Nitric oxide activates hypoglossal motoneurons by cGMP-dependent inhibition of TASK channels and cGMP-independent activation of HCN channels

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Submitted 9 September 2011; accepted in final form 30 November 2011

Nitric oxide activates hypoglossal motoneurons by cGMP-dependent inhibition of TASK channels and cGMP-independent activation of HCN channels. J Neurophysiol 107: 1489–1499, 2012. First published November 30, 2011; doi:10.1152/jn.00827.2011.—Nitric oxide (NO) is an important signaling molecule that regulates numerous physiological processes, including activity of respiratory motoneurons. However, molecular mechanism(s) underlying NO modulation of motoneurons remain obscure. Here, we used a combination of in vivo and in vitro recording techniques to examine NO modulation of motoneurons in the hypoglossal motor nucleus (HMN). Microperfusion of diethylamine (DEA; an NO donor) into the HMN of anesthetized adult rats increased genioglossus muscle activity. In the brain slice, whole cell current-clamp recordings from hypoglossal motoneurons showed that exposure to DEA depolarized membrane potential and increased responsiveness to depolarizing current injections. Under voltage-clamp conditions, we found that NO inhibited a Ba2+-sensitive background K+ conductance and activated a Cs+-sensitive hyperpolarization-activated inward current (Ih). When Ih was blocked with Cs+ or ZD-7288, the NO-sensitive K+ conductance exhibited properties similar to TWIK-related acid-sensitive K+ (TASK) channels, i.e., voltage independent, resistant to tetraethylammonium and 4-aminopyridine but inhibited by methanandamide. The soluble guanylyl cyclase blocker 1H-[1,2,4]oxadiazole[4,3-d]oxazine-1-one (ODQ) and the PKG blocker KT-5823 both decreased a NO modulation of this TASK-like conductance. To characterize modulation of Ih in relative isolation, we tested effects of NO in the presence of Ba2+ to block TASK channels. Under these conditions, NO activated both the instantaneous (Ihinst) and time-dependent (Iht) components of Ih. Interestingly, at more hyperpolarized potentials NO preferentially increased Iht. The effects of NO on Ih were retained in the presence of ODQ and blocked by the cysteine-specific oxidant N-ethylmaleimide. These results suggest that NO activates hypoglossal motoneurons by cGMP-dependent inhibition of a TASK-like current and S-nitrosylation-dependent activation of Ih.

Nitric oxide (NO) is a diffusible messenger produced by NO synthase (NOS1–3), and it plays important roles in a variety of physiological and pathophysiological processes (Calabrese et al. 2007). It is well established that soluble guanylyl cyclase (sGC) is the primary cellular NO receptor; activation of sGC by NO leads to increased cGMP production and subsequent activation of signaling mechanisms including cGMP-dependent protein kinases, phosphodiesterases, and activation of ion channels (Ahern et al. 2002; Garthwaite 2008). In addition, S-nitrosylation has recently emerged as an alternative avenue of NO signaling (Ahern et al. 2002). This cGMP-independent signal is conferred by the covalent addition of an NO moiety to the sulfhydryl group of cysteine residues. This reversible posttranslational modification regulates function of several channels including NMDA receptors (NMAD-R) (Choi et al. 2000), ATP-sensitive K+ (KATP) channels (Kawano et al. 2000), Ca2+-dependent K+ channels (Lang et al. 2000), and cyclic nucleotide-gated (CNG) channels (Briollet and Firestein 1996). However, despite compelling evidence that S-nitrosylation is an important component of NO signaling, physiological significance of this pathway remains uncertain.

