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Modulation of Hypoglossal Motoneurons by Nitric Oxide

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Modulation of Hypoglossal Motoneurons by Nitric Oxide

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ABSTRACT

Obstructive sleep apnea (OSA)- the occurrence of repetitive episodes of airway obstruction during sleep- is considered a major health problem affecting up to 9% of adults in the United States (Parish & Somers, 2004). The hypoglossal motor nucleus (HMN) controls genioglossus muscle tone and is critically important for maintaining airway patency; loss of excitatory input to the HMN during sleep results in disfacilitation of hypoglossal motoneurons, increased airway resistance and contributes to the development of OSA (Horner R. L., 2007). However, a fundamental question of sleep medicine that remains unresolved is what mechanisms help maintain airway patency during sleep? A potential source of sleep-activated compensatory drive is nitric oxide released from cholinergic terminals in the HMN (Pose et al. 2005; Vincent & Kimura, 1992). Here we show that NO functions as an excitatory transmitter in the HMN by a cGMP-dependent inhibition of a background TASK-like conductance and an S-nitrosylation-dependent activation of the instantaneous but not the time-dependent component of the hyperpolarization-activated current (Ih) generated by hyperpolarization-activated cyclic nucleotide gated (HCN) channels. These results suggest that sleep-induced nitrergic innervation of the HMN helps compensate for respiratory motoneuron disfacilitation and disruption of NO/cGMP signaling may contribute to the etiology of OSA. Although a causal link between disruption of NO/cGMP signaling and occurrence of OSA has yet to be established, it is well known that patients with metabolic syndrome have high levels of uric acid- a potent NO scavenger- and, perhaps consequently, are at much higher risk of developing OSA (Mota, 2010).

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE **PAGE**

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INTRODUCTION

Obstructive sleep apnea (OSA) is a problem that is on the rise year after year. Individuals with this health problem have repeated bouts of asphyxia because of upper airway narrowing and closure. Around 9% of the population suffers from OSA with a great deal of cases going undiagnosed (Grunstein et al. 2001). It is often cited as a comorbidity with stroke, cardiovascular disease and daytime somlence (Smith & Quinnell, 2004). The cause of OSA is not known but is thought to result in part from fundamental changes in upper airway motoneuron function during sleep. The awake-sleep state dependent control of airway motoneurons is poorly understood and thus pharmacological strategies designed to treat sleep apnea have been largely unsuccessful (Grunstein et al. 2001; Smith & Quinnell, 2004; Veasey et al. 2006). As part of my thesis work I identity a novel mechanism by which nitric oxide (NO), released during sleep, modulates the activity of respiratory motoneurons and in doing so offers new potential therapeutic targets for the treatment of OSA.

The hypoglossal motor nucleus (HMN) controls genioglossus muscle (tongue) tone and is critically important for maintaining upper airway patency; loss of this tone increases airway resistance and therefore contributes to the development of OSA (Horner, 2007). During wakefulness the HMN receives tonic serotonergic (5HT) and noradrenergic (NE) drive. It is well established that these neurotransmitters increase excitability of hypoglossal motoneurons, in part, by activation of G-protein coupled receptors (GPCRs) linked to the Gαq subunit inhibition of the TWIK-related acid-sensitive K^+ (TASK) member of the KNCK family of background K^+ channels (Abudara et al. 2002; Ahern et al. 2002). Activation of hyperpolarization activated cyclic nucleotide

gated channels (HCN) is mediated by NE stimulating adenylate cyclase (AC) which increases cAMP causing a shift in activation to more depolarized potentials (Walh-Schott & Biel, 2009). It has also been shown that AC stimulation can increase cAMP which can then activate protein kinase A (PKA). PKA may phosphorylate HCN channels directly to increase their activity in the SA node of the heart (Larsson, 2010).

In the brain, the majority of this excitatory input is lost during sleep resulting in disinhibition of TASK channels and loss of motoneuron activity, a process referred to as disfacilitation (Horner, 2000; Horner, 2006). The normal process of disfacilitation results in a narrowing of the airway, but it is not the only variable that leads to OSA. The focus of my thesis work is to explore the potential neuronal mechanisms that help keep the upper airway in a state of patency during sleep, thus rendering OSA more likely to be treated with novel pharmacological agents.

It has been shown that components of the sleep-activated cholinergic system project to the HMN and express high levels of neuronal nitric oxide synthase (nNOS) in terminals near hypoglossal neurons (Montero et al. 2008). Neuronal NOS will generate and release NO in an activity dependent manner (Williams, Vincent, & Reiner, 1997). However, levels of NO in the HMN have not been measured and a physiological role of NO in control of respiratory motoneurons has not fully understood. It is well known that nitric oxide (NO) can modulate neuronal activity by either activation of soluble guanylate cyclase (sGC) with the formation of cGMP or by a cGMP-independent pathway conferred by S-nitrosylation (Abudara et al. 2002; Ahern et al. 2002). I propose that NO compensates for loss of excitatory drive during sleep by activating hypoglossal motoneurons. I will use slice-patch electrophysiological techniques to determine if NO

functions as an excitatory transmitter in the HMN and to identify the signaling pathway (i.e., cGMP-dependent and/or –independent mechanisms) and downstream ion channel targets mediating NO-sensitivity.

BACKGROUND

The upper airway extends from the nasopharynx to the epiglottis and is supported primarily by the activity of pharyngeal skeletal muscles. The genioglossus muscle (GG) of the tongue is the largest upper airway dilator muscle; contraction of which results in anterior movement of the tongue and widening of the oropharyngeal airway (Kubin et al. 1998). Hypoglossal motoneurons innervate the GG and so their activity determines the position, shape and tone of the tongue. As such, the hypoglossal motor nucleus (HMN) is the principle focus for basic research aimed at understanding control of upper airway function.

The HMN receives inspiratory drive from pre-motor neurons in brainstem respiratory centers in order to counteract negative airway pressure generated during inspiration. This respiratory drive is mediated by glutamate acting on postsynaptic alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (Bocchiaro et al. 2003; Funk et al. 1997; Funk et al. 1993) and is considered relatively stable across sleep-wake cycles (Horner, 2007). In the absence of inspiratory drive (i.e., between breathes), upper airway diameter is determined primarily by tonic excitatory drive mediated by 5HT, thyrotropin-releasing hormone (TRH), substance P (SP) and NE (Kubin et al. 1998). Hypothalamic neurons also contribute to vigilance by releasing orexin throughout the neuroaxis, including the HMN (Sakurai, 2007). The effects of these neurotransmitters are well characterized, and collectively been shown to increase

hypoglossal activity through activation of Gαq subunit of G-protein coupled receptors inhibition of TASK channels (Bayliss et al.1994; Chen, et al., 2006; Fenik & Veasey, 2003; Mathie, 2007).

Tonic excitatory drive provided to the HMN is state dependent; raphe neurons (which release 5HT, TRH, SP), noradrenergic subcoeruleus and orexin-producing hypothalamic neurons are most active during wakefulness and minimally active during REM sleep (Aston-Jones & Bloom, 1981; Chan et al. 2006; Horner, 1996; Jacobs & Azmitia, 1992; Sakurai, 2007). Therefore, the onset of sleep initiates fundamental changes in the control of pharyngeal motoneurons that result in a loss of HMN activity, a decrease in responsiveness to glutamatergic inspiratory drive (Bellingham & Funk, 2000) and suppression of pharyngeal reflexes (Fogel et al. 2003). These changes in motoneuron activity during sleep correspond with an increase in airway resistance, development of hypoventilation and an increase in arterial P_{CO2} normally observed in sleeping humans (Horner, 2007; Kubin et al. 1998). In individuals with an already anatomically narrow upper airway, such as associated with obesity (Arens et al. 2003), the loss of pharyngeal muscle tone during sleep can cause airway obstruction and OSA (Horner, 1996; Horner, 2000; Horner, 2007; Jordan & White, 2008; Kubin et al. 1998).

If OSA is a consequence of an otherwise normal process of motoneuron disfacilitation then the condition should be amiable to pharmacological treatment. However, pharmacological strategies to compensate for disfacilitation by increasing tonic drive using 5HT or NE reuptake inhibitors, receptor specific agonists, or agents that increase production or slow transmitter breakdown, have been largely unsuccessful (Smith & Quinnell, 2004). These observations suggest that factors in addition to sleep-

induced loss of excitatory input to pharyngeal motoneurons contribute to the development of OSA. I hypothesize that the onset of sleep activates a compensatory mechanism that normally helps maintain airway patency by activating respiratory motoneurons through activation of HCN channels and inhibition of TASK channels. I propose that disruption of this mechanism will contribute to the development of OSA.

The collective loss of a wakeful stimulus (i.e., disfacilitation) to respiratory motor nuclei including the HMN is an enduring concept in respiratory medicine and is widely believed to be the primary mechanism of OSA (Horner, 1996; Horner, 2000; Horner, 2007; Jordan & White, 2008; Kubin et al. 1998). Numerous pharmacological treatments have been proposed for the amelioration of sleep apnea symptoms, such as tricyclic antidepressants, SSRIs, sex steroids, theophylline and hypertensive agents, yet none have proven successful (Grunstein et al. 2001). One treatment viewed as the "gold standard" is continuous positive airway pressure (CPAP). CPAP was addressed as a treatment for sleep apnea in 1981 (Smith & Quinnell, 2004). The apparatus contains a mask that contours to the nose and occasionally mouth of the individual. The mask is connected to a pump that continually generates positive pressure allowing the airway to remain in a state of patency or openness (Smith & Quinnell, 2004). The positive pressure effectively acts as a splint to ensure less resistance to airflow. However, compliance with the system is often low often due to patient discomfort. In light of the complications involved with using a mechanical apparatus to resolve the problems associated with sleep apnea, novel pharmacological approaches seem preferable. This manuscript proposes nitric oxide as potentially one of those treatments.

