THE EFFECTS OF HYDROGEN SULFIDE ON NEURONS IN THE NUCLEUS OF THE SOLITARY TRACT

By

Rishi Malik

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Abstract

Hydrogen sulfide (H_2S) is a gasotransmitter that has recently been described to affect the membrane potential of neurons in a number of brain areas. Using whole cell patch-clamp electrophysiological techniques, I investigated whether the H_2S donor, sodium hydrosulfide (NaHS) affects the membrane potential of neurons in the nucleus of the solitary tract, an autonomic nucleus where NaHS has previously shown to effect blood pressure and heart rate.

Bath application of NaHS (1 mM, 5 mM and 10 mM) to 300 µm coronal NTS brain slices led to a clear reversible depolarization in 95% of neurons tested (72/76), and in 64% (46/72) of these responding neurons was followed by a longer lasting hyperpolarization. These effects on membrane potential were found to be concentration-dependent.

Furthermore, in the presence of the voltage-gated sodium channel antagonist tetrodotoxin (TTX) and the glutamate receptor antagonist kynurenic acid (KA), the depolarizing effects of 5 mM NaHS (5.0 ± 2.2 mV (n=7)) were still observed, although they were significantly reduced compared to regular aCSF (7.7 ± 2.0 mV (n=7), p *< 0.05, paired t-test). These observations support the conclusion that NaHS has both pre and post-synaptic effects which contribute to controlling the excitability of NTS neurons. I also tested the hypothesis that hyperpolarizations in response to 5 mM NaHS resulted from modulation of the K_{ATP} channel by comparing effects observed following the K_{ATP} channel blocker glibenclamide (-1.9 ± 0.9 mV (n=8)) with those recorded in control conditions (-7.9 ± 1.2 mV n=8, p* < 0.05, paired t-test). This nearly complete loss of hyperpolarizing effects in the presence of glibenclamide identifies a primary role for the K_{ATP} channel in the NaHS induced hyperpolarization of NTS neurons.

Thus, the H_2S donor, NaHS causes two responses in NTS neurons. These effects were found to be in part post-synaptic and the K_{ATP} channel was found to play a role in the NaHS induced hyperpolarization. This study has for the first time described post-synaptic effects of this gasotransmitter on the membrane potential of NTS neurons and thus implicates this transmitter in regulating the diverse autonomic systems controlled by these NTS neurons.

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Abbreviations

3-MST……...……3-Mercaptopyruvate Sulfurtransferase

α-MSH……...…….….α-Melanocyte Stimulating Hormone

aCSF…….……...……...…….Artificial Cerebrospinal Fluid

AGII.....Angiotensin II

AgRP…….……Agouti-related Peptide

AMPA……..….. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AP-5……..……2-Amino-5-Phosphonopentanoic Acid

BK Channel…….…….…….. Large Conductance Potassium Channel

CAT……...…….Cysteine Aminotransferase

CBS…… Cysthathionine β-Synthase

CCK…….Cholecystokinin

CNQX…….……6-Cyano-7-Nitroquinoxaline-2,3-Dione

CNS……...……..Central Nervous System

CO……Carbon Monoxide

CSE…….…… Cysthathionine γ-Lyase

DTT……..……...…… Dithiothreitol

EPSP……. Excitatory Post-Synaptic Potentials

GABA…….……..*Gamma*-Aminobutyric Acid

H_2S……...……..Hydrogen Sulfide

HEK…….....……...……..Human Embryonic Kidney

HRP…….…….Horseradish Peroxidase

IPSP…...……...….Inhibitory Post-Synaptic Potentials

JAK-STAT3…….. Janus Kinase-Signal Transducer and Activator of Transcription 3

K_{ATP} Channel……...……Potassium Adenosine Triphosphate Channel

KA…… Kynurenic Acid

LH……Lateral Hypothalamus

LTP……Long-Term Potentiation

MC_3…… Melanocortin-3

MC_4……..Melanocortin-4

mEPSC……...Miniature Stimulated Excitatory Post-Synaptic Current

mRNA……Messenger Ribonucleic Acid

MSN……..Monosynaptic Neuron

MTII.....Melanotan II

Na_2S…….Sodium Sulfide

NaHS……..…….Sodium HydroSulfide

NMDA……*N*-Methyl-D-aspartate

NO……..……Nitric Oxide

NPY……..Neuropeptide Y

NTS……..……Nucleus of the Solitary Tract

OVT……..……..[$d(CH_2)_5$, Tyr $(Me)_2$ Orn^8]-Vasotocin

PBN……..…Parabrachial Nucleus

POMC……...…….Pro-Opiomelanocortin

PSN…….Poly-Synaptic Neuron

PVN……...……..Paraventricular Nucleus

RNA……..…….Ribonucleic Acid

RVLM……Rostral Ventrolateral Medulla

SAM……..…….. S-Adenosyl Methionine

SEM…….….Standard Error Mean

SFO…….……..Subfornical Organ

SK Channel……...…….. Small Conductance Calcium-Activated Potassium Channel

SNA……...……Sympathetic Nerve Activity

TRPA1……..……. Transient Receptor Potential Ankyrin 1

TRPV1……...…….Transient Receptor Potential Cation Channel Subfamily V Member 1

TS-EPSC……Solitary Tract Stimulated Excitatory Post-Synaptic Currents

TTX……..…….Tetrodotoxin

ZnDPBG…….. Zinc Deuteroprophyrin 2,4-Bis-Glycol

Chapter 1: Introduction

1.1 Central autonomic control

The autonomic nervous system plays critical roles in the regulation of homeostatic control systems, which collectively contribute to maintain the "milieu interior" in a state supportive of normal physiological functions and thus life itself. Such autonomic control is, for the most part, as the name suggests, autonomous, in that it is regulated as a background function associated with integrated physiological systems. The central nervous system (CNS) plays an important role in such autonomic regulation through the hierarchical control not only of individual systems (e.g., cardiovascular, immune, reproductive, and metabolic), which are necessary for survival, but also by integrating these autonomic functions to establish a combined autonomic state. These functions are controlled by multiple autonomic control centres in the CNS, specifically in the hypothalamus, mid-brain, and the medulla (Hetherington & Ranson, 1940; Schreihofer *et al.*, 1994).

The areas of the brain responsible for various autonomic functions were first identified through lesion studies, which characterized the hypothalamus as the "satiety centre" of the brain. Hetherington and Ranson (1940) were the first to investigate hypothalamic lesions in relation to obesity, and their studies demonstrated that bilateral lesions of the ventromedial hypothalamus led to hyperphagia and obesity, while similar lesions of the lateral hypothalamus (LH) resulted in marked hypophagia and weight loss, observations which led to the two centre hypothesis for understanding the hypothalamic control of energy balance (Hetherington & Ranson, 1940). Over half a century later, the methods for investigating the different hypothalamic nuclei and their roles in integrated autonomic function have greatly improved, leading to a better understanding of central autonomic control.

These later studies identified the arcuate nucleus as an additional critical region, playing important roles in the regulation of both energy balance and the cardiovascular system (Broberger *et al.*, 1998; Hahn *et al.*, 1998; Zheng *et al.*, 2010). In fact, they led to the development of the "the arcuate theory," which proposes a prominent role for this nucleus in the regulation of energy balance, which is modeled around functionally opposing roles for pro-opiomelanocortin (POMC)/cocaine amphetamine related transcript (CART)-expressing neurons and neuropeptide Y (NPY)/agouti-related peptide (AgRP)-expressing neurons (Hahn *et al.*, 1998). This theory leans heavily on the integration of connections between the arcuate nucleus, paraventricular nucleus (PVN), and LH and the role of POMC/CART and NPY/AgRP neurons in integrating the metabolic information important to this circuitry (Broberger *et al.*, 1998), and thus also adds these latter two hypothalamic nuclei to the list of critical players in autonomic control. The peptide neurotransmitters released from these arcuate neurons into the PVN and LH cause either orexigenic or anorexigenic effects. Specifically, arcuate POMC neurons release α-melanocyte stimulating hormone (α-MSH), which is a posttranslational product of POMC which acts at melanocortin 3/4 (MC_3 and MC_4) receptors to elicit anorexigenic effects when released into these areas (Schulz *et al.*, 2010). Importantly, these arcuate POMC neurons also project to the nucleus of the solitary tract (NTS), an important medullary autonomic control centre (Zheng *et al.*, 2010). On the other hand, AgRP/NPY neurons release AgRP, an orexigenic peptide which acts as a competitive antagonist for the same MC_3 and MC_4 receptors (Ollmann *et al.*, 1997; Hagan *et al.*, 2000). Similarly, NPY acts as a potent orexigenic peptide in the brain but in this case presumably through actions at Y_1 or Y_2 receptors (Clark *et al.*, 1984).

In addition to their roles in regulating energy balance, the hypothalamus and medulla are also involved in cardiovascular control (Kubo & Kihara, 1990; Horn *et al.*, 1994; Ohta &

Talman, 1994; Rahmouni & Morgan, 2007). The arcuate nucleus has been shown, through various experiments, to regulate the cardiovascular system through activation of POMC neurons (Li *et al.*, 1996) or activation of leptin receptors (Rahmouni & Morgan, 2007), although other hypothalamic nuclei such as the PVN have been suggested to play more important roles (Pyner & Coote, 2000; Yang *et al.*, 2002; Li *et al.*, 2006). In experiments where *N*-Methyl-D-aspartate (NMDA), angiotensin II (AGII), and the nitric oxide (NO) donor sodium nitroprusside were microinjected into the PVN, all resulted in changes in blood pressure and/or heart rate as well as changes in sympathetic nerve activity (SNA) (Bains *et al.*, 1992; Horn *et al.*, 1994; Li *et al.*, 2006). PVN efferents responsible for such effects likely include the PVN neurons, which project to the spinal cord (Hallbeck *et al.*, 2001), rostral ventrolateral medulla (RVLM), and the NTS (Pyner & Coote, 2000).