The hypoglossal motor nucleus (HMN) is an ideal model system to study NO modulation of neuronal activity because 1) properties of hypoglossal motoneurons are well characterized (Rekling et al. 2000); 2) the HMN receives nitrergic innervations (Pose et al. 2005; Travers et al. 2005); 3) hypoglossal motoneurons express sGC (Gonzalez-Forero et al. 2007); and 4) short-term manipulation of NO/cGMP signaling in the HMN can modulate rhythmic output of the region (Montero et al. 2008). These results provide compelling evidence that NO is an important modulator of hypoglossal motoneuron activity; however, our understanding of the role of NO in the HMN is far from complete. For example, the functional consequence of NO signaling on genioglossus muscle activity has not been described, and mechanism(s) by which acute NO modulates motoneuron activity remain unknown. Studies in hypoglossal and trigeminal motoneurons report that brief (≤10 min) NO exposure depolarized membrane potential with no change in input resistance (Abudara et al. 2002; Gonzalez-Forero et al. 2007), suggesting NO has offsetting effects on inward and outward currents. Long-term exposure to NO (hours) leads to motoneuron hyperexcitability by cGMP-dependent inhibition of TASK channels (Gonzalez-Forero et al. 2007), therefore, a similar mechanism may underlie acute NO responsiveness as well. It also has been proposed that hyperpolarization-activated cyclic nucleotide-gated (HCN) channels contribute to NO sensitivity of motoneurons (Abudara et al. 2002; Montero et al. 2008); however, this possibility has yet to be demonstrated. HCN channels produce a hyperpolarization-activated inward current (Ih) with two components: a slowly developing steady-
state current ($I_{h}$) and an instantaneous current ($I_{\text{inst}}$) (Proenza et al. 2002). It is well known that cyclic nucleotides bind HCN channels to cause a depolarizing shift in $I_{h}$ activation (Biel et al. 2009; DiFrancesco and Tortora 1991), and evidence suggests that in the heart and other brain regions, NO-mediated cGMP production can activate $I_{h}$ by this mechanism (Musialek et al. 1997; Pape and Mager 1992; Wilson and Garthwaite 2010). In addition to cyclic nucleotide modulation, it has been shown that HCN channels are targets of endogenous S-nitrosylation (Jaffrey et al. 2001); however, functional evidence supporting this possibility is currently lacking.

Here we show in vitro that NO functions as an excitatory transmitter in the HMN and in vivo that NO signaling in the HMN stimulates genioglossus muscle activity. The molecular mechanisms underlying NO sensitivity of hypoglossal motoneurons involve cGMP-dependent inhibition of a TASK-like conductance and activation of $I_{h}$ by a process consistent with S-nitrosylation. In addition, we find that NO preferentially increased $I_{\text{inst}}$ compared with $I_{h}$ at more hyperpolarized potentials, suggesting that $I_{\text{inst}}$ can be differentially regulated as a neuromodulatory mechanism.

**METHODS**

*In vivo preparation.* In vivo studies were performed on 9 adult male Wistar rats [mean body weight = 281 ± 6 (SE) g, range = 255–310 g; Charles River]. All procedures conformed to the recommendations of the Canadian Council on Animal Care, and the University of Toronto Animal Care Committee approved the experimental protocol. The surgical procedures and experimental preparation were similar to those previously described (Dubord et al. 2010).

All experiments were performed under isoflurane anesthesia (2.2–3.1%). After induction of surgical levels of anesthesia, as judged by the abolition of the hindlimb withdrawal and corneal blink reflexes, the rats were tracheotomized and the femoral artery and vein were cannulated. Throughout the surgery and the experiments, the rats spontaneously breathed a 50:50 mixture of room air and oxygen. In all experiments, core body temperature was maintained between 36 and 38°C with a water pump and heating pad (TPump heat therapy system; Gaymar, NY). The rats received continuous intravenous fluid (0.4 ml/h) containing 7.6 ml of saline, 2 ml of 5% dextrose, and 0.4 ml of 1 M NaHCO$_3$. For electromyogram (EMG) recordings of diaphragm activity, two insulated, multistranded stainless steel wires (AS636; Coorner Wire, Chatsworth, CA) were sutured into the costal diaphragm activity, two insulated, multistranded stainless steel wires (AS636; Coorner Wire, Chatsworth, CA) were sutured into the costal diaphragm via an abdominal approach. The rats were placed in a stereotoxic apparatus (Kopf model 962; Tujunga, CA), and the head was secured with blunt ear bars. To ensure consistent positioning between rats, the flat skull position was achieved with an alignment tool (Kopf model 944). To record the cortical electroencephalogram (EEG), two stainless steel screws attached to insulated wire were implanted onto the skull over the frontal-parietal cortex (Hajiha et al. 2009). Two stainless steel wire electrodes were also inserted bilaterally under direct observation into the body of the tongue, via an oral approach, to record genioglossus (GG) activity. We have shown that such electrode placements record activity from muscles innervated by the medial branch of the hypoglossal nerve (Morrison et al. 2002).

