absence of stimulation (107.6% ± 12.6%) (Figure 33).
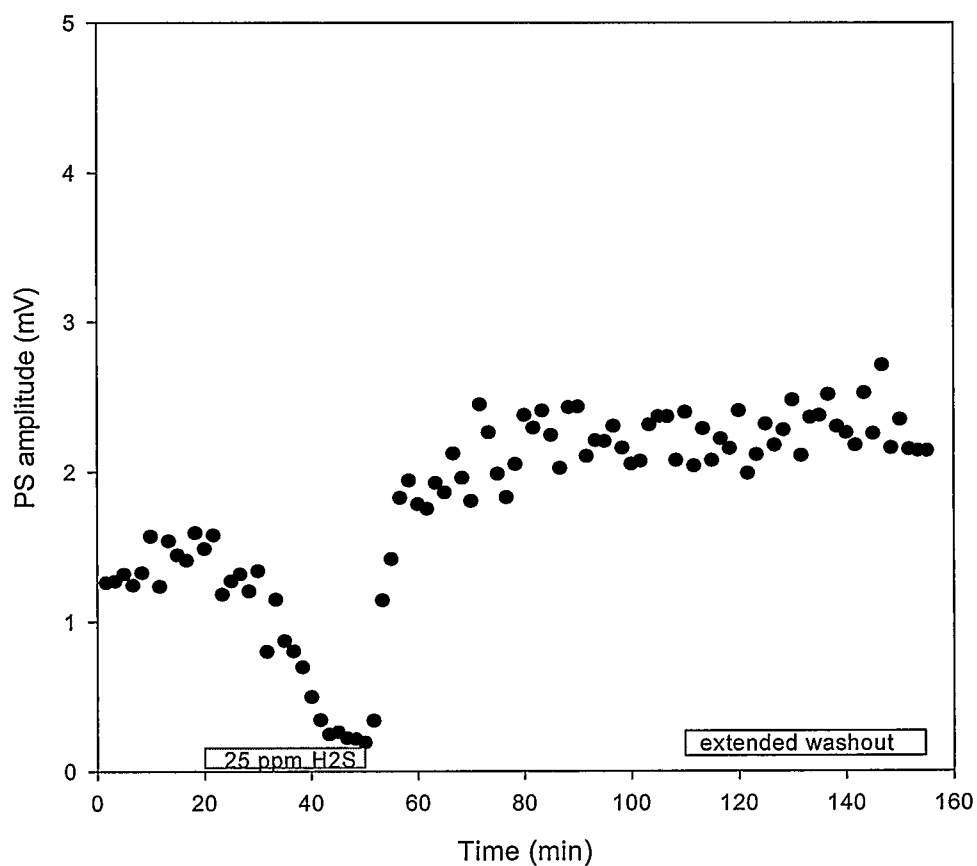
Figure 31. Results from a representative 25 ppm H₂S experiment with an extended washout period. The increase in PS2 amplitude (mV) following application of H₂S (shaded bar) was long-lasting when there was concomitant stimulation for the duration of the entire experiment. Enhancement of PS2 amplitude was still observed at the end of the extended washout period (white bar).
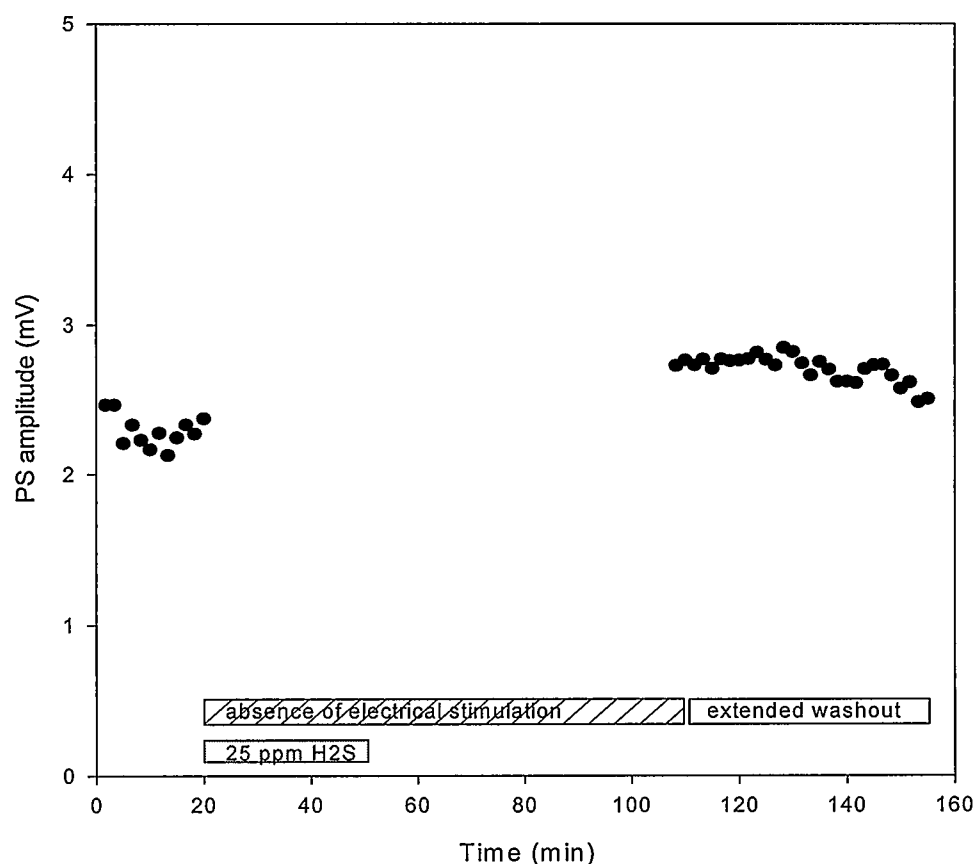
Figure 32. Results from a single 25 ppm H2S experiment in the absence of electrical stimulation. There was little effect on PS2 amplitude following application of H2S (shaded bar) with a lack of electrical stimulation during the gas application and initial one-hour washout periods (hatched bar). There was a slight increase in PS2 amplitude (mV) at the commencement of the extended washout period (white bar), followed by a gradual decrease in PS2 amplitude values to the pre-exposure values by the end of the extended washout period.
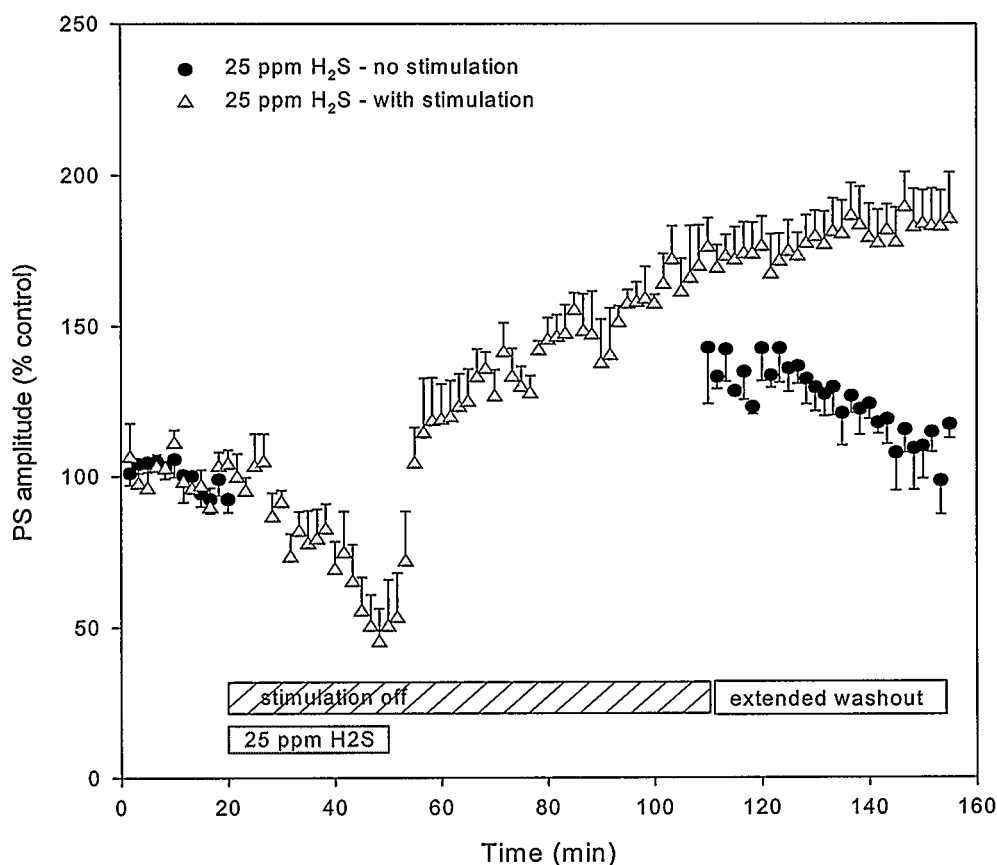
Figure 33. A comparison of the effects of 25 ppm H₂S in the presence and absence of electrical stimulation. With concurrent electrical stimulation (n=5 slices, shaded Δ represents average values, error bars show standard error), application of 25 ppm H₂S (shaded bar) resulted in depression of PS2 amplitude, followed by significant enhancement of PS2 amplitude during the washout phase that lasted through the extended washout period (white bar). In the absence of electrical stimulation (hatched bar), PS2 amplitude (n=5 slices, ● represents average values, error bars show standard error) was transiently elevated after application of 25 ppm H₂S, but values returned to pre-exposure levels during the extended washout, with no significant enhancement of PS2 amplitude compared to the pre-exposure value.