Nitric oxide (NO) is a potent neuromodulator involved in numerous physiological processes, such as sleep (Cavas & Navarro, 2006), memory (Esplugues, 2002) and respiratory control (Pierrefiche et al. 2007). The amino acid precursor L-arginine is needed to generate nitric oxide. Calcium dependent enzymes know as nitric oxide synthases (NOS) converts L-arginine into L-citrulline and nitric oxide (Gautier-Sauvigne et al. 2005). Three main isoforms of NOS are found within the body. Endothelial NOS (eNOS) is important in the relaxation of smooth muscle (Foster et al. 2003). Inducible NOS (iNOS) is expressed primarily by the immune system during times of inflammation and is the isoform that can function in a calcium independent manner (Calabrese et al. 2007). The focus of the current study in the brainstem pertains to isoform neuronal nitric oxide synthase (nNOS) located in cholinergic terminals in the HMN.

Unlike most neurotransmitters, NO is not stored in synaptic vesicles and released by exocytosis, rather, nNOS generates NO in a Ca^{2+}/calmodulin dependent manner and can readily diffuse and serve a paracrine function with neighboring cells. Therefore, synthesis of NO is key to regulating its activity. The main target of NO is the cytosolic enzyme soluble guanylyl cyclase (sGC). The sGC family has a multitude of isoforms, but NO predominately interacts with the α1β1 family (Murad, 2006). Upon NO binding, activation of sGC catalyzes the formation of cGMP from GTP (Ahern et al. 2002). Cyclic GMP (cGMP) initiates activation of three major types of downstream effectors. It could activate serine/threonine protein kinase G (PKG) which has been shown to contribute to the downstream effects of cGMP via phosphorylation (Ahern et al. 2002). cGMP also has an effect on cyclic nucleotide gated channels, including hyperpolarization-activated cyclic nucleotide-gated channels (HCN channels) that produce a hyperpolarization-

activated cationic current (Ih) (Esplugues, 2002). Finally, cGMP levels effect phosphodiesterases (PDE) that serve to limit the level and type of nucleotides present in the cytosol. In addition, NO has also been shown to affect neuronal activity by a sGC-independent mechanism conferred by direct protein S-nitrosylation (Ahern et al. 2002). S-nitrosylation is the process whereby cysteine thiols are modified via a reduction-oxidation (REDOX) reaction by nitric oxide (Foster et al. 2003). My results will show that both cGMP-dependent and –independent signaling contribute to NO-modulation of hypoglossal motoneurons.

The HMN is known to express sGC and evidence suggests that under normal conditions the HMN receives NO innervation from the medullary reticular formation and pontine cholinergic neurons of the laterodorsal and pedunculopontine tegmental nuclei (LDT/PPT) (Gautier- Sauvigne et al. 2005; Pose et al. 2005). Activity of neurons in the LDT/PPT and medullary reticular formation are controlled by inhibitory influence of 5HT and NE producing neurons. During sleep, the progressive loss of 5HT and NE drive to the LDT/PPT results in withdrawal of inhibition. Subsequent activation of LDT/PPT neurons increases acetylcholine release into the pontine and medullary reticular formation to trigger REM sleep. In addition to these cholinergic effects on REM sleep, activation of the LDT/PPT and reticular formation has been shown to cause the activity-dependent release of NO, e.g., nitrergic LDT projections to the thalamus release NO when stimulated or during REM sleep. NADPH-d studies further support this finding with varicosities localized near motoneurons in the hypoglossal nucleus (Montero et al. 2008). However, levels of NO in the HMN have not been measured and a physiological

role of NO in state-dependent control of respiratory motoneurons has not been established.

The exact molecular substrate conferring NO-sensitivity to hypoglossal motoneurons is not known at this time. There are several NO-sensitive voltage-dependent K^+ channels including K_{ATP} (Lei et al. 2004), Ca^{2+} activated (Lei et al. 2004), (Dong, et al., 2007), delayed rectifier (Han et al. 2006) and transient K^+ channels (Han et al. 2006). Cyclic nucleotide gated channels (CNG channels) may also represent common target of both arousal- and sleep-active signals.

The hypoglossal motoneurons expresses a NO modulated family of channels, known as HCN channels. These channels produce a hyperpolarization-activated current (Ih) that consists of: a fast instantaneous current (Iinst) and a slowly developing steady state current (Iss). This is a mixed cationic conductance with a reversal potential around -35 mV (Walh-Schott & Biel, 2009). Hypoglossal neurons express HCN1 and HCN2 channel subunits (Chen et al. 2005) and a prominent Ih current that can be activated by transmitters associated with wakefulness (e.g., 5HT, NE, TRH) (Walh-Schott & Biel, 2009). The Iss component of Ih can be activated directly by cAMP and cGMP in a protein kinase independent manner (Walh-Schott & Biel, 2009). Cyclic nucleotides bind directly to the cyclic nucleotide binding domain (CNBD) on the C-linker domain with a resulting shift in the activation curve and opening kinetics (Seifert et al. 1999). Binding of cyclic nucleotides differ between cAMP which is in the *anti* conformation and cGMP which is in the *syn* conformation of the C-terminus (Craven & Zagotta, 2006). In cardiac pacemaker cells which also express high levels of HCN2, NO has been shown to increase heart rate by increasing Ih (Musialek et al. 1997). Importantly, drugs and both

cAMP and cGMP increase Iss. There is little evidence to date that Iinst can be differentially regulated as a neuromodulatory mechanism.

It has been shown that the instantanous component can be increased without changing the overall voltage dependence with application of the drug Tanshinone IIA (Liang et al. 2009). This occurred due to a slowing of the activation and deactivation kinetics of the channel and increasing the minimum open probability (Liang et al. 2009). There was a more pronounced effect on the HCN channel with slower open kinetics, such as HCN2 channels, which are highly expressed in the hypoglossal motoneuron population (Liang et al. 2009). However, the exact mechanism of the phenomenon has not yet been elucidated. In addition, coexpression of HCN2 with the accessory protein MiRP1 (KCNE), a β subunit that is involved in numerous voltage-gated potassium channels, was shown to increase the Iinst while also decreasing max Ih (Proenza et al. 2002). These work support the concept that Ih and Iinst may be differentially modulated. As part of my thesis, I will present the first evidence that Iinst can be differentially regulated as a neuromodulatory mechanism involving S-nitrosylation.

Another potential method of NO modulation of channels is a post-translational modification though which NO can modulate proteins in a covelent manner directly, known as S-nitrosylation (figure 1). S-nitrosylation is a REDOX reaction involving oxidation of the thiol group on cysteine residues (Broillet, 1999). NO can mediate this reaction directly or via a nitrosium ion intermediate (Broillet, 1999). In either case, formation of the S-nitrosothiol (R-SNO) can confer conformational changes to the protein and thus change protein activity in a manner similar to other post-translational modifications including phosphorylation (Figure 1) (Gaston et al. 2003). S-nitrosylation

Figure 1. NO formation and modes of action. A, Formation of the nitrosonium ion (NO^+) can bind to the thiol group (S-H) located on cysteine residues forming an S-nitrosothiol. S-nitrosothiols can modulate channel activity. **B**, NO can bind to superoxide ($O2^-$) forming reactive nitrogen species (RNS) like peroxynitrite. RNS can cause S-nitrosylation of a multitude of targets. **C**, In aerobic conditions, NO can form nitrites and nitrogen dioxide. **D**, NO can bind to the enzyme soluble guanylyl cyclase (sGC) producing cGMP. This second messenger cGMP can activates channels, kinases and phosphodiesterases. **E**, NO can bind to transition metals.

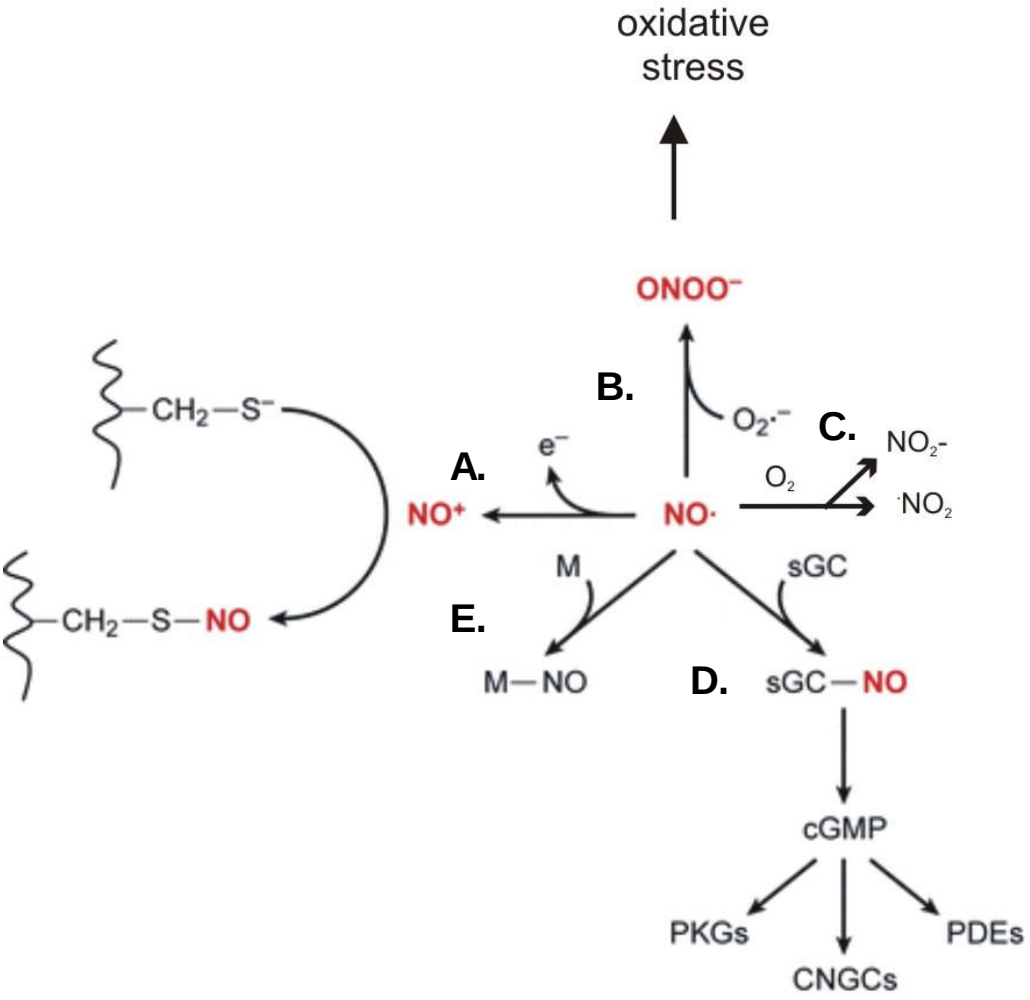
oxidative
stress
ONOO⁻
B.
O2·⁻
C.
NO2-
·NO2
O2
A.
e⁻
NO⁺
NO·
M
sGC
E.
M—NO
D.
sGC—NO
cGMP
PKGs
CNGCs
PDEs
CH2—S⁻
CH2—S—NO

Figure 1

can has been shown to increase and decrease the activity of other channels directly, such as, ryanodine receptors, NMDA receptors, cyclic-nucleotide-gated channels, Na^+ channels, Ca^{2+}-activated K^+ (BK) channels, among others (Ahern, Klyachko, & Jackson, 2002).