**Microdialysis.** A microdialysis probe (CMA/11 14/01; CSC, St. Laurent, QC, Canada) was placed through a small hole drilled at the junction of the interparietal and occipital bones. The probes were lowered into the HMN at the following final 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. In each rat, a brief burst of GG activity was observed when the probe initially penetrated the HMN, and then the probe was advanced another 0.5 mm before being left at the final coordinates. This burst of GG activity during probe insertion was transient (typically <5 min), did not affect diaphragm activity, blood pressure, or respiratory rate, and was useful as a preliminary indication of probe placement (Hajiha et al. 2009). The rats stabilized for at least 30 min before any interventions, after which the pharmacological interventions were undertaken without changing the level of isoflurane throughout the rest of the study.

The microdialysis probes were 240 µm in diameter with a 1-mm cuprophane membrane and a 6,000-Dalton cutoff. The probes were connected to FEP Teflon tubing (inside diameter = 0.12 mm) and connected to 1.0-ml syringes via a zero-dead space switch (Uniswitch, West Lafayette, IN). The probes were continually flushed with artificial cerebrospinal fluid (ACSF) at a flow rate of 2.0 µl/min using a syringe infusion pump (Pump 22; Harvard Apparatus; Holliston, MA). The lag time for fluid to travel to the tip of the probe at this flow rate was ~6 min. The composition (in mM) of the ACSF was 125 NaCl, 3 KCl, 1 KH$_2$PO$_4$, 2 CaCl$_2$, 1 MgSO$_4$, 25 NaHCO$_3$, and 30 glucose. The ACSF was made fresh each day, warmed to 37°C, and bubbled with CO$_2$ to a pH of 7.42 ± 0.01.

**Recording.** The electrical signals were amplified and filtered (Super-Z head-stage amplifiers and BMA-400 amplifiers/filters; CWE, Ardmore, PA). The EEG was amplified and filtered between 1 and 100 Hz, whereas the EMG signals were amplified and filtered between 100 and 1,000 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). In addition, the moving time average (time constant = 100 ms) of the GG and diaphragm signals were obtained (Coulbourn S76-01; Lehigh Valley, PA). Each signal, along with blood pressure (DT-XX transducer; Ohmeda, Madison, WI; and PM-1000 amplifier; CWE) were digitized and recorded on computer (Spike 2 software, 1401 interface; CED, Cambridge, UK).

**Protocol and analyses.** Signals were recorded continuously during microdialysis perfusion of ACSF into the HMN (control condition) and then during perfusion of the NO donor DEA at 10 and 100 mM. Each intervention lasted 15–25 min. For each agent delivered to the HMN, measurements were taken over 1-min periods at the end of each intervention, i.e., ACSF (baseline control period) and 10 and 100 mM DEA. Breath-by-breath measurements of GG and diaphragm activities were calculated and averaged in consecutive 5-s 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), quantified in arbitrary units, and expressed as a percentage of the respective ACSF controls. Electrical zero was the voltage recorded with the amplifier inputs grounded. 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-s period. The EEG was sampled by computer at 500 Hz and subjected to a fast-Fourier transform for each 5-s time bin, and the power within frequency bands spanning the 0.5- to 30-Hz range was calculated. The ratio of high ($\delta$, 20–30 Hz)- to low ($\beta$, 2–4 Hz)-frequency activity was calculated and used as a relative marker of EEG activation (Hajiha et al. 2009; Steenland et al. 2008). Each rat served as its own control.