3.4.3   PS1/PS2 Ratio

In an attempt to determine whether presynaptic mechanisms were involved in the long-lasting increase in PS2 amplitude in the slice during the washout period following exposure to 25 ppm H_2S, the interaction between enhanced synaptic transmission and paired pulse facilitation (PPF) was studied. Using recordings from slices exposed to 25 ppm H_2S, PS1 amplitudes were measured and compared to PS2 amplitudes from the same slices (PS1/PS2 ratios) in order to detect any changes in PPF in the slice after application of H_2S and subsequent washout. PS1/PS2 ratios for slices exposed to 25 ppm H_2S are presented in Table 1. For each slice, the last ten PS1 complexes from the pre-exposure period and the last five PS1 complexes from the washout period were measured and compared to the corresponding PS2 amplitudes. For each slice, there is an increase in the PS1/PS2 ratio during the washout period, indicating a decrease in PPF corresponding to the enhancement of PS2 amplitude (Table 1). Average ratios for the four slices show a significant increase in PS1/PS2 ratio during the washout period (0.40 ± 0.12) when statistically compared to the control period (0.12 ± 0.10) using the t-test (Table 1).

Slice	Pre-exposure PS1/PS2	Gas PS1/PS2	Washout PS1/PS2
1	0.02	N/A	0.56
2	0.17	N/A	0.30
3	0.24	N/A	0.41
4	0.06	N/A	0.32
Average	0.12 ± 0.10	N/A	0.40 ± 0.12

Table 1. PS1/PS2 ratios for the pre-exposure, gas application and washout periods for individual slices. No ratios could be reported during the gas application period due to complete depression of the evoked PS1 amplitude. Averages of the ratios show a significant decrease in PPF from the control period to the washout period for slices exposed to 25 ppm H_2S (p=0.012).

4.0   Discussion

4.1   Controls

Control experiments conducted with only carbogen, methane or nitrogen did not produce any significant changes in the PS2 amplitude during the 110-minute test period.   These results demonstrate that the hippocampal slice is a stable preparation and does not respond to exposures of methane or nitrogen at these levels.   Therefore, the changes in synaptic transmission that are observed are a result of exposure of the slice to the test gases (sour gas or H_2S).

4.2   Comparison of Sour Gas and Hydrogen Sulphide Effects

The toxic effects of sour gas mixtures have not been previously studied either *in vivo* or *in vitro*.   Despite this, H_2S is generally regarded to be the major toxic component of sour gas. The effects of high-level H_2S exposure have been extensively studied.   Exposure to high-level H_2S has been shown to cause headaches, vertigo, incoordination and intense fatigue and anxiety (Milby and Baselt, 1999a) that can lead to unconsciousness ("knockdown") and even death depending on duration of exposure and concentration of H_2S (Guidotti, 1996). Brain damage with serious neurological and psychiatric sequelae (e.g. amnesia, motor deficits, severe headaches, hallucinations, etc.) has been reported in resuscitated individuals following "knockdown" for up to five years post-exposure (Tvedt et al., 1991a, 1991b; Kilburn, 1993).

The research on low-level H_2S exposure is lacking. Recently, exposures to low, sub-lethal, ambient levels of H_2S have been associated with neurophysiological and neuropsychological symptoms, including memory loss, psychiatric disturbances and motor problems (Kilburn, 1999). Residents who live downwind from sour gas plants frequently complain of headaches, nausea, vomiting, dizziness, pain and numbness in the extremities, disorientation, depression, personality changes, nosebleeds and respiratory problems (Kilburn & Warshaw, 1995a; White et al., 1999). There is no evidence for a direct causal relationship between low-level exposure to H_2S and the symptoms associated with it. It is also not definitively known if the effects of sour gas are caused only by the presence of H_2S alone. Therefore, there was a need to study the effects of exposure to sour gas containing low-levels of H_2S and compare them to the effects of exposure to low-levels of H_2S alone.