S-nitrosylation has also been shown to activate a family of channels structurally related to HCN, know as cyclic nucleotide gated channels (Broillet & Firestein, 1996). These channels share their origin from a family of voltage gated K^+ channels with a tetrameric arrangement of proteins around a central pore (Walh-Schott & Biel, 2009). The transmembrane domain known as S4 contains the conserved voltage sensor which is more sensitive in HCN channels (Walh-Schott & Biel, 2009). The intracellular C-terminus contains the CNBD connected to the S6 linker domain in both channels (Craven & Zagotta, 2006). This S6 linker domain is important because CNGs have been shown to be directly modulated by S-nitrosylation (Broillet & Firestein, 1996). In addition to the direct activation of CNG by S-nitrosylation, HCN has been shown to be a nitrosylation target via a biotinylation assay (Jaffrey et al. 2001). However, there is currently no evidence that S-nitrosylation has any direct effect on HCN channels.

CNG and HCN channels are closely related but serve different physiological functions. CNG channels are located primarily in senory regions, such as photoreceptors and neurons used in olfaction. These channels are voltage independent and rely on direct binding of cyclic nucleotides (Broillet & Firestein, 1996). In contrast, HCN channels are important for the firing rates of neurons, as well as pacemaker properties of the sinoatrial node in the heart. Their modulation is by cyclic nucleotides

such as cAMP, cGMP and also hyperpolarizing voltage changes (Craven & Zagotta, 2006).

Another potential target of NO, are the KNCK family of two-pore or tandem pore domain (K2P) background K^+ channels which are necessary to establish a negative membrane potential (Kim, 2005; Patel & Honore, 2001). Inhibition of these channels promotes membrane depolarization, whereas their activation induces hyperpolarization. Of the KNCK family of background K^+ channels expressed in the brain, the only two that appear to be NO sensitive are TASK (Gonzalez-Forero et al. 2007) and TREK-1 (Dallas et al. 2008). There is also evidence that TALK-1 and TALK-2 are activated by NO (Duprat et al. 2005) but these background K^+ channels are not expressed in the brain and so are excluded as candidates for the NO-sensitive current in hypoglossal neurons. It is well documented that hypoglossal neurons express high levels of TASK-1 and TASK-3 channels, and that neurotransmitters released during arousal increase activity of hypoglossal neurons by activation of Gαq linked receptors and inhibition of TASK-like K+ currents (Chen et al. 2006). The TASK family of channels are characterized as "leakage" channels based on their voltage and time independence. Therefore, we consider TASK-1 and/or TASK-3 channels the most likely molecular substrate for determining NO-sensitive of the K^+ component in hypoglossal neurons.

As mentioned previously, TASK channels are shown to be vitally important in activities like establishing resting membrane potential and altering cell excitability (Talley et al. 2000). There is some controversy on how TASK channels are modulated during wakefulness. Some studies implicate the involvement of PLC, as well as its products generated during hydrolysis, such as Phosphatidylinositol 4,5-bisphosphate

(PIP_2) depletion or diacylglycerol (DAG) stimulation of Protein Kinase C (PKC) (Mathie, 2007). A recent study provides evidence of a direct modulation of TASK channels by the Gαq subunit of the activated GPCR (Chen et al., 2006). Experiments conducted in this paper provide compelling evidence that PIP_2 depletion can occur with no change in TASK conductance. Direct modulation was also shown via coimmunoprecipitation of the Gαq subunit of the GPCR and the TASK1-TASK3 heterodimer (Berg et al. 2004).

The physiological consequence of nitrergic drive to the HMN is not known. A recent *in vivo* study found that inhibition of endogenous NOS or sGC in the HMN decreased activity of hypoglossal neurons, whereas application of NO donors or a cell permeable and non-hydrolysable cGMP analog (8-Br-cGMP) increased activity of hypoglossal neurons (Gonzalez-Forero et al. 2007). In the brain slice, whole-cell recordings from guinea pig trigeminal neurons show that NO donors and cell permeable analog of cGMP both increase excitability with no change in input resistance, possibly by activating HCN channels and increased Ih, in addition to inhibiting the TASK component (Abudara et al. 2002). Similar results were observed in rat hypoglossal motoneurons where acute exposure to NO donors also increased excitability with no discernable change in input resistance (Gonzalez-Forero et al. 2007). These results indicate that NO can activate pharyngeal motoneurons, however the mechanism by which acute NO affects motoneuron excitability is undefined. Based on these results it was postulated that NO has a pathological effect on hypoglossal neurons by contributing to excitotoxicity and neurodegeneration (Gonzalez-Forero et al. 2007). For example, if produced in excess, NO damages normal cells and contributes to the pathogenesis of motoneuron degeneration (Calabrese et al. 2007; Strijbos et al. 1996).

However, clearly sub-toxic levels of NO can mediate numerous physiological processes (Cavas & Navarro, 2006; Esplugues, 2002; Pierrefiche et al. 2007). Therefore, we hypothesize that acute exposure to NO at physiological concentrations is not toxic; but rather confers a physiological signal to respiratory motoneurons during sleep to regulate activity in an adaptive, physiological capacity.

MATERIALS AND METHODS

In vivo preparation.

All of the *in vivo* experiments were done by Dr. Richard Horner at the University of Toronto. The surgical procedures and experimental protocols were similar to those previously described (Steenland et al. 2008); (Sirois et al. 2002) and conform to recommendations of the Canadian Council on Animal Care. Briefly, isoflurane (2.2 – 3.1%) anaesthetized adult rats (N=9) were instrumented with a microdialysis probe (CMA/11 14/01, CSC, St. Laurent, QC, CA) and electrodes to measure muscle (genioglossus and diaphragm) electromyogram (EMG) activity and cortical electroencephalogram (EEG) and genioglossus activity. The microdialysis probe will be placed into the HMN at the following coordinates: 13.7 ± 0.04 mm posterior to bregma, 0.1 ± 0.02 mm lateral to the midline and 10.1 ± 0.03 mm ventral to bregma. A brief burst of tongue activity was used to determine correct probe placement in the HMN. At the end of each experiment, we confirmed probe placement in the HMN using histology and by evoking a characteristic response to serotonin. To record cortical EEG, we implanted wire electrodes onto the skull of the frontal-parietal cortex (Liu, Sood, Liu, & Horner, 2005). To record genioglossus activity, we positioned two wire electrodes bilaterally under the body of the tongue (Horner R. L., 2007). All electrical signals including raw EEG and genioglossus activity will be amplified, filtered, digitized and recorded on a computer as previously described. For experiments in conscious animals, rats will be allowed ~1 week recovery from surgery.

Microdialysis.

During an experiment, the probe was continually perfused (2.0 ml min^{-1}) with artificial cerebrospinal fluid (ACSF) of the following composition (mM): 125 NaCl, 3 KCl, 1 KH_2PO_4, 2 $CaCl_2$, 1 $MgSO_4$, 25 $NaHCO_3$, and 30 glucose; bubbled with 95% O_2-5% CO_2 to pH 7.42 ± 0.003. Each animal was perfused for 1-2 hours with ACSF to establish a stable baseline prior to testing the effects of the NO donor (DEA). Once a stable baseline has been established, the perfusion medium was switched to perfusate containing DEA (10 and 100 mM).There was a lag time of ~ 6 min and each drug exposure lasted 15 min; only data from the last min will be used for analyses.

***In vivo* recording and analysis.**

The electrical signals were amplified and filtered (Super-Z head-stage amplifiers and BMA-400 amplifiers/filters, CWE Inc., Ardmore, PA). The EEG was amplified and filtered between 1 and 100 Hz, whereas the EMG signals were amplified and filtered between 100 and 1000 Hz. The GG and diaphragm signals were recorded at the same amplification across all experiments. It was not necessary to alter the gain of the recording apparatus between experiments and baseline EMG activity was similar across rats. The electrocardiogram was removed from the diaphragm signal using an oscilloscope and electronic blanker (Model SB-1, CWE Inc.). In addition, the moving time averages (time constant= 100 ms) of the GG and diaphragm signals were obtained (Coulbourn S76-01, Lehigh Valley, PA, USA). Each signal, along with blood pressure (DT-XX transducer, Ohmeda, Madison, WI, USA and PM-1000 Amplifier, CWE Inc.) were digitized and recorded on computer (Spike 2 software, 1401 interface, CED Ltd., Cambridge, UK).