While the PVN influences cardiovascular function through the aforementioned pathways, it receives much information regarding the current state of the cardiovascular system through afferent input originating in the medullary NTS, perhaps the most important integrative autonomic control centre in the hindbrain (Ricardo & Koh, 1978; Yin *et al.*, 1994), with critical roles in cardiovascular (Yin *et al.*, 1994), metabolic (Smith *et al.*, 1985; Emond *et al.*, 2001), and respiratory (Furuya *et al.*, 2014) control systems.

1.2 The NTS

Location and connections

The NTS is a bilateral structure located in the medulla, ventral to the area postrema, with extensive afferent and efferent connections to both the viscera and additional CNS autonomic control centres. Most of the afferent visceral connections arrive through the solitary tract, which is composed of the facial (VII), glossopharyngeal (IX), and vagus (X) nerves (Torvik, 1956),

with sensory fibers originating from the larynx, trachea, bronchus, lungs, heart, and stomach (Kalia & Mesulam, 1980). The NTS receives extensive afferents from other brain regions as well, such as the medial prefrontal cortex, bed nucleus of the stria terminalis, central nucleus of the amygdala, PVN, arcuate nucleus, and ventral and posterior agranular cortex (van der Kooy *et al.*, 1984). The NTS also sends extensive efferent projections to other areas of the brain, with some of the most important autonomic outputs being those to the dorsal motor nucleus of the vagus, PVN, arcuate nucleus, parabrachial nucleus (PBN), spinal cord, and the RVLM (Ricardo & Koh, 1978; Schreihofer & Guyenet, 2002). These extensive neural connections uniquely position the NTS for its role as a critical integrative medullary autonomic control centre.

The NTS and energy balance

The NTS has a well-established role in the regulation of energy balance, particularly as a centre that integrates information regarding satiety (Emond *et al.*, 2001; Berthoud *et al.*, 2001). A variety of peptides have been shown to act either directly or indirectly at the NTS to influence satiety, including the gastric satiety peptide cholecystokinin (CCK), which is secreted by endocrine cells in the intestine in response to gastric distension (Gibbs & Smith, 1977). CCK acts through vagal afferents to activate NTS neurons (Yuan *et al.*, 2000). Similarly, application of leptin to the gastrointestinal tract results in the activation of NTS neurons as a result of the activation of vagal afferents (Yuan *et al.*, 2000). Along with stimulation of NTS neurons through the vagus nerve, leptin also has been shown to increase gastric distension-induced activity in NTS neurons through direct actions on the nucleus (Schwartz & Moran, 2002). NPY, the melanocortin 3/4 ($MC_{3/4}$) receptor antagonist melanotan II (MTII) and ghrelin, all peptides involved in the regulation of energy balance, have also been shown to elicit effects in the NTS. NPY causes a decrease in gastric distension-induced activity in neurons when applied in the NTS

(Schwartz & Moran, 2002). Similarly, MTII causes a decrease in food intake when injected into the fourth ventricle (Grill *et al.*, 1998) while similar ventricular administration of ghrelin results in increased food intake (Kinzig *et al.*, 2006); both effects are most likely the result of actions of these peptides within the NTS.

While there are many peptides that act in the NTS to regulate energy balance, the two most extensively studied are CCK and leptin. Investigation of the roles of these two peptides have elucidated many of their cellular actions in the NTS in modulating energy balance as well as revealed the complex array of other peptides and receptors involved in these actions. The MC_4 receptor has been proven necessary for the CCK-mediated satiety effects, as mice lacking the MC_4 receptor and wild-type mice microinjected with the MC_4-receptor antagonist SHU9119 lack a satiation response to intraperitoneal (i.p.) injection of CCK (Fan *et al.*, 2004; Sutton *et al.*, 2005). This role for the MC_4 receptor is also supported by the demonstration that 30% of NTS POMC neurons (Bronstein *et al.*, 1992) were positive for c-Fos after i.p. injection of CCK (Fan *et al.*, 2004).

Leptin also has cellular effects on NTS neurons, some direct and others indirect. Intravenous injection of leptin, which reaches the CNS by crossing the blood brain barrier through a transporter (Banks *et al.*, 1996), results in leptin-induced activation of Janus kinase-signal transducer and activator of transcription 3 (JAK-STAT3) in the NTS (Hosoi *et al.*, 2002). Furthermore, leptin also has indirect effects on the NTS through actions at the arcuate nucleus and PVN of the hypothalamus (Blevins *et al.*, 2004; Morton *et al.*, 2005). Interactions between CCK- and leptin-induced satiety were suggested by studies showing that rats lacking the leptin receptor had a significant reduction in their satiety response to i.p. CCK injection. This reduction in CCK-induced satiety was reversed when leptin receptor expression in the arcuate nucleus was

restored through adenoviral gene therapy, thus suggesting an important role for leptin signaling in the arcuate nucleus for CCK-induced satiety in the NTS (Morton *et al.*, 2005). Additionally, oxytocin projections from the PVN to the NTS may have a role in the indirect effects of leptin on the NTS, as terminals associated with these projections are seen close to i.p. CCK-induced c-Fos-positive neurons in the NTS (Blevins *et al.*, 2004). Such interactions are supported by the demonstration that the effects of CCK on food intake are decreased with the application of an oxytocin receptor antagonist [$d(CH_2)_5$, Tyr $(Me)_2$ Orn^8]-vasotocin (OVT) into the fourth ventricle (Blevins *et al.*, 2003). The role of these oxytocin projecting neurons relies in part on leptin signaling in the PVN, as leptin administration in the third ventricle has been described to sensitize the NTS to the effects of i.p. CCK through these NTS-innervating PVN oxytocin neurons (Schwartz & Moran, 2002; Blevins *et al.*, 2004).

The NTS and the cardiovascular system

Beyond its role in energy balance, the NTS also has a role in cardiovascular control (Czachurski *et al.*, 1982; Yin *et al.*, 1994). Early studies investigating the NTS described decreases in blood pressure and heart rate following microinjection of the excitatory neurotransmitter glutamate into the nucleus (Talman *et al.*, 1980; Yin *et al.*, 1994). Additionally, rats with complete NTS lesions failed to elicit a baroreflex response to an increase in blood pressure produced by phenylephrine (Schreihofer *et al.*, 1994). The NTS also receives critical visceral cardiovascular information through the solitary tract; specifically, from the glossopharangeal nerve, which receives baroreceptor input from the carotid sinus and the vagus nerve, which, in turn, receives its input from the aortic arch baroreceptors (Torvik, 1956; Czachurski *et al.*, 1982). Other neuromodulators besides glutamate have also been shown to exhibit cardiovascular effects in the NTS, including NPY, neurotensin, L-arginine, and

angiotensin II, all of which cause a decrease in heart rate and blood pressure when microinjected into the NTS (Kubo & Kihara, 1990; Lin *et al.*, 1999). Likewise, microinjection of the MC_3 and MC_4 receptor agonist, MTII, into the fourth ventricle causes a decrease in heart rate (Zheng *et al.*, 2005). Conversely, microinjection of peptides such as nesfatin-1 and arginine vasopressin into the NTS caused an increase in heart rate and blood pressure (Kubo & Kihara, 1990; Mimee *et al.*, 2012). Interestingly, the neurotransmitter orexin caused different responses when microinjected into the NTS, depending on the concentration of peptide used. At a low dose (5 pmol), orexin causes a decrease in both heart rate and arterial pressure (Shih & Chuang, 2007) while at a higher dose (200 pmol or higher), the peptide caused these same parameters to increase (Smith *et al.*, 2002; Shih & Chuang, 2007). The effects of these various peptides highlight the complexity of the cardiovascular response within the NTS. Furthermore, this nucleus is able to affect these cardiovascular parameters through its efferent connections to the PBN, PVN (Ricardo & Koh, 1978), and RVLM, which in turn can affect SNA and lead to changes in heart rate and blood pressure (Schreihofer & Guyenet, 2002).

While application of peptides to the NTS can cause physiological changes in cardiovascular parameters, the NTS is also able to integrate the various signals from different visceral afferents at the cellular level. Single neurons within the NTS can receive input from the carotid sinus receptors, the aortic arch receptors, or both, illustrating that these inputs can converge at the level of single neurons within this nucleus (Donoghue *et al.*, 1985; Mifflin *et al.*, 1988). Stimulation of these cardiovascular inputs, which synapse with NTS neurons, can lead to either excitatory post synaptic potentials (EPSP), inhibitory post synaptic potentials (IPSP), or a combination of an EPSP followed by an IPSP (Mifflin & Felder, 1988; Mifflin *et al.*, 1988; Andresen & Yang, 1995). Furthermore, various glutamate receptors may potentially play

different roles at the cellular level in the NTS-mediated baroreflex (Ohta & Talman, 1994; Zhang & Mifflin, 1997; Zhang & Mifflin, 1998). The effects of aortic depressor nerve stimulation on monosynaptic neurons (MSNs) within the NTS can be blocked by selective non-NMDA receptor antagonists, such as 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), but not by selective NMDA receptor antagonists, such as 2-amino-5-phosphonopentanoic acid (AP-5). However, the effects of aortic depressor nerve stimulation on poly-synaptic neurons (PSN) in the NTS can be blocked by both non-NMDA and NMDA receptor antagonists, indicating a possible difference in the roles of the two receptors in the NTS and baroreflex regulation (Zhang & Mifflin, 1998). Experiments using specific non-NMDA and NMDA agonists also supported these findings as NMDA only weakly excited MSNs, while application of the non-NMDA receptor agonists α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate demonstrated a greater excitation of MSNs (Zhang & Mifflin, 1997).