When the effects of sour gas and H_2S on PS2 amplitudes (a measure of synaptic transmission) were compared at identical concentrations of H_2S, there were no significant differences observed during either gas application or washout periods (Figure 34). This observation lends support to the overall hypothesis for this thesis, that the neurotoxic effects of sour gas exposure are largely dependent on the H_2S component, the major toxic component of sour gas, and the effects of sour gas and H_2S would be very similar.
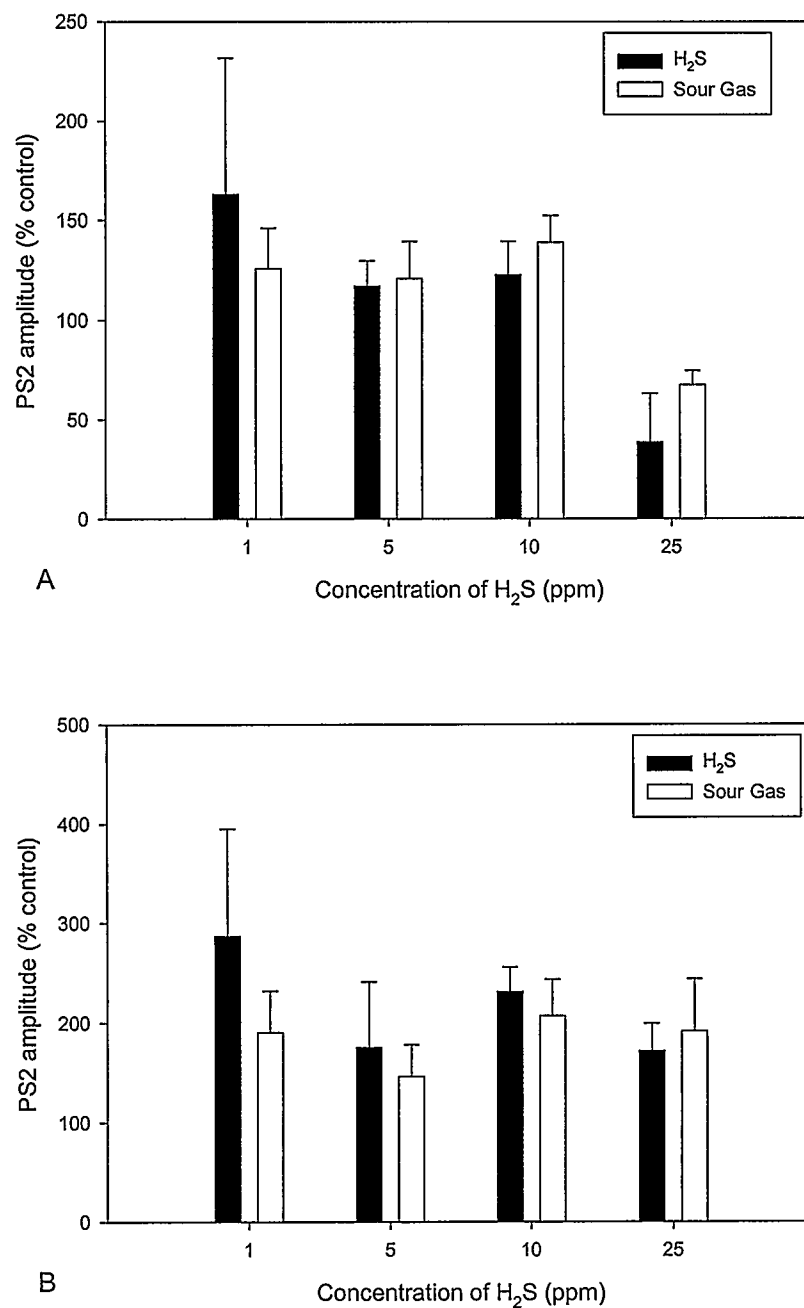
Figure 34.   Effects of sour gas (white bar) and H_2S (black bar) on PS2 amplitudes.   There were no significant differences between the effects of H_2S and sour gas at identical concentrations of H_2S during either (A) gas application or (B) washout periods.   Data are presented as mean ± SD (n=5 or 6 slices).

Application of sour gas (Figure 35) or H_2S alone (Figure 36) to the slice altered the amplitude of the PS2 response to electrical stimulation in a dose-dependent manner, suggesting that synaptic transmission in the CA3/CA1 pathway was changed by electrical stimulation in the presence of sour gas or H_2S. At the highest concentration studied (sour gas containing 25 ppm H_2S and 25 ppm H_2S alone) both gases caused a biphasic effect on synaptic transmission. There was an initial significant depression of PS2 amplitude observed during the gas application period (see Results Sections 3.2.4 and 3.3.4), followed by a return to pre-exposure levels and a further significant increase of PS2 amplitude during the washout period (see Results Sections 3.2.4 and 3.3.4). This increase was long-lasting and stable over an extended washout period (105 minutes; see Results Section 3.4.2).