Breath-by-breath measurements of GG and diaphragm activities were calculated and averaged in consecutive 5-sec time bins (Hajiha et al. 2009). All values were written to a spreadsheet and matched to the corresponding intervention at the HMN to provide a grand mean for each variable, for each intervention, in each rat. The EMG signals were analyzed from the moving time average signals (above electrical zero) and quantified in arbitrary units and expressed as a percentage (%) of the respective ACSF controls. GG activity was quantified as mean tonic activity (i.e. basal activity during expiration), peak activity and respiratory-related activity (i.e. peak inspiratory minus tonic activity). In practice there was no tonic GG activity in these experiments performed under anesthesia, so only respiratory-related GG activity is presented. Mean diaphragm amplitudes (i.e. respiratory-related diaphragm activity), respiratory rate and mean arterial blood pressure were also calculated for each 5-sec period. The EEG was sampled at 500 Hz and subjected to a fast-Fourier transform for each 5-sec time bin, and the power within frequency bands spanning the 0.5 to 30 Hz range was calculated. The ratio of high (β_2, 20-30 Hz) to low (δ_1, 2-4 Hz) frequency activity was calculated and used as a relative marker of EEG activation (Steenland, Liu & Horner, 2008); (Hajiha et al. 2009). Each rat served as its own control. The effects of DEA on each parameter of interest were compared by two-way RM-ANOVA using the Bonferroni corrected P value. Data are presented as means ± S.E.M. unless otherwise indicated.

***In vitro* brain slice preparation.**

Slices containing the HMN were prepared as previously described. All procedures were performed in accordance with National Institutes of Health and University of Connecticut Animal Care and Use Guidelines. Briefly, neonatal rats

(Sprague-Dawley, 7-12 days postnatal) were decapitated under ketamine/xylazine anesthesia and transverse brainstem slices (300 µm) cut using a microslicer (DSK 1500E, Dosaka, Japan) in ice-cold substituted Ringer's solution containing (in mM): 260 sucrose, 3 KCl, 5 $MgCl_2$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, and 1 kynurenic acid. Slices were incubated for ~30 minutes at 37ºC and subsequently at room temperature in normal Ringer's solution (in mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose. Both substituted and normal Ringer's solutions were bubbled with 95% O_2/5% CO_2.

Slice-patch electrophysiology.

Individual slices were transferred to a recording chamber mounted on a fixed-stage microscope (Zeiss Axioskop FS) and perfused continuously (~2 ml min^{-1}) with a bath solution composed of (mM): 140 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 N-2-hydroxyethylpiperazin-N'-2-ethanesulfonic acid (HEPES), 10 glucose; pH was adjusted between 6.9 and 7.5 by addition of HCl or NaOH. Hypoglossal motoneurons were visualized using differential interference optics and identified by their location (lateral and ventrolateral to the central canal) and by their characteristic size and shape (Sirois, Lynch, & Bayliss, 2002). All recordings were made with an Axopatch 200B patch-clamp amplifier, digitized with a Digidata 1322A analog-to-digital converter, and recorded using pCLAMP 10.0 software (Molecular Devices). Recordings were obtained at room temperature with patch electrodes pulled from borosilicate glass capillaries (Warner Instruments Inc.) on a two-stage puller (Sutter Instruments, P89) to a DC resistance of 3–5 MΩ when filled with internal solution containing the following (in mM): 120 KCH_3SO_3, 4 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, 10 EGTA, 3 Mg-ATP, 0.2% biocytin, and 0.3

GTP-Tris, pH 7.2; electrode tips were coated with Sylgard 184. Voltage-clamp recordings were made at a holding potential of -60 mV and in the presence of tetrodotoxin (TTX, 0.1 µM). Exposure to DEA varied between 3-5 minutes durations with concentrations determined by generation of an EC_{50} (figure 2). We follow the time course of the NO induced changes in holding current and conductance with intermittent steps (0.2 Hz) to -100 mV. Current-voltage (*I-V)* relationships were determined for various treatment protocols from 'instantaneous' currents (i.e. immediately following the capacitive transient, before activation of the time-dependent I_h current) obtained by hyperpolarizing the cell in 10 mV steps (to −150 mV). The maximal amplitude of I_h was quantified as the size of the time-dependent current during a 1 second long step to −150 mV. A liquid junction potential of 10 mV was corrected off-line.

Statistical Analysis.

Data were reported as means ± SEM and were analyzed by the paired *t* test or two-way RM-ANOVA followed by Bonferroni's multiple comparisons test when appropriate ($p<0.05$). Data are presented as means ± S.E.M. unless otherwise indicated.

Figure 2. Bath NO measurements. Nitric oxide was measured polarographically (+860 mV). **A,** trace of bath NO and summary data. **B**, show the level of NO produced by DEA. **C,** trace of holding current shows that 1-100 μM DEA reversibly decrease holding current. **D,** average (N=5) DEA dose response curve. Note that our working DEA concentration (20 μM) is near the estimated EC_{50}.

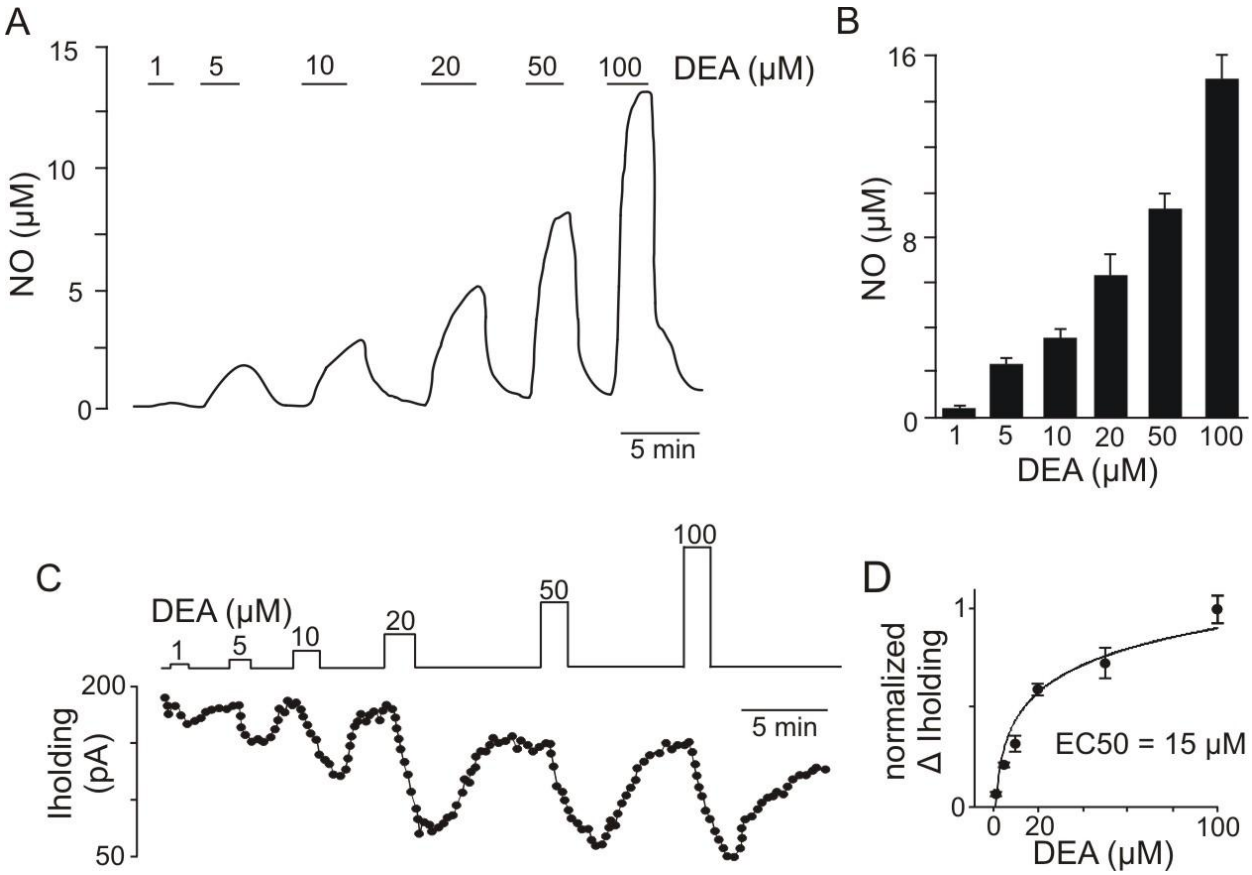
A
15
10
5
0
NO (μM)
1
5
10
20
50
100
DEA (μM)
5 min
B
16
8
0
NO (μM)
1
5
10
20
50
100
DEA (μM)
C
DEA (μM)
1
5
10
20
50
100
200
50
Iholding (pA)
5 min
D
1
0
normalized Δ Iholding
EC50 = 15 μM
0
20
100
DEA (μM)

Figure 2

RESULTS

To test the hypothesis that NO helps maintain airway patency by increasing genioglossus activity, Dr. Horner applied Diethylamine NONOate (DEA/NO, a NO donor) to the HMN of anesthetized animals while recording genioglossus and diaphragm activity, blood pressure and respiratory rate. Under control conditions blood pressure averaged 75.7 ± 3.1 mmHg, respiratory-related GG and diaphragm muscle activities averaged 97.1 ± 17.8 and 689.7 ± 79.2 arbitrary units respectively, and respiratory rate averaged 47.5 ± 2.7 min^{-1}. Microdialysis of DEA (10 mM) into the HMN caused a robust increase in GG activity with no change in amplitude of diaphragm activation or blood pressure (Fig 3A and B), i.e., the effects on GG activity were specific to the interventions at the hypoglossal motor pool and did not significantly affect these other variables. Likewise, there was no effect of DEA on the ratio of high (β_2, 20-30 Hz) to low (δ_1, 2-4 Hz) EEG frequency activity (not shown). However, higher concentrations of DEA (100 mM) were not specific to the HMN as evidenced by the increase in respiratory rate (Fig 3B). In the brain slice preparation, exposure to 20 µM DEA, which corresponds with an NO concentration of 6.2 ± 0.4 µM (Fig S1A and B), depolarized membrane potential (7.1 ± 1 mV; N= 8) (in the presence of 0.1 µM TTX to block action potentials) and increased the firing rate response (in TTX free media) of hypoglossal motoneurons to depolarizing current injections (Fig. 3D-F) but with no parallel change in input resistance. Decomposed DEA that is no longer able to release NO had no effect, indicating that effects of DEA on hypoglossal motoneurons are mediated by NO rather than potential reactive byproducts. These results are consistent with reported effects of NO donors on trigeminal (Abudara et al. 2002) and hypoglossal motoneurons