In addition to peptidergic and amino acid-derived neurotransmitters, there is an additonal class of neurotransmitters, known as gasotransmitters, which affect autonomic cardiovascular control in the NTS. There are three gasotransmitters which have been identified: NO, carbon monoxide (CO), and the most recently discovered, hydrogen sulfide (H_2S). In the NTS, two enzymes which produce NO, endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS), have been localised (Lin *et al.*, 2007), and NO has also been suggested to have a role in regulating baroreflex sensitivity, as nNOS knockout mice have a blunted baroreflex (Carvalho *et al.*, 2006). Additionally, inhibitors of the NO producing enzymes attenuate the effects of both L-arginine (Lin *et al.*, 1999) and low-dose orexin (Shih & Chuang, 2007) microinjections into the NTS, suggesting that NO plays a role as a cellular messenger in peptide-mediated cardiovascular effects in the NTS. Similarly to NO, CO has been shown to play a role

in the NTS in controlling the cardiovascular system, though there have been significantly fewer studies investigating the role of this gasotransmitter in this nucleus. NTS administration of hematin, a molecule that is catalyzed into CO by heme oxygenase, causes bradycardia and a decrease in blood pressure. Furthermore, zinc deuteroporphyrin 2,4-bis-glycol (ZnDPBG), a molecule which inhibits heme oxygenase and thus the production of CO, has been found to decrease the baroreflex when microinjected into the NTS (Lo *et al.*, 2000; Lo *et al.*, 2004). As well as having direct effects in the NTS, CO has been shown to act as a cellular messenger in amino acid and peptide-mediated cardiovascular effects. Pre-application of ZnDPBG was found to attenuate the cardiovascular effects of microinjection of glutamate into the NTS (Silva *et al.*, 1999).

The third and most recently discovered gasotransmitter is H_2S, and recent studies intriguingly found one of the enzymes responsible for its production, cysthathionine β-synthase (CBS), to be expressed in the NTS (Austgen *et al.*, 2011) In addition, application of the H_2S donor, sodium hydrosulfide (NaHS), into the NTS has been shown to cause bradycardia and depressor effects through modulation of glutamate actions and the regulation of the potassium ATP (K_{ATP}) channel (Qiao *et al.*, 2011), data which suggest potentially important actions for this gasotransmitter within the NTS in the regulation of autonomic function .

1.3 Hydrogen sulfide

H_2S for many years has been known primarily as a deadly and noxious gas (Warenycia *et al.*, 1989; DeLeon *et al.*, 2012). However, considerable recent work has focussed attention on the potential physiological effects of H_2S in both peripheral and central nervous system sites (Abe & Kimura, 1996; Qiao *et al.*, 2011).

Production and enzymes

H_2S is endogenously produced in tissue by three different enzymes: CBS (Abe & Kimura, 1996), 3-mercaptopyruvate sulfurtransferase (3-MST) (Shibuya *et al.*, 2009), and cystathionine γ-lyase (CSE) (Stipanuk & Beck, 1982). These three enzymes are not uniformly expressed throughout the body, but all use homocysteine, cysteine, or 3-mercaptopyruvate as a substrate to create H_2S (Abe & Kimura, 1996; Shibuya *et al.*, 2009). Northern blot analysis of rat brain homogenates found that brain tissue expressed CBS RNA but not CSE RNA, indicating that the brain preferentially expresses CBS. Further experiments using whole brain homogenates and two inhibitors of CBS, hydroxylamine and amino-oxyacetate, demonstrated that these inhibitors decrease H_2S production in the CNS, effects that were found to be dose dependant (Abe & Kimura, 1996). On the other hand, the application of D,L-propargylglycine (Uren *et al.*, 1978) or β-cyano-L-alanine (Pfeffer & Ressler, 1967), two inhibitors of CSE, resulted in only small decreases in H_2S in whole brain homogenates (Abe & Kimura, 1996), leading to the conclusion that while CBS plays an important role in H_2S production in the brain, CSE likely does not. With this in mind, the observation that CBS knockout mice had brain levels of H_2S similar to that of wild-type animals suggests an additional significant role for 3-MST in CNS production of H_2S (Shibuya *et al.*, 2009). Shibuya et al. (2009) looked at rat brain homogenates and found that fractions of the mitochondria, synaptosomes, and cytosol all contained cysteine aminotransferase (CAT) and 3-MST. Even though all three fractions contained both enzymes, the synaptosomal and mitochondrial fractions required the cytosol fraction in order to produce H_2S. The investigators found the critical component in the cytosol to be α-ketoglutarate, one of the substrates for CAT. By adding α-ketoglutarate to the synaptosomal and mitochondrial fractions, they were able to produce H_2S in a dose-dependent manner. Furthermore, by using HEK293-F cells, the authors were able to determine that 3-MST makes a greater contribution to

bound sulfur stores than CBS. They transfected HEK293-F cells with either an empty control vector, a vector containing CBS DNA or a vector containing 3-MST and CAT DNA, then used dithiothreitol (DTT), a liberator of bound sulfur, to release these H_2S stores. Using this experimental design, the authors found that cells transfected with 3-MST and CAT DNA released far greater amounts of H_2S (up to 220%) than were released under control conditions in response to DTT. In addition, cell lines containing the CBS vector did not release a significantly different amount of H_2S compared to control (Shibuya *et al.*, 2009), providing evidence that H_2S created by 3-MST may more likely be stored as bound sulfur compared to H_2S created by CBS.

Cellular stores and endogenous concentrations

The identification of H_2S as an endogenous gasotransmitter with a vast array of biological effects led to studies investigating its endogenous concentrations with reports indicating levels from the nanomolar to micromolar range (Warenycia *et al.*, 1989; Furne *et al.*, 2008; Ishigami *et al.*, 2009). H_2S can be found in three forms within tissue: as free H_2S gas, stored as acid-labile sulfur, or as bound sulfur (Ogasawara *et al.*, 1994). Acid-labile sulfur is bound to enzymes within the mitochondria and is released when the mitochondrial pH is reduced to 5.4 or lower (Ishigami *et al.*, 2009). Given that the internal environment of mitochondria is not normally acidic, it is unlikely that these stores are used in physiological conditions (Ishigami *et al.*, 2009). Alternatively, bound sulfur can be found within the cytosol, as divalent sulfur bound to protein (Ogasawara *et al.*, 1994), and may be released by reducing molecules in the cell when intracellular pH increases (Ishigami *et al.*, 2009).

An early study attempting to measure endogenous free H_2S found levels in the rat brain to be 54 μM on average (Warenycia *et al.*, 1989), a value which was surprising considering the authors also found toxic levels of the compound to be only twice as great as these reported

endogenous levels. This study and others using similar experimental designs employed hydrochloric acid or reducing agents in the process of preparing the samples, making it likely that artificially elevated levels of free H_2S gas were reported due to the liberation of acid-labile sulfur or bound sulfur (Ishigami *et al.*, 2009). Ishigami et al. (2009) conducted an experiment that used silver particles to trap H_2S molecules, thus avoiding the use of compounds that would otherwise liberate acid-labile or bound sulfur. Using this strategy and a minimum detection protocol of 9.2 μM, they were unable to detect free H_2S in the brain, leading them to conclude that the level of free H_2S in the rat brain must be less than 9.2 μM (Ishigami *et al.*, 2009). Furne et al. (2008), using a protocol similarly designed to avoid the liberation of bound and acid-labile sulfur analyzed the gas space above whole brain tissue homogenates and found levels of endogenous H_2S in the brain to be 14 ± 3 nM, well below the 54 μM concentration reported by Warenycia et al. (1989). These varying reported levels highlight the lack of consensus in the literature with regard to the endogenous concentration of H_2S in the brain.

H_2S actions in the central nervous system

A growing literature highlighting the effects of H_2S in the CNS has evolved since the first study identifying a role for H_2S as a modulator of long-term potentiation (LTP) in hippocampal slices (Abe & Kimura, 1996). Administration of NaHS during weak tetanic stimulation was reported to enhance LTP, an effect which was shown to be NMDA dependent as it was blocked by preadministration of an NMDA receptor antagonist (Abe & Kimura, 1996). NaHS has also been shown to protect against oxidative stress by increasing cysteine transport into neurons, with intracellular cysteine then being converted into γ-glutamylcysteine and ultimately the antioxidant glutathione, which is believed to provide this protection (Kimura & Kimura, 2004). As in neurons, there is evidence of H_2S having effects on other CNS cells such as microglia (Lee *et al.*,

2006) and astrocytes (Nagai *et al.*, 2004). NaHS was found to increase intracellular calcium in cultured microglial cells, an effect which was inhibited by thapsigargin, a sarcoplasmic reticulum calcium pump ATPase inhibitor (Lee *et al.*, 2006). Similarly, a study investigating astrocytes found NaHS led to an increase in intracellular calcium in astrocytes, although in this case, the increase was found to be because of an influx of extracellular calcium rather than a release of intracellular calcium stores (Nagai *et al.*, 2004). Astrocytes are known to release signaling molecules such as glutamate in response to an increase in intracellular calcium (Parpura & Haydon, 2000), and the H_2S-mediated increase in intracellular calcium creates the potential for this gasotransmitter to have a role in astrocyte signaling.