The initial significant depression of PS2 amplitude observed during the gas application period is in agreement with previous reports that demonstrated that high-level H_2S produces depressant effects on synaptic transmission (Beauchamp et al., 1984; Warenycia et al., 1989b; Reiffenstein et al., 1992; Abe & Kimura, 1996). It has been suggested that the depressant effect of H_2S on synaptic transmission in the brain may be partly responsible for neurological symptoms (e.g. dizziness, unconsciousness) reported following exposures to sub-lethal levels of H_2S (Reiffenstein et al., 1992; Abe & Kimura, 1996). Previous studies in hippocampal CA1 neurons showed depression of
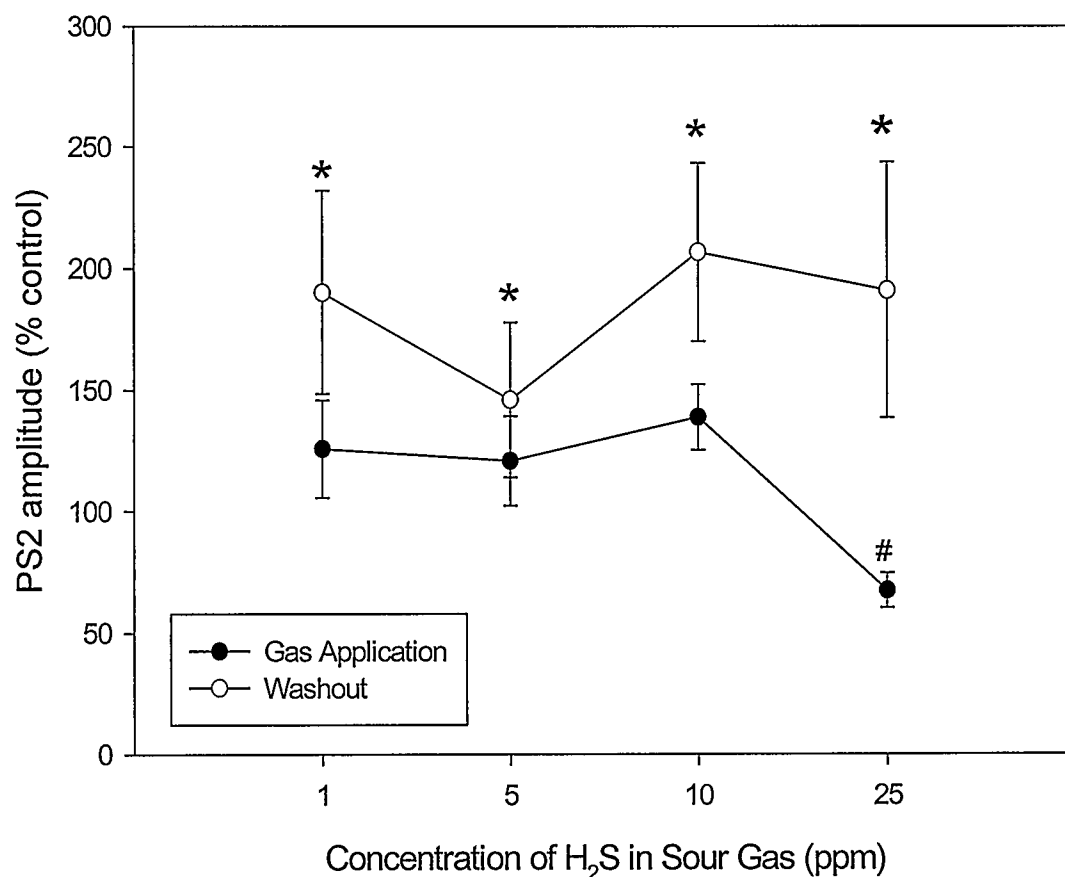
Figure 35. Sour gas has a dose-dependent effect on PS2 amplitude. Sour gas containing 1, 5 and 10 ppm H_2S had no significant effects on PS2 amplitudes during gas application, but increased PS2 amplitude significantly from pre-exposure levels during washout. However, the increase was not significantly different when comparing these different concentrations of sour gas. Sour gas containing 25 ppm H_2S produced a significant depression during gas application and a significant enhancement during washout that was not significantly different than the washout of the other concentrations of sour gas. Data points represent average effects on PS2 amplitudes (n=5 or 6 slices); significance indicated with * by two-way repeated ANOVA and with # by t-test (p<0.05).
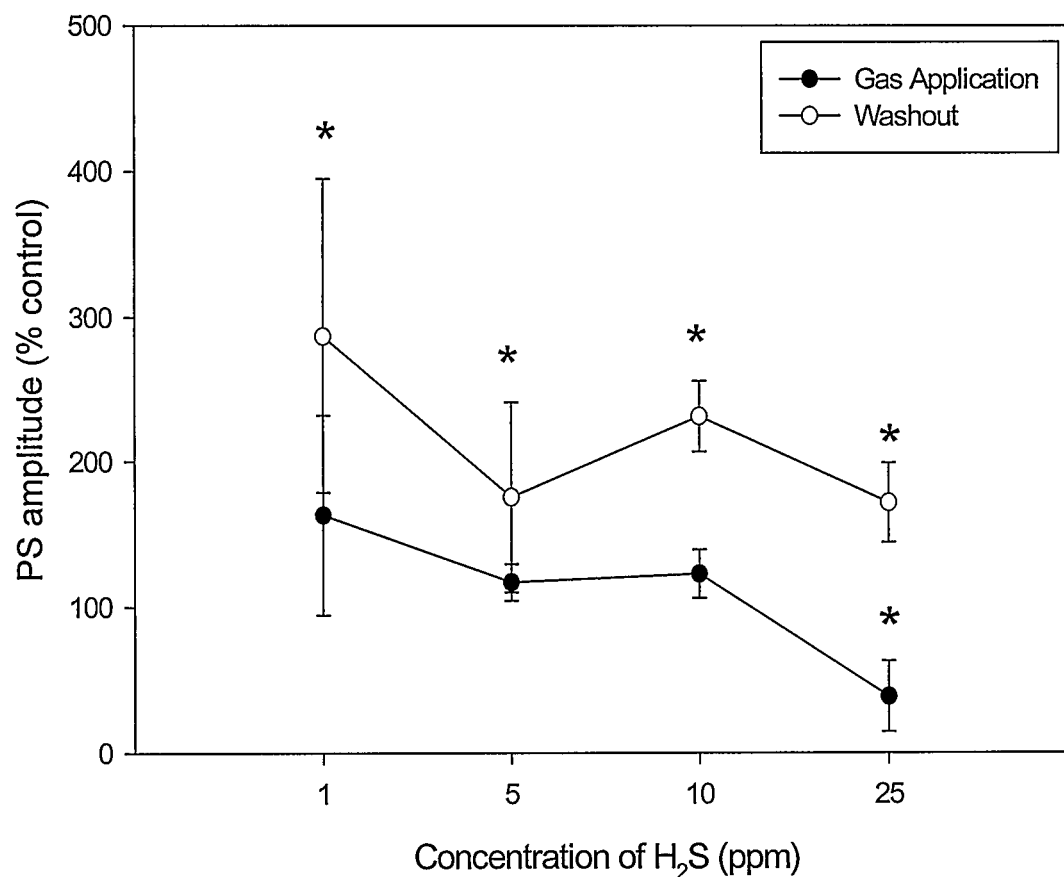
Figure 36. H_2S has a dose-dependent effect on PS2 amplitude. 1, 5 and 10 ppm H_2S had no significant effects on PS2 amplitudes during gas application, but increased PS2 amplitude significantly from pre-exposure levels during washout. However, the increase was not significantly different when comparing these different concentrations of H_2S. 25 ppm H_2S produced a significant depression during gas application and a significant enhancement during washout that was not significantly different from the washout of the other concentrations of H_2S. Data points represent average effects on PS2 amplitudes (n=5 or 6 slices); significance indicated with * by two-way repeated ANOVA and with # by t-test (p<0.05).