Figure 3. NO functions as an excitatory transmitter in the HMN *in vivo* and *in vitro*. **A**, traces of blood pressure (BP) and integrated diaphragm and genioglossus (GG) EMG activity show that microdialysis of the NO donor (DEA, 10 mM) into the HMN of an anesthetized adult rat caused a doubling of respiratory-related GG activity with no change in other parameters of interest. **B**, summary of *in vivo* data (N=9) showing effects of 10 and 100 mM DEA on GG EMG, diaphragm EMG, BP, and respiratory rate. Note that a small increase in respiratory rate was observed in response to 100 mM DEA. **C**, slice-patch current-clamp recording of membrane potential shows that exposure to DEA (20 µM) reversibly depolarized membrane potential ~8 mV. **D**, summary of *in vitro* data (N=7) showing DEA-induced depolarization. **E**, steady-state frequency-current relationships under control conditions and in the presence of DEA show that DEA increases the firing rate response to depolarizing current injections > +190 pA. These results show that NO can stimulate hypoglossal motoneurons *in vitro* and increases airway patency *in vivo*.

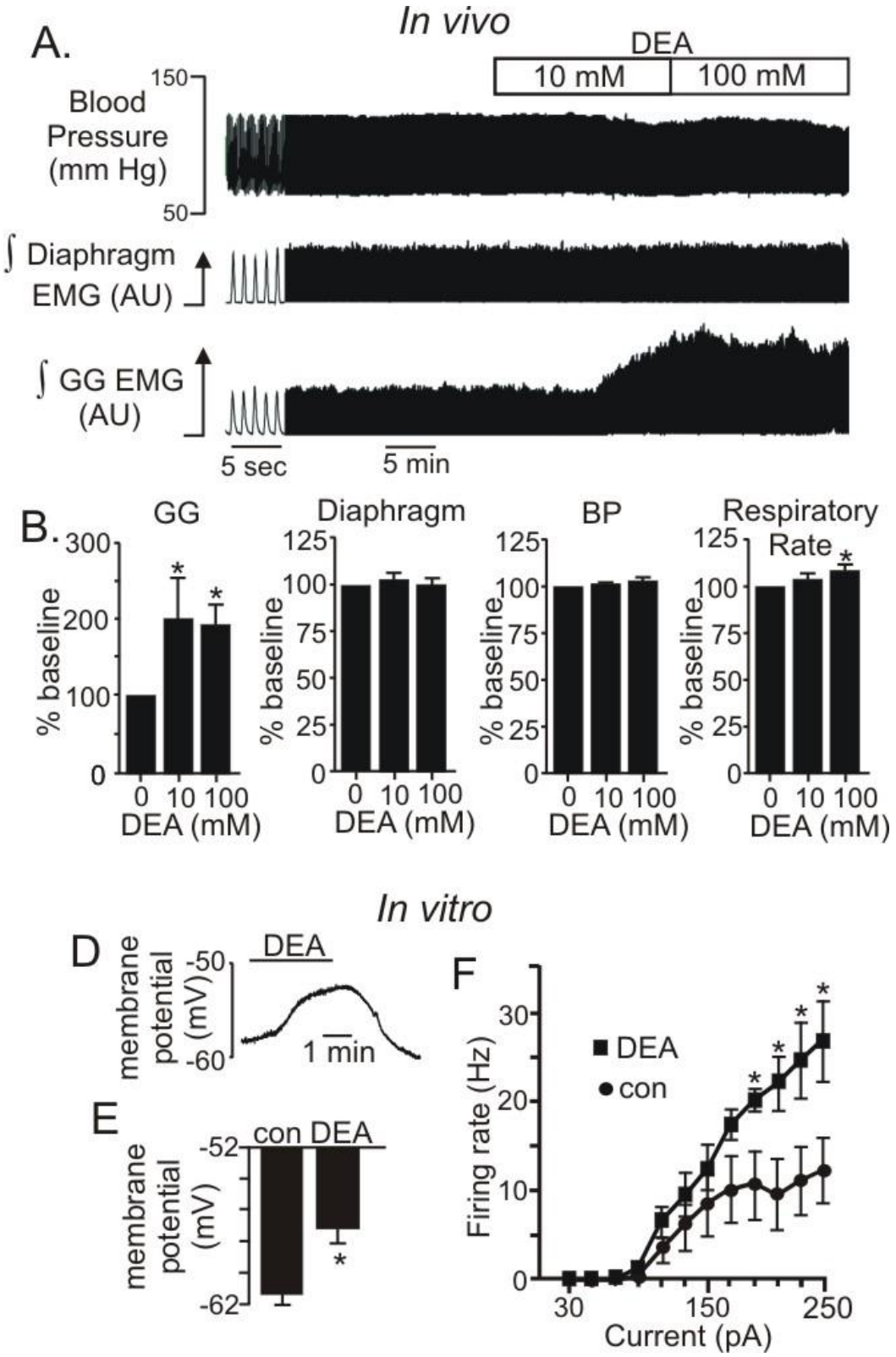
In vivo
A.
DEA
10 mM
100 mM
Blood Pressure (mm Hg)
150
50
∫ Diaphragm EMG (AU)
∫ GG EMG (AU)
5 sec
5 min
B.
GG
Diaphragm
BP
Respiratory Rate
% baseline
DEA (mM)
0 10 100
In vitro
D
membrane potential (mV)
-50
-60
DEA
1 min
E
con DEA
-52
-62
F
Firing rate (Hz)
DEA
con
Current (pA)
30
150
250

Figure 3

(Montero et al. 2008), and suggest that NO increases excitability of hypoglossal motoneurons by modulating multiple channels, possibly inhibition of background TASK channels (Gonzalez-Forero et al. 2007) or activation of HCN channels (Abudara et al. 2002).

To explore this possibility further, we made voltage-clamp recordings (holding potential of -60 mV; 0.1 µM TTX) from hypoglossal motoneurons during exposure to DEA under experimental conditions designed to block contributions of either TASK or HCN channels. In the first set of experiments, we found that DEA inhibited a resting K^+ conductance when HCN channels were blocked with Cs^+. For example, exposure to DEA in the continued presence of Cs^+ decreased outward current and conductance by 35.8 ± 2.4 pA (N=25) and 0.95 ± 0.06 nS (N=25), respectively (Fig. 4A1). The current-voltage (I-V) relationship of the NO-sensitive current in Cs^+ is reminiscent of TASK, i.e., relatively voltage-independent, active at resting membrane potential and reversed near E_K (Fig 4A2). To confirm that TASK channels contribute to NO-sensitivity of hypoglossal neurons, we retested DEA in Cs^+ this time in the presence of methanandamide, a blocker of TASK-1, TASK-3 and heteromeric TASK1/3 channels (Kim et al. 2009). Methanandamide (10 µM) decreased effects of DEA on holding current by 40% and virtually eliminated the residual NO-sensitive current in Cs^+ (Fig. 4B1-3). In addition, we found that the selective soluble guanyl cyclase (sGC) blocker 1*H*-(1,2,4)oxadiozolo(4,3-*a*)quinoxaline-1-one (ODQ, 10 µM) decreased effects of DEA on holding current and conductance (Fig. 4C1-3), thus indicating that the signaling pathway responsible for NO modulation of TASK channels in hypoglossal motoneurons.

Figure 4. NO inhibits a TASK-like conductance by a cGMP-dependent mechanism. **A1**, traces of holding current and conductance show that, in Cs^+ (2 mM) to block HCN channels and tetraethyl-ammonium (TEA,10 mM) and 4-aminopyridine (4AP, 50 µM) to block voltage-gated K^+ channels, exposure to DEA (20 µM) decreased outward current and conductance. **A2**, average (N=6) current-voltage (I-V) relationship of the NO-sensitive current (determined by subtracting I-V relationships obtained during exposure to DEA from those recorded in the absence of DEA) in Cs^+ is similar to a TASK-like conductance, i.e., relatively voltage-independent current that is active at resting membrane potential and reverses near the equilibrium potential for K^+. **A3**, summary data (N=6) show DEA-induced change in holding current in Cs^+ alone and in Cs^+ with TEA and 4AP. **B1**, traces of holding current and conductance show that in Cs^+ exposure to DEA decreases outward current and conductance. However, responsiveness to a second DEA exposure was reduced by methanandamide (Met, 10 µM), a TASK channel blocker. **B2-B3**, average I-V relationships in Cs^+ alone and during exposure to DEA in Cs^+ with and without Met. **B4**, summary data (N=6) showing that in the continued presence of Cs^+ subsequent inhibition of TASK channels with Met decreased the DEA-induced change in holding current. **C1**, traces of holding current and conductance show that the sGC blocker ODQ (20 µM) decreased DEA sensitivity. The asterisk designates a 10 min break. **C2-C3**, average I-V relationships in Cs^+ alone and during exposure to DEA in Cs^+ with and without ODQ. **C4**, summary data (N=13) showing that ODQ decreased the DEA-sensitivity TASK-like conductance. These results strongly suggest that TASK channels contribute to NO-sensitivity of hypoglossal motoneurons by a cGMP-dependent mechanism.