More recently, a number of studies have described the effects of H_2S in various autonomic control regions in the brain, specifically the PVN (Gan *et al.*, 2012; Khademullah & Ferguson, 2013), the subfornical organ (SFO) (Kuksis *et al.*, 2014), the NTS (Austgen *et al.*, 2011; Qiao *et al.*, 2011), and the hypoglossal rootlets in the medulla (Hu *et al.*, 2008). In our lab, we have identified H_2S-producing enzyme CBS in the PVN, and then used patch clamp recording techniques in hypothalamic slices to show that NaHS caused a biphasic depolarization with changes in input resistance, effects that were dose dependent, with neurons responding to concentrations ranging from 0.1 mM to 50mM (Khademullah & Ferguson, 2013). Additionally, a separate study found that application of nanomolar concentrations of the H_2S donor, GYY4137, into the PVN facilitated the cardiac sympathetic afferent reflex and increased mean arterial pressure, heart rate, and renal SNA (Gan *et al.*, 2012). H_2S has also been shown to have effects on SFO neurons, with current-clamp recordings from dissociated SFO neurons showing that the vast majority of these cells depolarize in response to NaHS (88/90 of neurons tested) (Kuksis *et al.*, 2014). While no study thus far has reported effects of H_2S on the membrane potential of NTS

neurons, studies investigating the cardiovascular effects of microinjections of NaHS into the NTS of rats have found that NaHS causes bradycardia and hypotension in these animals (Qiao *et al.*, 2011), an effect which was replicated with microinjections of an allosteric activator of CBS known as S-adenosyl metionine (SAM). Attempts to identify the channel facilitating these cardiovascular changes found that this effect can be significantly attenuated by the K_{ATP} channel blocker glibenclamide or the non-specific glutamate receptor blocker kynurenic acid (Qiao *et al.*, 2011). Furthermore, Austgen et al. (2011) examined the effects of NaHS on individual NTS neurons. Patch-clamp electrophysiological recordings showed that application of NaHS increases the amplitude of both solitary tract evoked EPSCs and the frequency of miniature excitatory post synaptic currents (mEPSC) (Austgen *et al.*, 2011). These studies both support a role for NaHS as a neuromodulator in the brain and specifically as an important signaling molecule within the NTS.

H_2S administration using Donors

In the past two decades there have been many studies investigating the effects of H_2S in physiological systems, most of which have used use one of two H_2S donors, either NaHS or sodium sulfide (Na_2S) (Olson, 2012). Dissolved in solution, H_2S acts as a weak acid, with Na_2S having a greater effect on pH because it requires two hydrogen ions from the solution to form H_2S while NaHS requires one (Olson, 2012). According to the Henderson-Hasselbalch equation, at physiological pH and temperature, H_2S exists in equilibrium with HS^-, with approximately 18.5% existing as H_2S (Dombkowski *et al.*, 2004). Furthermore, the volatility of H_2S must be considered in experiments that use this compound to understand the effective concentration that reaches the cells (DeLeon *et al.*, 2012). Using three different common biological experimental setups, including tissue culture plates, muscle myograph baths, and the Langendorff perfused

heart system, the rate at which H_2S dissipates has been assessed both with and without oxygen (DeLeon *et al.*, 2012). The half-life for the oxygenated tissue culture plates is 5.3 ± 0.33 minutes, the oxygenated myograph bath has a half-life of 2.46 ± 0.17 minutes, while the oxygenated Langendorff system shows half-life that can be split into fast (0.070 ± 0.003 minutes) and slow (1.85 ± 0.23 minutes) components (DeLeon *et al.*, 2012). These short half-lives emphasize the importance of applying the H_2S donor to the tissue shortly after its preparation. A further complication in performing biological experiments is that H_2S can be oxidized in the presence of biological tissue (Olson *et al.*, 2010). Previous studies have reported that exogenously applied H_2S first binds to sulfur molecules in proteins (bound sulfur), which acts as a buffer when there is an influx of the gas, thus decreasing the H_2S concentration available for physiological effect (Ishigami *et al.*, 2009). The complex nature and volatility of both H_2S and its donors emphasizes some of the experimental challenges faced when using these compounds.

1.4 Research aims

H_2S is a gasotransmitter implicated in various functions throughout the body. It has effects on a variety of CNS nuclei and has been described to affect numerous ion channels (Kombian *et al.*, 1993; Telezhkin *et al.*, 2009; Strege *et al.*, 2011). Furthermore, microinjection of H_2S into the NTS has been shown to effect blood pressure and heart rate (Qiao *et al.*, 2011), suggesting this gasotransmitter may have effects on the membrane potential of NTS neurons. The presence of CBS, one of the enzymes which catalyze the production of H_2S (Austgen *et al.*, 2011), and ion channels known to be modulated by H_2S (Kombian *et al.*, 1993; Telezhkin *et al.*, 2009; Qiao *et al.*, 2011) within the NTS suggests a possible role for H_2S in this medullary autonomic control centre as a gasotransmitter. Patch-clamp electrophysiological techniques were

used to investigate the ability of H_2S to modulate the membrane potential of the neurons in the NTS. The following hypotheses were tested:

i. H_2S influences the membrane potential of NTS neurons

ii. Changes in membrane potential are due to post-synaptic modulation

iii. Modulation of specific ion channels cause these changes in membrane potential

Chapter 2: Materials and Methods

Male Sprague-Dawley rats (Charles River, QUE, Canada) were used in all experiments. All animal procedures were approved by the Queen's University Animal Care Committee.

2.1 Brain Slice Preparations

21-28 day old male Sprague Dawley rats were sacrificed, the brainstem was removed and placed in ice cold slicing solution which contained (in mM): 87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 1.25 NaH_2PO_4, 25 glucose, 75 sucrose and was bubbled with carbogen (95% O_2/5% CO_2). The brainstem was blocked and fixed to a stage using superglue and a vibratome (Leica, Nussloch, Germany) was used to cut 300μm coronal slices through the region of NTS. These slices were then placed in 31^{0}C artificial cerebrospinal fluid (aCSF) which contained (in mM): 126 NaCl, 2.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 10 glucose and left to incubate for one hour.

2.2 Electrophysiology

Cells were visualized using an upright differential interference contrast microscope (Scientifica, Sussex, United Kingdom) at 400× magnification. Brain slices were placed in a recording chamber and perfused at a rate of 1-2 mL/min using a gravity perfusion system with aCSF heated to 37^{0}C using an inline heater (SC-20, Warner Instruments Corp., Hamden, Connecticut, USA). Recording electrodes were fabricated from borosilicate glass tips (World Precision Instruments, Sarasota, Florida, USA) and pulled to have a resistance of 2-5MΩ using a flaming brown micropipette puller (P97, Sutter Instruments Company, Novato, California, USA). These glass recording electrodes were filled with internal solution which contained (in mM): 125 potassium gluconate, 10 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 5.5 EGTA, 10 HEPES, 2 NaATP (adjusted to pH 7.2 with KOH). The estimated free calcium within the internal solution was

calculated to be 11.3 nM. Whole cell recordings were obtained using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, California, USA) filtered at 2.4 kHz, and acquired at a sampling rate of 10 kHz using a Micro 1401 (Cambridge Electronic Design, Cambridge, United Kingdom). A micromanipulator (ACCi UI, Scientifica, East Sussex, United Kingdom) was used to navigate the recording electrode tip to an individual NTS neuron. Light suction was used to make a gigaseal with the cell, and then a gentle pulse of suction was applied to rupture the membrane in order to gain access to the internal environment of the cell and achieve the whole cell recording configuration. Only neurons with an input resistance of >700MΩ which fired action potentials >60mV in amplitude were included in our experiments. A liquid junction potential of -15 mV is corrected for in all reported values. NaHS and all other agents were applied through the same gravity perfusion system as the aCSF. Resting membrane potential was determined for each neuron using all digital points obtained in the 100s period prior to any drug administration, this calculation yielding a mean as well as a standard deviation of the mean for each cell. If the mean membrane potential over a 50s period changed by greater than 2 standard deviations of the baseline after application of drug, it was considered a response.

2.3 Preparation of NaHS

NaHS was made up as a 50mM stock solution and kept on ice in a dark insulated box for up to 2 hours. This stock solution was then used to produce the lower concentrations immediately (within 5 mins) prior to testing of each individual neuron.

2.4 Data Analysis

All data was analyzed in GraphPad Prism 6.01 (La Jolla, California, USA). The average of 100 seconds of baseline measurements were compared with average of 50 second measurements of the peak effect for all experiments. The one exception to this was the

experiment investigating changes in conductance. For this experiment conductance was measured as the average of three 10 millisecond, -10 mV steps during the control period and compared to the average of three 10 millisecond, -10 mV steps during the peak effect. All data was analyzed with either an unpaired or paired student's t-test as indicated. All values are reported as mean ± standard error of the mean.