extracellular PSs and EPSPs in slices exposed to 320 and 640 μM NaHS. Other studies of hippocampal CA1 neurons documented depression of extracellular PSs and EPSPs as well as intracellular EPSPs in response to $\geq$ 60 μM NaHS exposure (Baldelli et al., 1989). Upon application of NaHS ($\geq$ 80 μM), CA1 cells showed either a hyperpolarization or depolarization (caused by an increase in internal calcium, followed by a sustained hyperpolarization upon washout (Baldelli et al.,1989). Similar effects have been documented in dorsal raphe serotonergic neurons (Kombian et al., 1993). It is believed that the hyperpolarization was caused by an increase in potassium (K^+) channel conductance (Baldelli et al., 1989; Kombian et al., 1993). High levels of NaHS (5-10 mM) have been shown to inhibit sodium channel conductance in mouse neuroblastoma cells in the presence of taurine (Warenycia et al., 1989b).

While there is no clear explanation for the biphasic response observed at 25 ppm H_2S, it is possible that separate mechanisms may be involved. As mentioned previously, high-level H_2S causes depression of synaptic transmission that may occur through modulation of K^+ channels. During washout of 25 ppm H_2S, the concentration of H_2S in the circulating atmosphere above the slices decreased quickly to 0 ppm within 5 minutes. It was not possible to measure the concentration of H_2S in the slice, but it is speculated that the concentration in the slice also decreased so that the effect on K^+ channel conductance was lessened. Therefore, the depression of synaptic transmission was reversed and the PS2

amplitude returned to pre-exposure levels quickly as well (within 5 minutes). However, it is possible that there was a sufficiently low concentration of H_2S remaining in the slice that was able to cause enhancement of synaptic transmission through a different mechanism discussed in the following section.

The biphasic response observed at higher levels of sour gas and H_2S was unique and interesting, but the more significant findings of the sour gas and H_2S experiments were the enhancement effects observed at lower concentrations. Application of sour gas containing 1, 5 and 10 ppm H_2S produced slight increases of PS2 amplitudes observed during the gas application periods, with significant enhancement of PS2 amplitudes during the washout periods (see Figure 36). Enhancement of PS2 amplitudes was also observed in slices exposed to corresponding concentrations of H_2S during washout periods, with a gradual increase in PS2 amplitudes during the gas application period (see Figure 37). The increases in PS2 amplitudes are consistent with the enhancement of synaptic transmission in the CA3/CA1 pathway in the hippocampus that has been reported in slices exposed to subanaesthetic concentrations of pentobarbital (Archer et al., 2001). It is also interesting to note that elderly patients recovering from anaesthesia also report cognitive problems (Archer et al., 2001) that are similar to those reported in individuals exposed to low-level H_2S. Another remarkable similarity between H_2S and anaesthetics is that at high levels, both have been found to cause depressant effects on synaptic

transmission. High-level H_2S has been described previously to be an anaesthetic-like agent (Beck et al., 1983; Roth, 1999)

The increases in PS2 amplitude during washout for slices exposed to 1, 5, 10 and 25 ppm H_2S (alone or as a component of sour gas) were not significantly different from each other (two-way repeated measures ANOVA). The enhancement response during washout for all concentrations of H_2S also reached a plateau around the variable 200% response level, as was observed with sour gas. There were no significant differences found when PS2 amplitudes of slices exposed to sour gas containing 1, 5 and 10 ppm H_2S were compared for the gas application periods (two-way repeated measures ANOVA) (Figure 35). There were also no significant differences in PS2 amplitudes when the washout periods for slices exposed to sour gas containing 1, 5, 10 and 25 ppm H_2S were compared. All concentrations of sour gas and H_2S produced approximately 200% enhancement of PS2 amplitude during washout over the pre-exposure period (since the pre-exposure values are ideally at half the maximum PS2 amplitude, a 200% increase would represent an enhancement to maximal response). However, the enhancement responses were variable, as indicated by the standard deviation (see Figure 36 and 37). One explanation for this variability is that pre-exposure levels for some slices may not have been exactly at half-maximal amplitude. This would produce variable levels of increase, above or below 200%. However, the fact that the increase in PS2

amplitudes reached 200% would suggest that the enhancement effect during washout reached a plateau or maximum for all concentrations of H_2S alone or in sour gas. This effect is similar to the LTP effect observed in slices exposed to H_2S in the presence of a weak tetanus (Abe & Kimura, 1996):

There is a concentration-dependent trend in the data suggesting that sour gas containing 1, 10 and 25 ppm H_2S produces greater effects on PS2 amplitudes during washout when compared to sour gas containing 5 ppm H_2S. There is a similar trend with H_2S alone. There are a couple of possible explanations for the reduced effects at 5 ppm; there may be a different or additional mechanism(s) of action (for example, 5 ppm may affect a different receptor), it may be a function of biological variability of the tissue, rendering some slices more vulnerable to the effects of sour gas exposure, or there may be a small difference in concentrations (1, 5, 10 ppm) therefore any slight fluctuation in flow rate may have caused variable concentrations of H_2S that went undetected. It is interesting that this reduced effect was seen for both sour gas and H_2S alone (see Figure 35), suggesting that there may be a different action occurring in slices exposed to 5 ppm H_2S. However, at the present time there is no evidence of this.

4.3   Possible Mechanisms of Action of Hydrogen Sulphide

Due to the fact that H_2S is the most toxic component of sour gas, and that the results obtained from slices exposed to sour gas and equivalent levels of H_2S

were not significantly different, studies on the mechanisms of action were conducted using H_2S alone. Since sour gas is a complex mixture, it is difficult to draw any conclusions regarding a mechanism of action for its effects on synaptic transmission. Therefore, all theories discussed in this section will be limited to the effects of H_2S on synaptic transmission alone.

It is possible to suggest that both postsynaptic and presynaptic mechanisms of action of H_2S are involved in producing enhancement of PS2 amplitudes. However, interpretations of results from extracellular studies are problematic, as indicated by Archer et al. (2001), because it is difficult to determine the components of the pathway that have been affected. Therefore, future investigations into the mechanisms of action would need to include intracellular techniques.