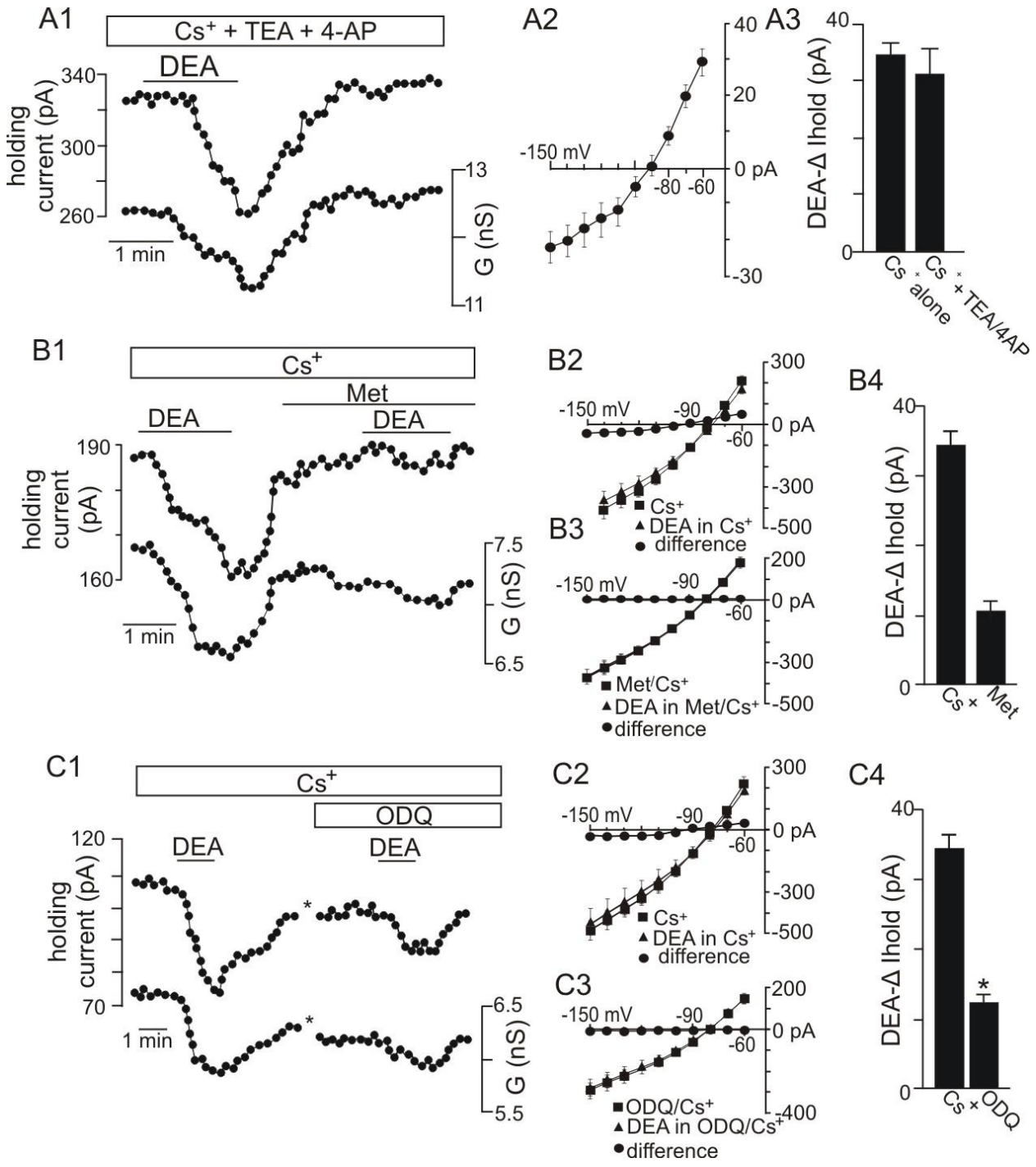
A1
Cs+ + TEA + 4-AP
DEA
holding current (pA)
340
300
260
1 min
13
G (nS)
11
A2
40
20
0 pA
-30
-150 mV
-80 -60
A3
DEA-Δ Ihold (pA)
40
0
Cs
Cs + TEA/4AP
alone
B1
Cs+
Met
DEA
DEA
holding current (pA)
190
160
1 min
7.5
G (nS)
6.5
B2
300
-150 mV
-90
-60
0 pA
-300
-500
Cs+
DEA in Cs+
difference
B3
200
-150 mV
-90
-60
0 pA
-300
-500
Met/Cs+
DEA in Met/Cs+
difference
B4
DEA-Δ Ihold (pA)
40
0
Cs
Met
C1
Cs+
ODQ
DEA
DEA
holding current (pA)
120
70
1 min
6.5
G (nS)
5.5
C2
300
-150 mV
-90
-60
0 pA
-300
-500
Cs+
DEA in Cs+
difference
C3
200
-150 mV
-90
-60
0 pA
-400
ODQ/Cs+
DEA in ODQ/Cs+
difference
C4
DEA-Δ Ihold (pA)
40
0
Cs
ODQ

Figure 4

These results are consistent with the reported effects of NO/cGMP on TASK channels in hypoglossal motoneurons (Gonzalez-Forero et al. 2007).

Hypoglossal neurons also express HCN channels which can be directly activated by cGMP so we considered the possibility that the hyperpolarization activated current (Ih) generated by HCN channels also contributes to NO-sensitivity of hypoglossal neurons. To study NO modulation of HCN channels in relative isolation, we used Ba^{2+} (2 mM) to block background K^+ channels (i.e., TASK) with minimal effect on HCN channels (Biel et al. 2009). Under these conditions exposure to DEA decreased holding current by 60.3 ± 3.1 pA (N= 20) and increased conductance by 1.32 ± 0.1 nS (N= 20), indicating that DEA/NO activated an inward current (Fig. 5A) with ODQ (20 μM) (Fig. 5A), suggesting that DEA-sensitivity is not dependent on formation of cGMP. In addition, effects of Cs^+ on holding current and conductance were 94.7 ± 22% and 75.4 ± 8.3% (N=9; Fig. 5A) larger in DEA plus Ba^{2+} compared to Ba^{2+} alone, indicating that DEA/NO activates a Cs^+-sensitive inward current similar to Ih. To determine the effects of DEA on time- and voltage-dependent properties of Ih, we delivered 1 second long hyperpolarizing voltage steps from -60 to -150 mV (Δ 10 mV). The DEA difference current, generated by subtracting current responses to hyperpolarizing voltage steps recorded in DEA plus Ba^{2+} from those recorded in Ba^{2+} alone, show that DEA preferentially increased Iinst with little change in the time dependent component of Ih (Fig. 5B). This can also be seen in summary data plotted as amplitude of total Ih at -150 mV (Fig. 5F) or as the ratio of Iinst/Iss across voltages ranging from -60 to -150 mV (Fig. 5G).

Figure 5. NO selectively activates Iinst. A, traces of holding current and conductance show that in Ba^{2+} (2 mM) to block background K^+ channels, exposure to DEA (20 µM) decreased outward current and increased conductance. In addition, DEA increased effects of Cs^+ on holding current and conductance by 55% and 58% (N=9), indicating that DEA increased a Cs^+-sensitive inward current. Inset, Cs^+-sensitive difference currents, generated by subtracting current responses to hyperpolarizing voltage steps recorded at the indicated times (scale bars are 500 ms and 500 pA). **B,** current responses to hyperpolarizing voltage-steps recorded in Ba^{2+} alone, Ba^{2+} plus DEA, and the corresponding difference current show that DEA preferentially increases Iinst with little change in Iss. This can also be seen in the superimposed current responses to a -150 mV step in Ba^{2+} alone and Ba^{2+} plus DEA plotted in the lower portion of panel B. **C,** traces of holding current and conductance show that DEA was without effect when background K^+ channels are blocked with Ba^{2+} (2 mM) and HCN channels are blocked with the selective HCN channel blocker ZD7288 (50 µM). **D,** summary data showing effects of DEA on conductance in Ba^{2+} alone (N=7) and Ba^{2+} plus ZD7288 (N=7). **E,** Iinst evoked by hyperpolarizing voltage steps in Ba^{2+} alone and in Ba^{2+} plus DEA. The data were fitted with linear regression and the extrapolated intersection of the regression lines is near the reversal potential for Ih. **F,** average Ih amplitude plotted as Iinst and Iss evoked by a -150 mV step in Ba^{2+} alone (N=8), Ba^{2+} plus DEA (N=8), and Ba^{2+} plus DEA and Cs^+ (N=3) or ZD7288 (N=5). **G,** average Ih amplitude plotted as a ratio of Iinst/Iss across voltages ranging from -60 to -150 mV. Note that decomposed DEA was without effect (not shown), indicating that DEA responses are mediated by NO rather than reactive byproducts.