2.5 Chemicals

TTX was obtained from Alamone Laboratories (Jerusalem, Israel), glibenclamide was obtained from Tocris (Bristol, United Kingdom) and all other chemicals were obtained from Sigma-Aldrich (Oakville, Ontario, Canada).

Chapter 3: Results

3.1 NaHS affects the membrane potential of NTS neurons in a concentration dependent manner

I first examined the effects of NaHS on the membrane potential of NTS neurons, by bath applying NaHS to 300μm coronal NTS brain slices while whole-cell current clamp recordings were obtained from NTS neurons. Recordings were obtained from neurons in the medial and commissural NTS, and a total of 76 NTS neurons were tested with NaHS, of which 72 were found to show clear rapid and reversible depolarizing responses to NaHS. These responding NTS neurons could be split into two different groups, one of which showed a simple depolarization followed by a return to baseline (36%, n=26/72; Figure 1A), while in the second group, the initial depolarization was followed by a larger hyperpolarization which eventually returned to baseline (64%, n=46/72; Figure 2A). In the first group, the length of the response was 369.9 ± 46.6s (n=17), while in the second group the depolarization was shorter in duration (87.1 ± 8.6s, n=23), and was followed by a long hyperpolarization (533.2 ± 62.2s, n=23).

These effects of NaHS on NTS neurons were concentration dependent with 100% (n=52/52) of neurons tested responding at 10mM NaHS, 96% (n=22/23) responded at 5mM NaHS, 54% (n=14/26) responded at 1mM NaHS, and no cells responded at 100μM (n=0/8). The proportion of neurons which were observed to hyperpolarize was similar across the 10mM, 5mM, and 1mM NaHS concentrations (62%, 68%, and 50%, respectively). Additionally, the mean magnitudes of the depolarization were: 13.2 ± 1.2 mV at 10mM (n=52), 7.8 ± 0.8 mV at 5mM (n=19), and 3.6 ± 0.8 mV at 1mM (n=14) (Figure 1B). The mean magnitudes of the hyperpolarizations were: -13.0 ± 1.0 mV at 10mM (n=32), -14.7 ± 1.5 mV at 5mM (n=15), and -8.0± 2.2 mV at 1mM (n=7) (Figure 2B). The depolarizations increased in magnitude with

increasing concentration of NaHS, as did hyperpolarizations although these reached peak magnitude at 5mM NaHS.

3.2 NaHS induces post-synaptic effects on the membrane potential of NTS neurons

Previous studies investigating the effects of NaHS on NTS neurons described modulation of pre-synaptic glutamate release but did not observe direct effects on the membrane potential of NTS neurons (Austgen *et al.*, 2011). We therefore carried out experiments to determine if the effects on membrane potential previously mentioned resulted from such actions on synaptic glutamate release, or were mediated through different mechanisms, by evaluating the effects of NaHS in the presence of the glutamate blocker kynurenic acid (KA) and the voltage-gated sodium channel blocker tetrodotoxin (TTX). These recordings from synaptically isolated NTS neurons showed that depolarizing effects of NaHS (5 mM) were still observed in the presence of TTX and KA (5.0 ± 2.2 mV (n=7); Figure 3A), although these effects were smaller than those seen in regular aCSF (7.7 ± 2.0 mV (n=7), $p^* < 0.05$, paired t-test; Figure 3B). Depolarizations observed in the presence of TTX and KA clearly are neither pre-synaptic nor glutamate receptor mediated, and therefore represent post-synaptic actions of NaHS on the membrane potential of NTS neurons.

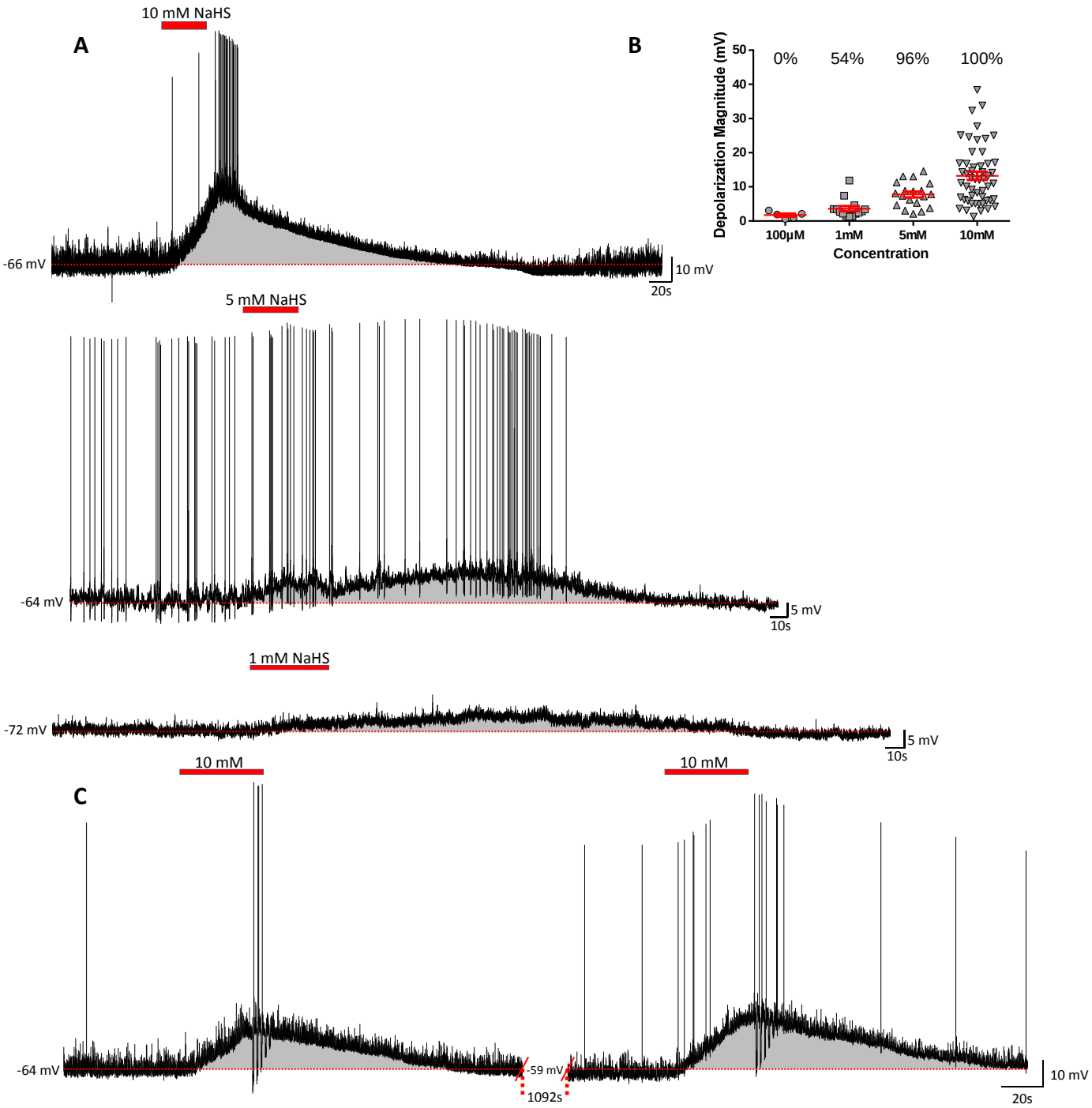

Figure 1: NTS neurons depolarize in response to NaHS

A. Current-clamp recordings from NTS neurons showing the effects of 10 mM, 5 mM and 1mM NaHS (period of administration indicated by the red bars) on membrane potential. Notice the rapid depolarizing effects of NaHS which are followed by return to baseline membrane potential. **B.** This scatterplot shows the depolarization magnitude for every neuron tested at four different concentrations of NaHS, while the black lines and error bars represent the mean ± SEM for each individual group. Percentage of responders at each concentration are indicated at the top of the graph. **C.** A current-clamp recording from a NTS neuron showing the repeatability of the effects of NaHS within a single neuron. Hyperpolarizing current steps were applied in 10 pA increments from -50 pA to -10 pA at the peak of both responses.