4.3.1 Postsynaptic Mechanisms - The NMDA Receptor

It has been suggested that H_2S is endogenously produced in the body and can be made from cysteine by pridoxal-5'-phosphate-dependent enzymes, including cystathionine β-synthase (CBS) found in the brain and cystathionine γ-lyase (CSE) expressed in the ileum, portal vein and thoracic aorta (Abe & Kimura, 1996; Kimura, 2000). H_2S produced by CSE is thought to play a role in relaxation of smooth muscle (Kimura, 2000). CBS is concentrated in the hippocampal and cerebellar regions of the brain and produces H_2S in relatively

high concentrations (50 – 160 µM), suggesting that H_2S may function as a neuromodulator (Abe & Kimura, 1996; Kimura, 2000). Abe and Kimura (1996) conducted extracellular experiments with NaHS (source of H_2S) using a single pulse paradigm (single pulse delivered every 20 seconds) on the CA3/CA1 pathway of the hippocampus. A weak tetanic stimulation (15 pulses at 100 Hz) alone did not cause long-term potentiation (LTP), but in the presence of endogenous levels of NaHS (10 - 130 µM) induced LTP of both field EPSPs and PSs (Abe & Kimura, 1996). Occlusion experiments demonstrated that the LTP induced by H_2S in the presence of a weak tetanus and LTP induced by a strong tetanus share similar mechanisms (Abe & Kimura, 1996). The authours concluded that H_2S "facilitates" LTP at active synapses and therefore is involved in associate learning as defined by Hebb (Abe & Kimura, 1996).

Long-lasting (over one hour) increases in the efficacy of synaptic transmission in the hippocampus produced by a strong tetanus are referred to as long-term potentiation (LTP), a form of synaptic plasticity associated with learning and memory processes (Zamanillo et al., 1999). It is thought that induction of LTP in the Schaffer collateral/CA1 pathway in the hippocampus requires activation of the postsynaptic NMDA receptor (Bliss & Collingridge, 1993; Malenka & Nicoll, 1993; Collingridge & Bliss, 1995; Malenka & Nicoll, 1999; Nicoll & Malenka, 2000).

Activation of the NMDA receptor occurs through the nearly simultaneous timing of two events: glutamate must bind to the NMDA receptor and the postsynaptic membrane must be depolarised, leading to relief of the voltage-dependent Mg^{2+} block of the associated ion channel (Bliss & Collingridge, 1993; Akhondzadeh, 1999). Removal of Mg^{2+} activates the ion channel that is permeable to calcium ions (Ca^{2+}), leading to an influx of Ca^{2+} into the cell that increases synaptic strength and triggers LTP (Bliss & Collingridge, 1993; Malenka & Nicoll, 1999; Nicoll & Malenka, 2000). There are many pathways that have been suggested as being responsible for transforming the influx of Ca^{2+} into an increase in synaptic strength (Malenka & Nicoll, 1999). There is convincing evidence that suggests that an increase in intracellular Ca^{2+} leads to activation of Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) (and other protein kinases), the last step in the induction of LTP (Malenka & Nicoll, 1999).

Abe and Kimura (1996) also conducted intracellular whole-cell patch clamp experiments to determine the effects of NaHS (a source of H_2S) on NMDA receptors. They observed a significant increase of NMDA-induced inward currents in response to physiological concentrations of NaHS (10 - 130 μM) that was blocked by AP5, confirming the involvement of the NMDA receptor (Bliss & Collingridge, 1993). NaHS did not cause any effect on currents induced by the non-NMDA agonist α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) (Abe & Kimura, 1996). They concluded that endogenous levels of H_2S

selectively enhance NMDA-receptor function and H_2S does not induce LTP when NMDA function is blocked (Abe & Kimura, 1996), and therefore H_2S is probably not functioning as a retrograde messenger, unlike nitric oxide and carbon monoxide (two endogenously-produced gases that have been proposed as retrograde messengers in hippocampal LTP).

The effects of H_2S on NMDA receptor function have also been examined in *Xenopus* oocytes (Kimura, 2000). Voltage-clamped oocytes expressing NMDA receptors were exposed to NaHS (1 – 30 μM) for five minutes, and then 100 μM NMDA with 10 μM glycine was applied (Kimura, 2000). All concentrations of NaHS tested decreased the response time of the NMDA receptor, with the greatest effect at 30 μM NaHS (Kimura, 2000).

In support of the Kimura results, results of the present studies do not show enhancement of PS2 amplitude during the washout period in slices exposed to AP5 and 25 ppm H_2S concurrently (see Results Section 3.4.1), suggesting modulation of the NMDA receptor during enhancement of synaptic transmission for slices exposed to 25 ppm H_2S. However, it is not possible to conclude that the increase in PS2 amplitude that was observed is, in fact, LTP. Present studies differ from those conducted by Abe and Kimura in several ways: 1) present experiments utilised a paired pulse protocol (Abe and Kimura used a single pulse); 2) experiments conducted by Abe and Kimura involved application

of a weak tetanus concurrently with application of NaHS; 3) the present studies examined the effects of H_2S (Abe and Kimura looked at the effects of NaHS); and 4) from the present studies it can not be concluded that LTP could be induced by tetanus after the enhancement of PS2 amplitude by H_2S, or if enhancement by H_2S could be induced following tetanus-induced LTP (occlusion or saturation studies were not conducted). As suggested by Archer et al. (2001), occlusion studies are necessary in order to clarify the relationship between LTP and enhancement.

Preliminary experiments conducted in slices exposed to AP5 and 1 ppm H_2S (Strosher & Roth, unpublished data) also revealed that NMDA receptor function was modulated by H_2S. No significant increase in PS2 amplitudes was observed when 1 ppm H_2S was applied to the slice in the presence of AP5. The fact that low-level H_2S modulates NMDA receptor function *in vitro* is extremely interesting, and may provide some insight into the neurological symptoms, specifically the cognition and memory problems, that are reported in individuals exposed to low-level H_2S from industrial and environmental sources (see Introduction Section 1.2.7.2.1).

The current findings support previous studies (Abe & Kimura, 1996; Kimura, 2000) demonstrating that low (endogenous) levels of H_2S modulate the function of the NMDA receptor. There are a couple of possible mechanisms that explain

how H_2S may modify NMDA receptor function. One mechanism involves H_2S interacting with the NMDA receptor directly. It has been demonstrated that disulfide bonds participate in altering the function of different proteins, including NMDA receptors (Aizenman et al., 1989; Tang and Aizenman, 1993). The NMDA receptor has disulfide bonds and free thiols where H_2S may interact. Abe & Kimura (1996) conducted experiments on the ability of dithiothreitol (DTT), an irreversible thiol-protecting agent, to block LTP facilitated by NaHS. The results showed that NaHS was able to induce LTP in the presence of a weak tetanus even after DTT treatment, suggesting that thiol redox sites do not contribute to the ability of NaHS to modulate NMDA receptors (Abe & Kimura, 1996). H_2S has been compared to cyanide, another toxic agent, and both have been reported to inhibit cytochrome oxidase at high levels (see Introduction Section 1.2.2). Cyanide has also been found to potentiate NMDA receptors responses in neurons, however this effect has been attributed to the observation that it interacts directly with the receptor at thiol-specific sites (Arden et al., 1998).