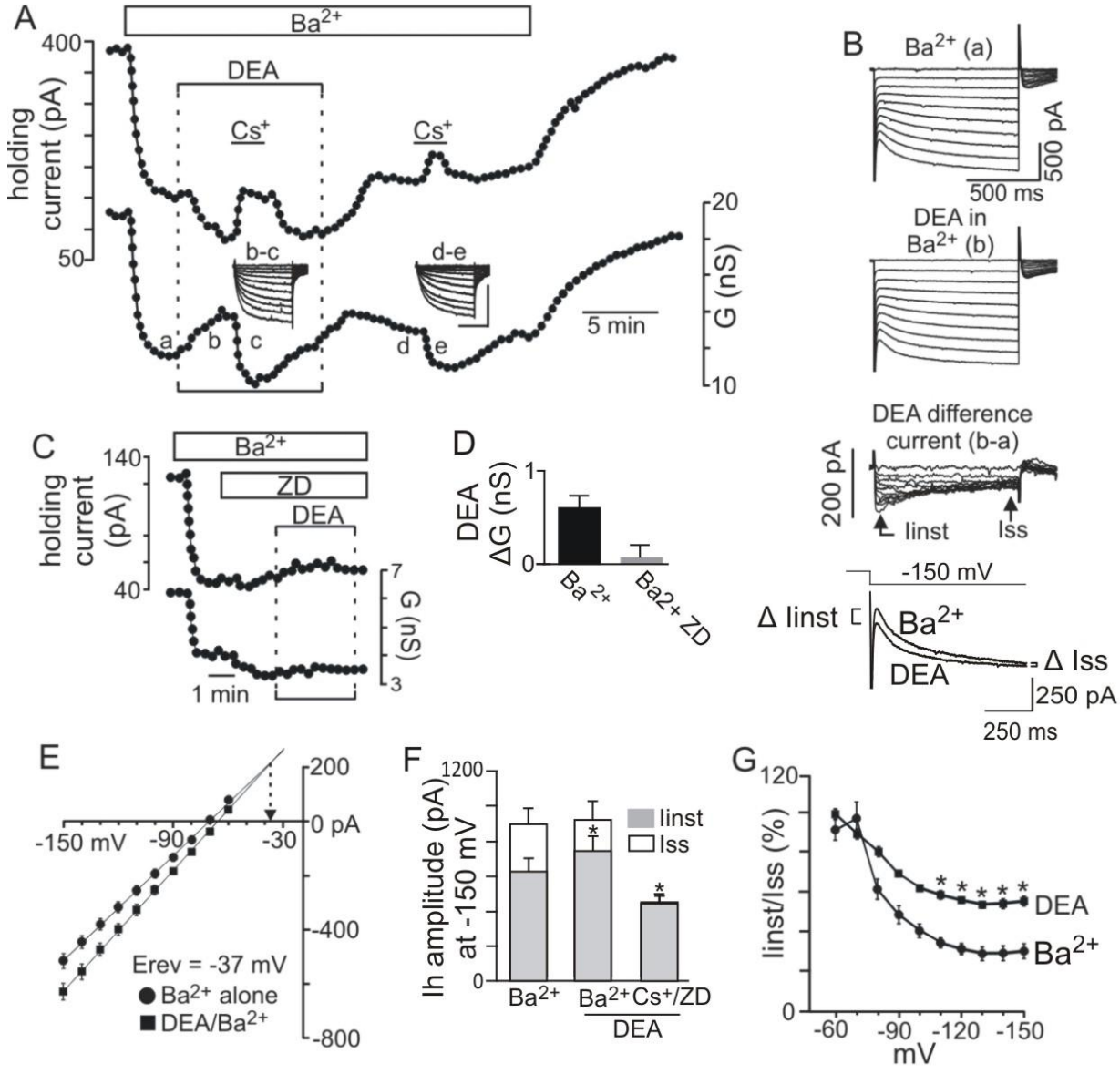
A
Ba2+
DEA
Cs+
Cs+
holding current (pA)
400
50
b-c
d-e
a
b
c
d
e
5 min
20
10
G (nS)
B
Ba2+ (a)
500 pA
500 ms
DEA in Ba2+ (b)
C
Ba2+
ZD
DEA
holding current (pA)
140
40
7
3
G (nS)
1 min
D
DEA ΔG (nS)
1
0
Ba 2+
Ba2+ ZD
DEA difference current (b-a)
200 pA
Iinst
Iss
-150 mV
Δ Iinst
Ba2+
DEA
Δ Iss
250 pA
250 ms
E
200
0 pA
-400
-800
-150 mV
-90
-30
Erev = -37 mV
Ba2+ alone
DEA/Ba2+
F
Ih amplitude (pA) at -150 mV
1200
0
Iinst
Iss
Ba2+
Ba2+Cs+/ZD
DEA
G
Iinst/Iss (%)
120
0
DEA
Ba2+
-60
-90
-120
-150
mV

Figure 5

The I-V relationships of Iinst in Ba^{2+} alone and in Ba^{2+} plus DEA intersect near the reversal potential for HCN channels (Fig 5E) and the effects of DEA were completely blocked with ZD7288 (50 μM), a selective HCN channel blocker (Fig 5C-D). These are the first evidence that NO can preferentially increase instantaneous Ih and in doing so have profound effects on neuronal excitability.

The results described above differ from known effects of cyclic nucleotides on HCN channels (Biel et al. 2009); therefore we postulate that this novel form of HCN channel modulation is conferred a cGMP-independent mechanism involving S-nitrosylation. Consistent with this possibility, we found that effects of DEA (in Ba2+) on holding current and conductance were fully retained when activity of sGC was blocked There are no selective blockers of S-nitrosylation signaling, however, this is a reduction-oxidation (REDOX) pathway that requires the thiol group of cysteine residues to be in the reduced state. Therefore, we used oxidative stress in the form of hyperoxia (95% O2) or the cysteine specific oxidant N-ethylmaleimide (NEM; 300 μM) to oxidize cysteine residues and thus occlude subsequent S-nitrosylation. We found that hyperoxia (N=8) and NEM (N=4) decreased the DEA-induced inward current by 69.6 ± 3.2% and 69 ± 3%, respectively (Fig. 6B). In addition, we used uric acid, the most abundant plasma antioxidant and preferential scavenger of NO (Gersch et al. 2008), to buffer NO and limit S-nitrosylation signaling. We found that uric acid (1 mM, pH = 7.3) decreased DEA-induced inward current by 54.3 ± 3.6 % (N=7) (Fig. 6C). Together, these results strongly suggest that NO increases instantaneous Ih by an S-nitrosylation dependent mechanism. It is important to note that the level of hyperoxia used here to block S-nitrosylation signaling is commonly used to set control PO2 in many *in vitro*

Figure 6. NO modulates HCN channels by an S-nitrosylation dependent mechanism. A, traces of holding current and conductance show that exposure to DEA in Ba^{2+} activated an inward current with properties similar to Ih (Fig. 3). In the continued presence of Ba^{2+} subsequent inhibition of sGC with ODQ did not affect DEA-sensitivity. Bar graph on right shows summary data (N=5) plotted as DEA-induced change in holding current in Ba^{2+} alone and Ba^{2+} plus ODQ. **B,** traces of holding current and conductance show the characteristic DEA response in Ba^{2+}. A second exposure to DEA after 7 mins of incubation in hyperoxic (95% O_2) medium this time had no effect on holding current or conductance. In addition, the cysteine specific oxidant NEM (300 µM) also decreased DEA-sensitivity, suggesting that the inward current activated by NO (i.e., Iinst; Fig. 3) is mediated by S-nitrosylation-dependent signaling. **C,** traces of holding current and conductance show that uric acid (1 mM), antioxidant and preferential scavenger of NO, decreased the effects of DEA.

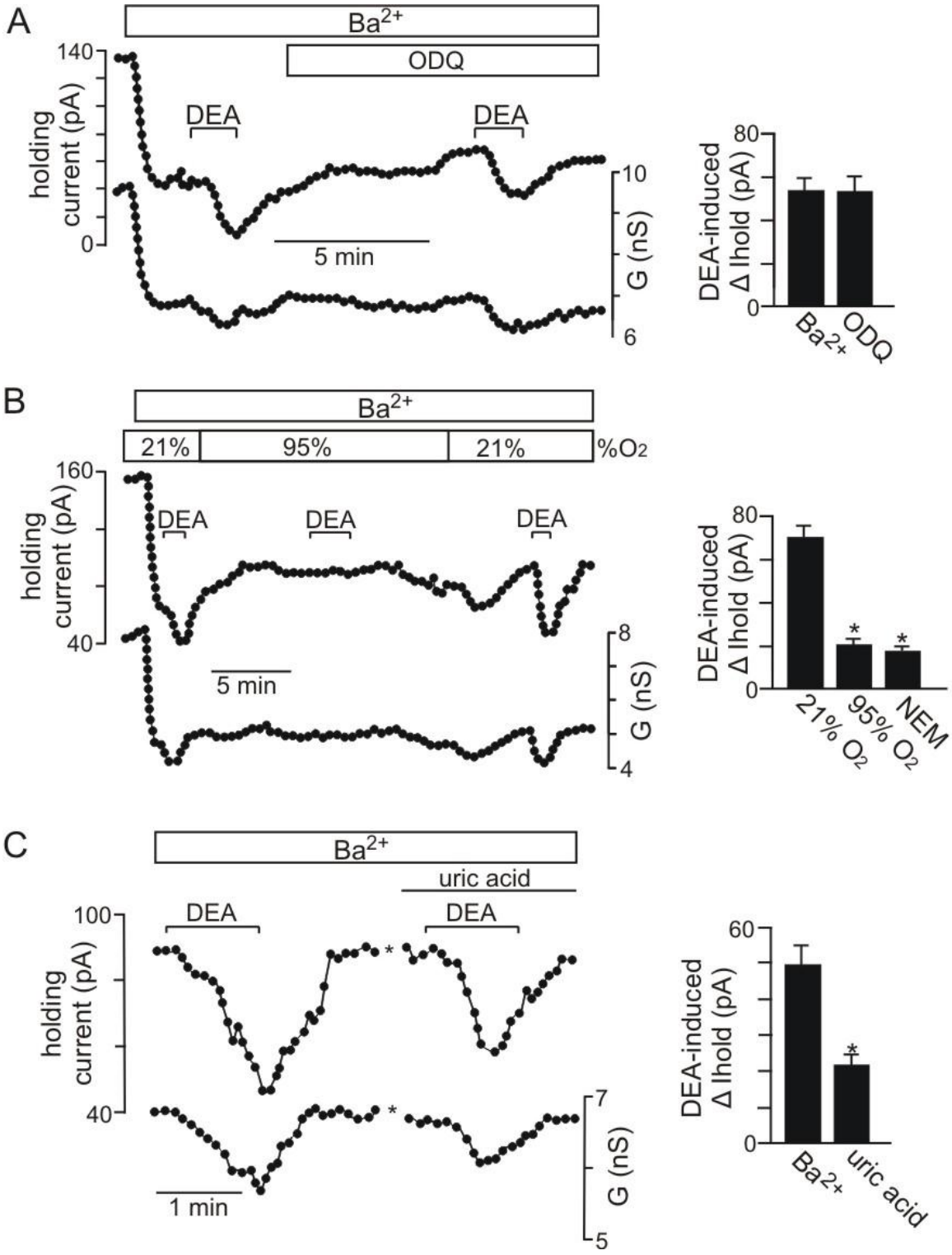
A
Ba2+
ODQ
140
holding current (pA)
0
DEA
DEA
5 min
10
G (nS)
6
80
DEA-induced Δ Ihold (pA)
0
Ba2+
ODQ
B
Ba2+
21%
95%
21%
%O2
160
holding current (pA)
40
DEA
DEA
DEA
5 min
8
G (nS)
4
80
DEA-induced Δ Ihold (pA)
0
21% O2
95% O2
NEM
C
Ba2+
uric acid
100
holding current (pA)
40
DEA
DEA
1 min
7
G (nS)
5
60
DEA-induced Δ Ihold (pA)
0
Ba2+
uric acid

Figure 6

preparations (Mulkey et al. 2001). Our evidence indicates that under these conditions NO-mediated S-nitrosylation would not occur, and so it is not surprising that this novel form of HCN channel modulation has not been previously observed.