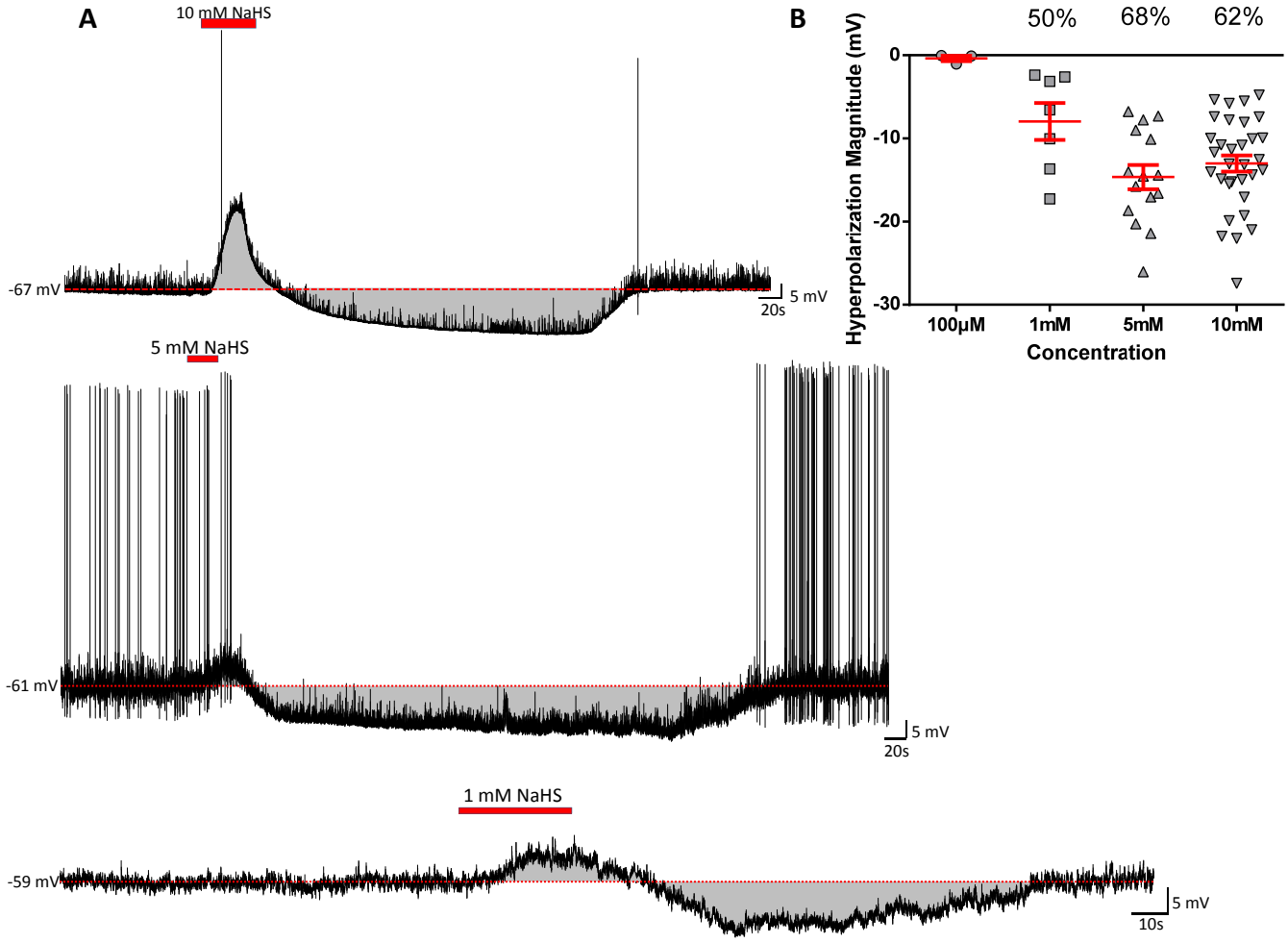

Figure 2: A significant proportion of NTS neurons respond to NaHS with a prolonged hyperpolarization after the initial depolarization

A. Current-clamp recordings from NTS neurons showing the effects of 10 mM, 5 mM and 1 mM NaHS in cells where the initial depolarization was followed by a longer lasting hyperpolarization. Red bars indicate NaHS application. **B.** A scatterplot of hyperpolarization magnitudes for every neuron tested at the four NaHS concentrations, black bars indicated mean hyperpolarization magnitudes and error bars represent SEM. Percentage of responding neurons which hyperpolarized are indicated at the top of the graph.

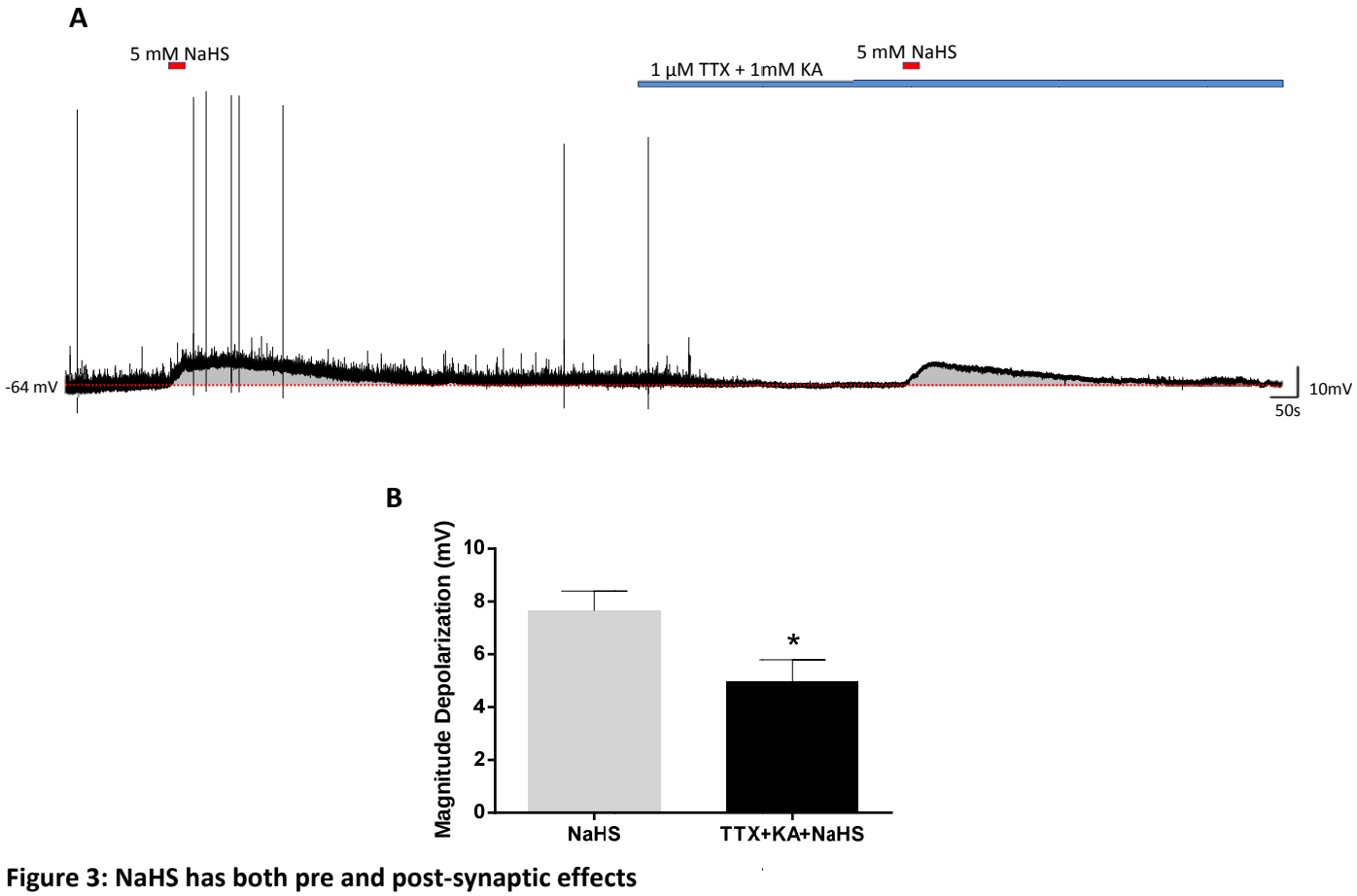

Figure 3: NaHS has both pre and post-synaptic effects

A. The upper panel is a current-clamp recording from an NTS neuron which shows a clear depolarizing response to NaHS in aCSF. A similar but slightly smaller depolarizing response is seen in this same neuron in response to the same concentration of NaHS during tetrodotoxin and kynurenic acid application. **B.** A bar graph depicting the magnitude of the depolarization response to NaHS in the different treatment groups (n=7). Error bars indicate SEM, $p^* < 0.05$, paired t-test.

3.3 Increases in conductance underlie both depolarizing and hyperpolarizing effects of NaHS on NTS neurons

We next used voltage clamp techniques to examine the ion channels underlying effects of NaHS on the membrane potential of NTS neurons. We examined changes in conductance in NTS neurons before during and after bath administration of 5mM NaHS by measuring currents evoked in response to 10 mV (10ms) hyperpolarizing voltage steps applied at 10s intervals before application of NaHS, during the early inward (depolarization) and later outward (hyperpolarization) currents (Figure 4A). NaHS led to increases in conductance from control values of 1.0 ± 0.1 nS, (n=5) to 1.8 ± 0.2 nS, (n=5) during the initial inward current, and to 6.0 ± 1.5 nS, (n=5, p *< 0.05, paired t-test; Figure 4C), indicating that the opening of different ion channels is responsible for these two effects.

We next examined the reversal potential of the whole cell current elicited by NaHS by measuring currents evoked by 5mM NaHS administration in NTS neurons at holding potentials of either -60 mV or -95 mV (Figure 4B). Mean inward currents evoked at these two potentials were -13.9 ± 2.8 pA, (n=6) at the holding potential of -60 mV and -19.0 ± 4.1 pA, (n=4) at the holding potential of -95 mV which when extrapolated suggests a reversal potential of 35 mV. In contrast, mean outward currents were 155.8 ± 35.4 pA, (n=7) at the holding potential of -60 mV and -76.2 ± 12.6 pA, (n=4) at the holding potential of -95 mV suggesting a reversal potential for these effects of -84 mV (Figure 4C), a value very close to the reversal potential for potassium under these conditions.