Another possible mechanism for H_2S modulation of NMDA receptor function may involve effects on a second messenger system. One important observation of LTP is that many different protein kinases, including protein kinase C (PKC) and cyclic adenosine monophosphate (cAMP)- dependent protein kinase (PKA), play a critical role in both induction and expression of LTP (Roberson and Sweatt, 1996; Bortolotto & Collingridge, 2000; Soderling & Derkach, 2001; Winder &

Sweatt, 2001). While there is extensive evidence supporting a role for PKC in NMDA receptor activation leading to LTP, this idea has been recently challenged based on non-specific PKC inhibitors used in studies (Bortolotto and Collingridge, 2000). As well, although the idea is not universally accepted, PKA has been implicated in NMDA receptor-dependent LTP (Frey et al., 1993; Matthies & Reymann, 1993; Blitzer et al., 1995; Leonard & Hell, 1997; Tingley et al., 1997; Bortolotto and Collingridge, 2000; Soderling & Derkach, 2001).

PKA has been shown to phosphorylate the NMDA receptor (Tingley et al., 1997), activating the receptor and thereby inducing LTP in CA1 of the hippocampus; PKA inhibitors block this effect (Kimura, 2000). Previous studies also show an increase in levels of cAMP, the second messenger activator of PKA, during the initiation of LTP (Roberson & Sweatt, 1996), suggesting that the cAMP pathway may modulate the NMDA receptor (Kimura, 2000). Results from one study (Kimura, 2000) showed an increase in levels of cAMP in response to physiological concentrations of H_2S. Kimura (2000) also reported that application of NaHS produced enhancement of NMDA receptor currents in *Xenopus* oocytes. Voltage-clamped oocytes expressing NMDA receptors were exposed to 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor used to prevent breakdown of cAMP into AMP) and NaHS (1 – 30 μM) for five minutes, and then 100 μM NMDA with 10 μM glycine was applied (Kimura, 2000). The enhancement effect was effectively blocked by cis-n-(2-phenylcyclopentyl)

azacyclotridec-1-en-2-amine (MDL-12,330A), an adenylyl cyclase inhibitor. These results suggest that H_2S induces production of cAMP, either by affecting adenylyl cyclase directly or interacting with unknown receptors for H_2S leading to cAMP production. Increased levels of cAMP would in turn activate PKA, an enzyme that phosphorylates the NMDA receptor, thereby increasing activity of the receptor (Kimura, 2000). This is a possible mechanism of action for the results of this research, and it may explain how H_2S affected NMDA receptors and increased PS2 amplitudes, in a manner similar to LTP.

4.3.2 Absence of Electrical Stimulation

When paired pulse stimulation was discontinued during application of 25 ppm H_2S and washout, there was no significant increase in PS2 amplitude observed at the end of the extended washout. These results suggest that enhancement of synaptic transmission caused by H_2S required concomitant stimulation of the CA3/CA1 pathway, an effect that has also been observed with anaesthetics (Archer et al., 2001). Abe and Kimura (1996) reported that LTP was induced only when H_2S was applied simultaneously with a weak tetanus. These results are in agreement with the suggestion that H_2S enhances synaptic transmission only in the presence of electrical stimulation, which together may depolarise the postsynaptic cell, leading to removal of the Mg^{2+} block that in turn causes an influx of Ca^{2+} into the postsynaptic cell, and various downstream effects responsible for enhancement of synaptic transmission.

4.3.3   Presynaptic Mechanisms - Paired Pulse Facilitation (PPF)

PPF is the simplest, short-term form of synaptic plasticity that may be involved in learning (Kuhnt & Voronin, 1994; Papatheodoropoulos & Kostopoulos, 1998; Bi & Poo, 1999; Archer et al., 2001).   It is probably caused by an increase in neurotransmitter released by the second pulse (Zucker, 1989; Papatheodoropoulos & Kostopoulos, 1998).   Studies of the interaction between LTP and PPF may provide some insight into the role of presynaptic factors for LTP (Kuhnt & Voronin, 1994; O'Mara et al., 2000) and therefore the current observed enhancement effects.

As mentioned previously, PPF is generally considered to be a presynaptic event that is based on presynaptic modifications that may increase the probability of glutamate release (Nicoll & Malenka, 1999; Li et al., 2000) or decrease the release of GABA (Archer et al., 2001).   Postsynaptic activation of calcium/calmodulin (Ca^{2+}/CaM)- dependent protein kinase II has also been suggested to contribute to PPF (Wang & Kelly, 1996).   It has been reported that PPF is not altered during LTP (Nicoll & Malenka, 1999) and the role for presynaptic factors in the induction of LTP is controversial (Nayak & Browning, 1999).   However, a decrease in PPF after the induction of LTP has been reported *in vitro* in the CA1 regions of the guinea pig and rat hippocampus (Kuhnt & Voronin, 1994; Archer et al., 2001).   It has been suggested that the decrease in PPF after the induction of LTP is due to an alteration in transmitter release

(Kuhnt and Voronin, 1994). As well, PPF has been studied in the CA1 to subiculum pathway of the hippocampus *in vitro* (O'Mara et al., 2000). Paired pulses delivered to the CA1 region with an interstimulus interval (ISI) of 50 ms produced maximal PPF in the subiculum that decreased significantly after induction of LTP (O'Mara et al., 2000). *In vivo* paired pulse studies have also been conducted in the CA1 region of the intact hippocampus, and results showed a persistent reduction in PPF during LTP (Li et al., 2000). Results from the current PS1/PS2 studies showing a decrease in PPF during enhancement of PS2 amplitudes (see Results Section 3.4.3) are consistent with these studies, and suggest a presynaptic mechanism of action for H_2S. It has been shown that PS2 increased significantly during the washout period for slices exposed to 25 ppm H_2S (see Results Section 3.3.4). However, when the PS1 amplitudes were measured and compared to PS2 (see Results Section 3.4.3), PS1 amplitudes increased more than PS2, leading overall to the decrease in PPF.