DISCUSSION

In this study, we tested the hypothesis that NO increased excitability of hypoglossal motoneurons *in vivo* and *in vitro.* Our results indicate that NO increased activity of hypoglossal motoneurons by a cGMP-dependent inhibition of TASK channels. We also demonstrated that HCN channels are activated in a cGMP-independent manner, specifically the linst component of Ih is selectivly activated by an S-nitrosylation dependent mechansim. We also show that NO increases activity of hypoglossal motoneurons *in vitro* and increases tongue activity *in vivo*. These observations are consistent with the possiblity that NO may help maintain airway patency.

Our *in vivo* data strongly suggests that NO activates hypoglossal neurons and increases genioglossus activity. Micro-perfusion of DEA into the HMN of anesthetized rats increased tongue activity consistent with no effect on respiratory rate or EEG. As described previously, during wakefulness the HMN receives 5HT and NE drive that increased excitability of hypoglossal motoneurons. Therefore, we proposed that regardless of arousal state (i.e., in the presence of wakeful transmitters or sleep-activated NO/cGMP signaling), exogenous application of NO donors will activate hypoglossal neurons and increase genioglossus activity.

A limitation associated with the micro-perfusion preparation is that it typically requires 10-100 times higher drug concentrations then used *in vitro* because of reduced tissue access and limited drug diffusion across the probe membrane (Chan, Steenland, Liu, & Horner, 2006). Despite the use of high drug concentrations, previous studies show that there is minimal spreading of drugs to the adjacent nucleus and effects are restricted to the HMN (Chan, Steenland, Liu, & Horner, 2006). We did however see an

increase in respiratory rate with 100 mM DEA. With such high concentrations of NO donor, it is entirely possible to have uncontrolled diffusion to other respiratory centers. It should also be noted that application of exogenous NO donors does not exactly mimic the transient and discrete synthesis of NO by activated nNOS-expressing fibers in the HMN. Nevertheless, these experiments support our *in vitro* experiments that will be discussed shortly and confirm that NO can modulate activity of the HMN *in vivo*.

We also showed *in vitro,* NO donors depolarized motoneuron membrane potential and increased excitability with no overall change in input resistance. Increased excitability in conjunction with no change in input resistance strongly suggests that NO is having opposing effects on the conductance two or more channels. Indeed, we show that NO inhibits a resting K^+ channel and activates an inward cesium-sensitive current similar to Ih.

Nitric oxide can affect neuronal excitability by cGMP-dependent and cGMP-independent mechanisms. Cyclic GMP-dependent signaling can involve activation of PKG (PKG-I or PKG-II) and phosphorylation of serine and/or threonine residues on target proteins (e.g., background K^+ channels), or direct activation of HCN channels that produce Ih current. We showed that the potential contribution of HCN, identified pharmacologically by inhibition with ZD7288, decreased the effects of NO on membrane potential and excitability of hypoglossal neurons. In addition, hypoglossal neurons also express a TTX-insensitive persistent Na^+-current (Fenik & Veasey, 2003) and evidence suggests that NO can activate persistent Na^+ channels in cardiac myocytes by a cGMP-independent mechanism involving S-nitrosylation (Ahern, Klyachko, & Jackson, 2002)

The cGMP-independent pathway is a reduction-oxidation mechanism involving S-nitrosylation of sulfhydryl groups (e.g., cysteine amino acids) on target proteins including ion channels or second messenger signaling molecules (Ahern, Klyachko, & Jackson, 2002). There are no known specific blockers of S-nitrosylation which makes it a difficult thing to test in an *in vitro or in vivo* system. The process of S-nitrosylation can be disrupted by increased oxidative stress in the external environment. High oxidative stress would oxidize the sulfhydryl groups of the cysteine which would prevent the nitrosothiol from forming. We exposed the hypoglossal neurons to 95% O_2 and subsequently applied DEA which had a much smaller effect than the control. We also demonstrated that the DEA effect in the presence of the sulfhydryl blocker, NEM was greatly decreased as well. Collectively, our data shows that the DEA response was occluded in the presense of oxidative stresses supporting the prospect that a S-nitrosylation mechanism is involved. Our data demonstrates that acute NO exposure with HCN channels has different effects on hypoglossal neurons by cGMP-independent mechanism which we believe is S-nitrosylation.

Another potential method to block S-nitrosylation is the presence of plasma antioxidants, the most abundant of which is uric acid. Uric acid comes into contact with NO forming 6-aminouracil which then depletes plasma NO levels (Gersch et al. 2008). The uric acid buffering system works preferentially for NO rather than other radicals, such as peroxynitrite (Gersch et al. 2008). A uniqure property of uric acid is that is can also act as a prooxidant in conditions where oxidative stresses are imposed (Gersch et al. 2008). Our data shows a diminished effect of DEA in the presence of uric acid. Perhaps an individual with elevated uric acid levels which is the cause in gout, have a

greater predisposition to OSA. It would therefore be beneficial to clinically use xanthine oxidase inhibitors which have been shown to lower uric acids levels (Gersch et al. 2008). In the future, further studies into using xanthine oxidase inhibitors, possibly in conjunction with NO donors could amerloriate the patholgical effects of OSA.

Ih is composed of an instantaneous (Iinst) and steady state component (Iss). It has been documented that these components of the channel can be modulated independently of one another (Liang et al. 2009). A study has shown that mutation of the S4 domain of the HCN2 isform in cell culture diminished overall Ih while leaving the Inst component unaffected (Proenza et al. 2001). This modulation could result by differential gating of one population of HCN channels. Consistent with this data, we demonstrated that application of our NO donor increased the Iinst component independently of the overall Ih.

The results from experiments on the effects of NO and cGMP signaling on hypoglossal neurons in brain slices will provide strong evidence that NO can increase excitability of hypoglossal neurons by a cGMP-mediated inhibition of a background K^+ channel and cGMP-independent activation of HCN channels. All *in vitro* experiments were conducted in the medullary brain slice preparation because it allows considerable control of the neuronal environment and easy access to neurons for patch clamp recording. However, it is important to recognize that the brain slice preparation has several limitations. Perhaps most importantly, slices are removed from a blood supply and receive O_2 and nutrients solely by diffusion from the bath. Therefore, most brain slice studies use bath solutions saturated with O_2 (95% O_2), and consequently subject cells on the surface of the slice to nonphysiologically high O_2 tension. High O_2 at the

slice surface, where most patch clamp recordings are made, may interfere with NO signaling; especially S-nitrosylation signaling which requires sulfhydryl groups to be in the reduced state. However, we used that to our advantage to show that oxidizing the sulfhydryl groups decreased the responsiveness to NO exposure. Other limitations of the slice preparation include traumatized tissue and a lack of most network connections, as well as effector muscles. Despite these limitations, the brain slice preparation is considered well suited for characterizing intrinsic properties of neurons as well as inspiratory drive to the HMN.

The effects of hypoxia on NO-mediated modulation of TASK channels are particularly interesting in the context of OSA where hypoxia (which results from OSA) may disrupt NO signaling and contribute to the onset of sleep disordered breathing. The mechanism by which hypoxia inhibits TASK channels may be linked to decreased ATP production and/or activation of AMP-activated protein kinase (AMPK) but our lab has shown that activation of AMPK does not affect activity of TASK-1 or TASK-3 channels (Kreneisz et al. 2009). We see more of an involvement of HCN channels when exposed to DEA as opposed to TASK. We have an alternative hypothesis which contends that reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced during hypoxia can cause the oxidation of cysteine residues on HCN channels resulting in decreased channel activity. In addition, ROS (e.g., superoxide) can react with NO to form peroxynitrite (Esplugues, 2002), a highly reactive and toxic ROS. Therefore, it is possible that peroxynitrite will be formed during periods of intermittent hypoxia and contributes to motoneuron damage associated with OSA. In the future, we plan to

explore potential interaction between hypoxia and NO on native TASK channels and hypoglossal motoneurons in the brain slice preparation.

It is our hope that these results help establish a novel modulatory role of NO in the HMN that may contribute to the state-dependent control of airway patency. Specifically, we determine that i) NO increased excitability of genioglossus in the *in vivo* animal; ii) NO activates hypoglossal neurons by inhibition of a background K^+ channel by a cGMP-dependent pathway; iii) NO depolarizes hypoglossal neurons by activation of an inward HCN like current by a cGMP-independent pathway by shifting Iss to Iinst.

CONCLUSION

Our results indicate that NO selectively increases Iinst by an S-nitrosylation-dependent mechanism. Considering that the HMN receives nitrergic innervation from the sleep-activated cholinergic system (Vincent & Kimura, 1992), this signaling pathway may partially compensate for loss of wakeful excitatory drive to respiratory motoneurons during sleep; conversely disruption of NO signaling may contribute to the etiology of obstructive sleep apnea (OSA). Although a causal link between disruption of NO signaling and OSA has yet to be established, it is well known that patients with metabolic syndrome have high levels of uric acid and, perhaps consequently, have a high prevalence of OSA (Mota, 2010). In addition, this novel mechanism may also influence HCN channels in cardiac pacemaker cells since NO has been shown to stimulate heart rate by activation of Ih (Musialek et al. 1997).

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