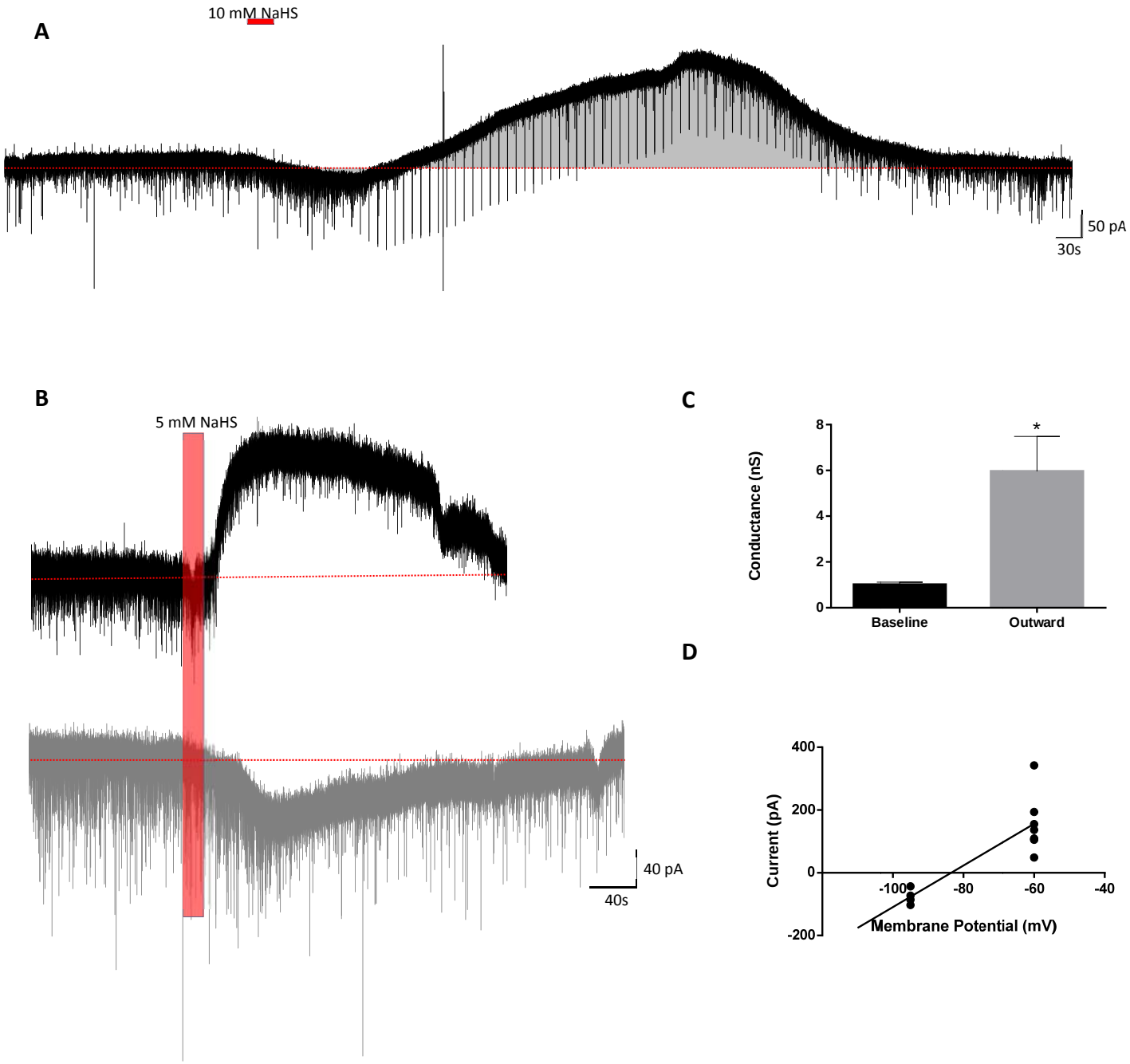

Figure 4: NaHS opens a potassium channel

A. A voltage-clamp recording of a neuron responding to NaHS while running 10 mV steps every 10s through the entirety of the trace. **B.** A voltage-clamp recording of a neuron responding to NaHS at two holding potentials, the black trace indicates a holding potential of -60 mV (n=7) and the grey trace indicates a holding potential of -95 mV (n=4). **C.** A bar graph depicting the change in conductance during the outward current compared to baseline in response to NaHS. Error bars represent SEM, $p^* < 0.05$, paired t-test. **D.** A scatter-plot with a line of best fit of a 50 second average of the peak current while the cell was held at -95 mV (n=4) and -60 mV (n=7).

3.4 NaHS hyperpolarizes NTS neurons through modulation of the K_{ATP} Channel

Our results suggest that the opening of a potassium channel plays a role in the NaHS induced hyperpolarization, observations which, in conjunction with work reporting that K_{ATP} channels have a role in NaHS mediated cardiovascular effects in the NTS (Qiao *et al.*, 2011), led us to investigate the involvement of the K_{ATP} channel in the NaHS induced hyperpolarization. We compared the NaHS mediated hyperpolarization in NTS neurons in regular aCSF (-7.9 ± 1.2 mV n=8) to that observed in the presence of the K_{ATP} channel antagonist glibenclamide (-1.9 ± 0.9 mV (n=8)), and found that glibenclamide significantly reduced the magnitude of this hyperpolarization (p *< 0.05, paired t-test; Figure 5B). In fact, this hyperpolarization was completely blocked in 6 of 8 cells, and in these cases a prolonged depolarization was observed (Figure 5A). Neurons in which the hyperpolarization was blocked and replaced by a depolarization were assigned a value of 0 mV for the size of the hyperpolarization. Furthermore, cells which only depolarized in response to NaHS were not included in this analysis. These observations suggest that NaHS induced hyperpolarization occurs as a result of the modulation of K_{ATP} channels.

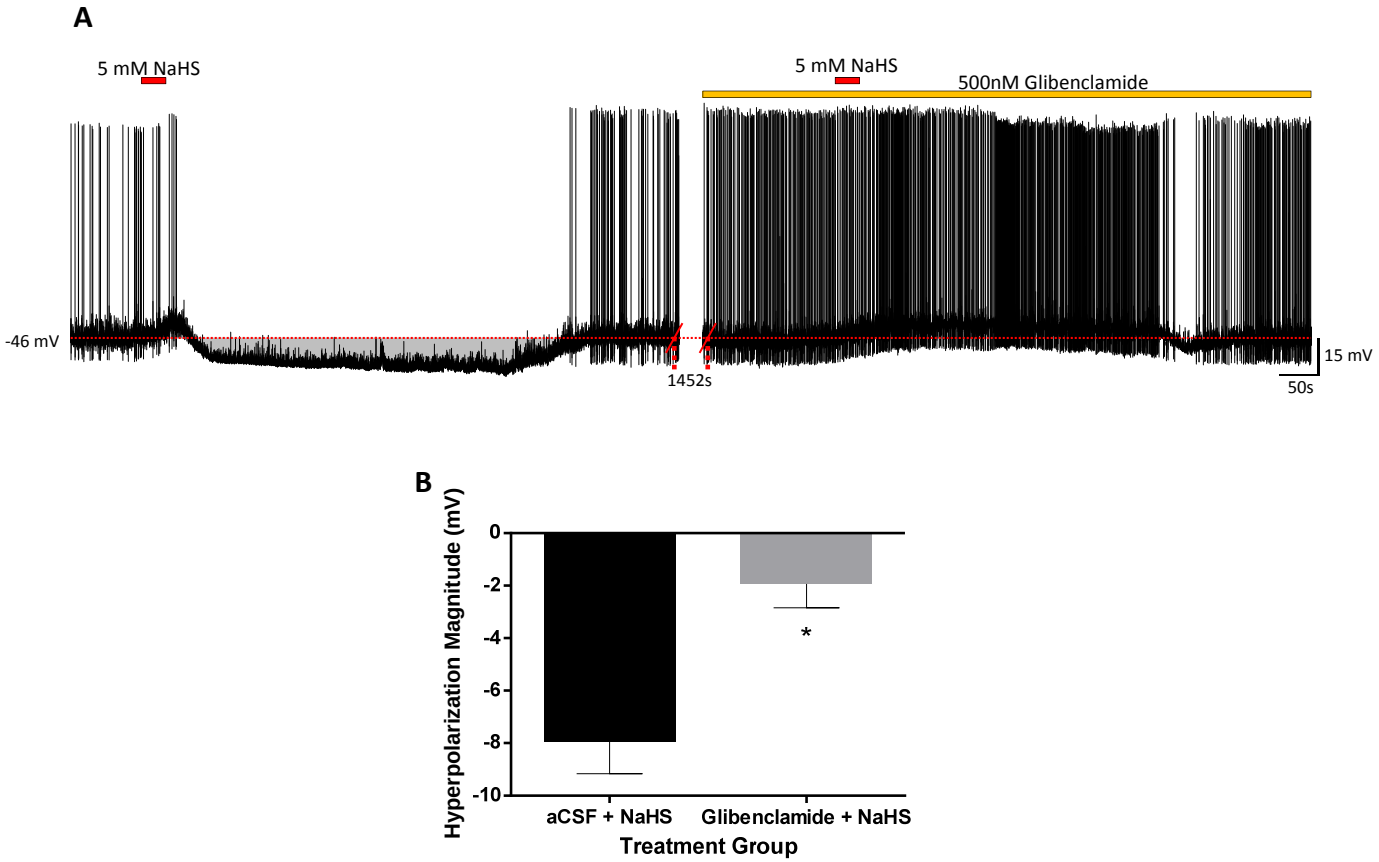

Figure 5: The NaHS induced hyperpolarization is blocked by the K_{ATP} blocker glibenclamide

A. A current-clamp trace of a neuron responding to NaHS before and during glibenclamide application. **B.** A bar graph depicting the magnitude of the hyperpolarization response to NaHS in the different treatment groups (n=8). Error bars indicate SEM, $p^* < 0.05$, paired t-test.