5.0   Conclusions

5.1   General Conclusions

Sour gas and H_2S have similar effects on the *in vitro* hippocampal slice model studied, therefore it is proposed that the effects of sour gas are largely attributable to the H_2S component. The occupational limit for H_2S exposure is 10 ppm, therefore any observed effect on synaptic transmission at these low levels is potentially important. Low-levels of H_2S (1 to 10 ppm) have been found in the present study to enhance synaptic transmission in the CA1 region of the hippocampus, and this enhancement effect appears to be long-lasting (greater than one hour), an effect that is similar to tetanus-induced LTP. Since the effects on PS2 amplitude suggest that H_2S can affect synaptic transmission, this observation may provide a possible link between low-level exposure to H_2S and the symptoms of cognitive dysfunction that are reported in exposed individuals.

It is possible that both presynaptic (changes in neurotransmitter release) and postsynaptic (modulation of the NMDA receptor) factors may function together to increase PS2 amplitudes in the CA3/CA1 pathway. Modulation of NMDA receptor function has also been implicated in LTP, an experimental model for synaptic modifications that may affect learning and memory (Malenka & Nicoll, 1999). It is significant that H_2S modulates NMDA receptor function, an effect that has also been observed with subanaesthetic concentrations of anaesthetics that

have been associated with long-term cognitive dysfunction reported in patients after anaesthesia (Archer et al., 2001). Individuals exposed to low-levels of H_2S also report cognitive problems, along with various other symptoms (see Introduction Section 1.2.7.2.1). H_2S has been reported to modulate the NMDA receptor through an increase in cAMP levels, leading to activation of PKA that in turn phosphorylates proteins, including NMDA receptors (Kimura, 2000). Since the NMDA receptor is involved in LTP (Nicoll & Malenka, 2000), NMDA receptor modulation by H_2S may be responsible for the cognitive dysfunction reported following low-level H_2S and sour gas exposures.

5.2 Implications

5.2.1 Future Directions

One limitation of the results of this research is that only the effect on PS2 amplitude was used as a measure of change in synaptic transmission. Other factors may need to be considered when making conclusions regarding H_2S exposure effects on synaptic transmission. Further studies are needed to address the change in the PS1/PS2 ratio, therefore it is suggested that effects on the PS1 amplitude be considered for future experiments. As well, the effects on EPSP of the first and second evoked potentials should be determined when discussing effects on synaptic transmission.

Since effects have now been established at low concentrations of H_2S (down to 1 ppm) *in vitro*, it is important to determine if effects on synaptic transmission can be detected at even lower concentrations. One of the limitations of the current experimental methods is the sensor detection limit for H_2S. Once the sensor can be modified, adjustments can be made so that lower concentrations applied to the slice can be monitored. It is also important to determine if these low levels of H_2S have effects on neurological function *in vivo*. Therefore, an appropriate animal model needs to be developed to conduct low-level H_2S exposure experiments and to study the subsequent effects on hippocampal function. As well, the effects of low levels of H_2S on different enzyme systems, including cytochrome oxidase and adenylyl cyclase, should be further studied.

It would also be interesting to look at other regions of the brain, such as the olfactory region, due to the reported symptoms thought to be associated with the repulsive odour of H_2S. Individuals detect H_2S at low concentrations (5 ppb) (Au and Legator, 1999) and report numerous complaints (e.g. headaches and nausea), thereby associating undesirable symptoms with H_2S exposure. However the health effects due to H_2S odour are not well understood (Roth, 1999) nor accepted. It has been suggested that the adverse effects associated with H_2S exposure are influenced by preconceived beliefs about the toxicity of H_2S rather than by the odour itself (Dorman et al., 1999) and malodourous chemicals (e.g. H_2S) have been shown to elicit pronounced psychological

reactions regardless of the degree of toxicity (Knasko, 1993). Since the presence of the H_2S odour has been reported to impact quality of life, the effects of H_2S odour should be a focus of future research.

5.2.2   Guideline Regulations

Since there are effects on synaptic transmission *in vitro* at levels of H_2S that are occupationally relevant, it is important to conduct *in vivo* experiments to either support or dispute the relevance of these observations. It is possible that occupational limits for H_2S may, in future, need to be changed to recognize the potential for the effects of low-level exposures. With respect to acceptable limits for ambient levels of H_2S, it is imperative that more research into the effects of ambient levels of H_2S be examined to determine whether the symptoms reported in individuals living around H_2S-producing facilities have any physiological basis.

In order to extrapolate the results and apply them to humans in risk assessment, a number of uncertainty factors must be considered (Illing, 2001). First, there is a 10-fold factor that is used to extrapolate from *in vitro* to *in vivo* models. Second, the interspecies variation (10-fold factor) is used to account for differences between the test species (rat) and the target species (human) for which the risk assessment is being conducted (Illing, 2001). Third, there is also interindividual variation (10-fold factor) that accounts for variability in response between different groups within humans (Illing, 2001). Both of these variations

take toxicodynamics and toxicokinetics into account. Therefore, there is at least a 1,000-fold safety factor that needs to be applied in order to extrapolate the observed results for H_2S to humans. H_2S has been found to affect synaptic transmission as low as 1 ppm *in vitro*. Consequently, it is reasonable to suggest that 0.001 ppm (1 ppb) may the minimum acceptable level in humans, a concentration of H_2S that is close to the odour threshold of H_2S. Given that the occupational limit for H_2S is 10 ppm over an 8-hour workday, this guideline may need to be adjusted considerably. Since the acceptable ambient level for H_2S is 10 ppb, it is suggested that this guideline may also need to be adjusted.

6.0   References

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Appendix A. Composition of Sour Gas Sample

Sour Gas Sample Donated by Shell Canada Ltd.

Extended Gas Analysis provided by Maxxim Analytical

Sample Point: Overhead Gas

Location: Hope Creek Gas Plant

Sample Date: 1999/03/25

Analysis Date: 1999/03/31

Composition	Mole Fraction Air Free As Received	Mole Fraction Air Free Acid Gas Free	Liquid Volume ml/m^3 Air Free As Received
H_2	N/A	N/A	
He	0.0006	0.0006	
N_2	0.0271	0.0289	
CO_2	0.0233	0.0000	
H_2S	0.0400	0.0000	
C1	0.8457	0.9028	
C2	0.0265	0.0283	94.73
C3	0.0167	0.0178	61.38
IC4	0.0035	0.0037	14.95
NC4	0.0084	0.0089	35.15
IC5	0.0024	0.0025	11.57
NC5	0.0030	0.0032	14.40
C6	0.0017	0.0018	9.39
C7+	0.0012	0.0013	6.66
C10+	0.00003	0.00003	0.22
C12+	0.00000	0.00000	0.00
Total	1.0000	1.0000	248.23

Onsite H_2S by Tutwiler: 4.00 mole percent

N/A = H_2 not reported due to possible degradation of H_2S