Chapter 4: Discussion

4.1 NaHS concentration

The available literature estimating endogenous levels of hydrogen sulfide in the brain describes these concentrations to be from the 10nM to 160μM (Warenycia *et al.*, 1989; Furne *et al.*, 2008; Ishigami *et al.*, 2009). In the current study we chose to administer H_2S using NaHS as a rapid donor, the delivery of which results predominantly in the formation of HS^- and H_2S (Levitt *et al.*, 2011), with studies suggesting that this donor produces between 6 - 33% H_2S in solution in a closed system (Dombkowski *et al.*, 2004; Levitt *et al.*, 2011); Wallace – personal communication). Thus, the amount of H_2S in the system is likely only a fraction of the NaHS concentration. A second important consideration in the administration of H_2S relates to the volatility of this gas in solution, especially in open oxygenated systems such as the one used in our experiments. DeLeon et al. (2012) measured the half-life of H_2S in such a system to be approximately 5 minutes which corresponds to the time it usually takes our NaHS solution to reach the recording chamber through our gravity perfusion system. Thus, in our experiments we would predict an initial concentration of 1mM NaHS would result in 60-330μM H_2S in solution, which would be reduced by a further 50% to 30-165μM due to the 5 minute time period it takes to reach the recording chamber. We would estimate such concentrations to be in the high physiological to low pharmacological range, but well in line with the high micromolar to low millimolar concentrations which have been shown to influence ion channels in cell expression systems (Streng *et al.*, 2008; Telezhkin *et al.*, 2009; Andersson *et al.*, 2012; Sekiguchi *et al.*, 2014).

4.2 Effects of NaHS in the NTS

While previous studies on the effects of NaHS in the NTS have described NaHS as having effects on the amplitude of solitary tract stimulated excitatory post synaptic currents and

on the frequency of miniature post synaptic currents (Austgen *et al.*, 2011), this is the first study to demonstrate that NaHS does in fact have post-synaptic effects on the membrane potential of NTS neurons. 95% of NTS neurons tested in this study showed a rapid reversible depolarization in response to administration of NaHS. An effect which was followed in 64% of responding neurons by a longer latency and duration, reversible hyperpolarization. Both of these effects were shown to be concentration dependent. Importantly, these effects were still observed when neurons were placed in synaptic isolation by blocking voltage-gated sodium channels and glutamate receptors (using TTX and KA), although the depolarizations were reduced in magnitude. These observations demonstrate for the first time clear post-synaptic effects of NaHS on the membrane potential of NTS neurons.

While in our studies all effected NTS neurons responded to NaHS with depolarizations, it was also interesting that in 64% of cases this initial depolarization was followed by a longer latency and duration hyperpolarization. We do not know the reason for this difference in responsiveness, but speculate that these different groups of neurons may be anatomically distinct in terms of their connectivity (Kalia & Mesulam, 1980), chemically distinct utilising different neurotransmitters (Grill *et al.*, 2002; Kishi *et al.*, 2003; Baskin *et al.*, 2010), or functionally distinct in that serve different physiological functions (Altschuler *et al.*, 1989). We also speculate that all NTS neurons will likely express the ion channels through which NaHS causes depolarization, while the cells showing the later hyperpolarization will likely show a differential expression of ion channels underlying this response.

4.3 Ion channels affected by NaHS in the NTS

Our voltage clamp recordings examining the reversal potential for currents induced in NTS neurons by NaHS provided evidence that the outward current induced (corresponding to the

hyperpolarization) reversed at approximately -84 mV. In contrast inward currents did not reverse within the range of holding potentials we tested, although extrapolation of these data suggested the reversal of the inward current at far more positive values of +35 mV. Although this suggests potential roles for calcium or even sodium channels such an interpretation is at best speculative, and additional experiments will obviously be necessary in order to isolate the ion channels responsible for these depolarizing effects.

However, the outward currents underlying the hyperpolarization showed a reversal potential very close to the calculated reversal potential of potassium for the solutions we used in these experiments (-95 mV). This in conjunction with the discovery of an increase in the conductance of the membrane during the outward current compared to baseline led to the conclusion that the opening of a potassium channel is likely the cause of the hyperpolarization. Functional roles for a number of different potassium channels have been identified in the NTS including both SK channel (Butcher *et al.*, 1999), and the BK channel, both of which have been suggested to be modulated by H_2S (Kombian *et al.*, 1993; Telezhkin *et al.*, 2009; Sitdikova *et al.*, 2010). However, we decided to investigate the K_{ATP} channel because of a previous *in-vivo* study implicating this channel in the NaHS induced cardiovascular effects of H_2S in the NTS (Qiao *et al.*, 2011). Our data showing that K_{ATP} channel blocker, glibenclamide, profoundly reduced the hyperpolarization in response to NaHS in NTS neurons and completely abolished it in 6/8 neurons tested suggests the K_{ATP} channel to be the primary conductance through which NaHS elicits these hyperpolarizing effects.

4.4 Physiological significance of H_2S signaling in the NTS

Although the exact role of endogenous H_2S in the NTS has yet to be discovered, we know from previous studies and the work presented here that exogenous H_2S does indeed have

effects in this nucleus (Austgen *et al.*, 2011; Qiao *et al.*, 2011). There has yet to be a reliable probe created for H_2S that can directly detect its release or production, and subsequently be used to discern its role within various areas of the body. Currently, only indirect markers of H_2S production, such as the presence of enzymes that produce this gasotransmitter, can be used to help uncover its local actions. A reliable method to detect H_2S may help to better understand its role within the NTS.

The effects of H_2S on the membrane potential of neurons in the NTS may ultimately result in the modulation of several physiological systems. With regard to the cardiovascular system, previous research has demonstrated that application of NaHS in the NTS leads to decreases in heart rate and blood pressure (Qiao *et al.*, 2011), effects that are minimized after blocking glutamate receptors in the nucleus. Considering almost all neurons tested in this study responded with a depolarization to NaHS, and other studies have found that NaHS facilitates glutamate transmission in the NTS (Austgen *et al.*, 2011), these cardiovascular effects are not surprising, as it is well known that stimulation of the NTS with the excitatory neurotransmitter glutamate leads to decreases in blood pressure and heart rate (Talman *et al.*, 1980; Yin *et al.*, 1994). Additionally, the cardiovascular effects of NaHS in the NTS could be mitigated by blocking K_{ATP} channels (Qiao *et al.*, 2011). As our current findings have implicated K_{ATP} channel involvement in NaHS-induced hyperpolarization of the membrane potential of a subset of NTS neurons, this hyperpolarizing effect may provide a second mechanism by which NaHS can ultimately modulate the cardiovascular system. Furthermore, as the cardiovascular system can quickly respond to dynamic fluctuations in blood pressure and heart rate, the immediate or near immediate effects of NaHS on the membrane potential of NTS neurons make H_2S well suited for such rapid modulation of heart rate and blood pressure.

The NTS also has a role in the gastrointestinal system, specifically related to satiety (Emond *et al.*, 2001; Berthoud *et al.*, 2001). While there have been no specific studies looking at the effects of H_2S in the NTS in relation to the gastrointestinal system, H_2S can potentially have effects on satiety through actions on neurons in this nucleus. Since 95% of neurons in the NTS depolarize to H_2S, the existence of H_2S-mediated gastrointestinal effects through NTS modulation of gastrointestinal neurons appears feasible. However, it is difficult to predict what this effect might be without the appropriate experimentation.

4.5 Summary and conclusion

Following the publication of what is largely considered the first paper implicating H_2S as an endogenous gasotransmitter (Abe & Kimura, 1996), there has been a significant increase in the literature on H_2S. Its effects on inflammatory modulators (Hu *et al.*, 2007; Lee *et al.*, 2009), reactive oxygen species (Jiang *et al.*, 2013), and transmitters such as glutamate (Austgen *et al.*, 2011) have been well described. Furthermore, it has been shown to affect a large range of ion channels such as potassium (Kombian *et al.*, 1993; Dawe *et al.*, 2008; Telezhkin *et al.*, 2009), sodium (Strege *et al.*, 2011), calcium (Nagai *et al.*, 2004; Yong *et al.*, 2010), and chloride channels (Kimura *et al.*, 2006). More recently, the potential therapeutic effects of H_2S have been explored by testing slow releasing H_2S compounds to treat both CNS and gastrointestinal injuries (Wallace *et al.*, 2010; Marutani *et al.*, 2012). The work presented here further elucidates the role of H_2S in the NTS by revealing the postsynaptic mechanism of membrane potential modulation by this gasotransmitter. Future studies will likely reveal further actions of H_2S in the NTS in relation to the autonomic functions of this nucleus, leading to a more complete picture of the functions of this gasotransmitter in terms of cardiovascular, gastrointestinal, and respiratory functions.

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