**Tests of function of HMN and histology.** At the end of each experiment, 10 mM serotonin (creatinine sulfate complex) was applied to the HMN as a positive control to confirm that it was still functional and able to respond to manipulation of neurotransmission as judged by the expected increase in GG activity (Hajiha et al. 2009; Jelev et al. 2001). At the end of each study, the rats were then
euthanized under anesthesia by intravenous injection of 3–5 ml of saturated KCl and a high dose of isoflurane. The rats then were perfused intracardially with 0.9% saline and 10% formalin, after which the brain was removed and fixed in 10% formalin. Medullary regions containing the HMN were blocked and transferred to a 30% sucrose solution for cryoprotection. The tissue was then sectioned at 50 μm using a cryostat (CM1850; Leica, Nussloch, Germany). Sections were mounted and stained with neutral red, and the lesion sites left by the microdialysis probes were recorded on a corresponding standard cross section using a stereotaxic atlas of the rat brain (Paxinos and Watson 1998).

**In vitro brain slice preparation.** Slices containing the HMN were prepared as previously described (Sirois et al. 2002). 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 brain stem slices (300 μm) were cut using a microslicer (DSK 1500E; Dosaka, Japan) in ice-cold substituted Ringer solution containing (in mM) 260 sucrose, 3 KCl, 5 MgCl₂, 1 CaCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, and 1 kynurenic acid. Slices were incubated for ~30 min at 37°C and subsequently at room temperature in normal Ringer solution containing (in mM) 130 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 glucose. Both substituted and normal Ringer solutions were bubbled with 95% O₂-5% CO₂.

**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) with a bath solution composed of (mM) 140 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 glucose; pH was adjusted to 7.3 by addition of 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 et al. 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) on a two-stage puller (P89; Sutter Instruments) to a direct current (DC) resistance of 3–5 MΩ when filled with internal solution containing (in mM) 120 KCH₂SO₄, 4 NaCl, 1 MgCl₂, 0.5 CaCl₂, 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. In current clamp, repetitive firing was evoked from a potential of approximately ~60 mV by a series of 1-s depolarizing current pulses of increasing amplitude (Δ10 pA), and the number of evoked spikes was plotted against step current. Depolarizing sag was determined from the difference in membrane potential at the beginning of a ~120-pA hyperpolarizing step (i.e., just after the capacitance artifact) and end of the step. 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- and 5-min durations. We followed 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 Iᵥ current) obtained by hyperpolarizing the cell in 10-mV steps (to ~150 mV). We paused for 5–10 s between voltage steps to ensure that currents activated during hyperpolarizing steps have deactivated before the next step as evidenced by superimposed current traces at ~60 mV. The maximal amplitude of Iᵥ was quantified as the size of the time-dependent current during a 1-s-long step to ~150 mV. The voltage dependence of Iᵥ activation was obtained from tail currents measured at ~80 mV following a series of hyperpolarizing steps; those data were normalized and fitted with a Boltzmann function (Sirois et al. 2002). A liquid junction potential of 10 mV was corrected off-line.

**Bath NO measurements.** To determine the concentration of NO generated by various concentrations of DEA, we measured NO using a polarographic (+860 mV) electrode (ISO-NOP30; WPI) positioned at a depth of ~200 μm in the tissue bath. The electrode was calibrated before and after each experiment by decomposition of S-nitroso-N-acetyl-l-d,l-penicillamine. As shown in Fig. 1A, the concentration of NO increased linearly in response to DEA concentrations ranging from 1 to 100 μM. We also used a mathematical model developed by Schmidt et al. (1997) based on the first-order decomposition of the NO donor (Eq. 1) in association with a third-order reaction of NO with oxygen (Eq. 2) to calculate the concentration of NO generated by ≤20 μM DEA.

![Graph](http://jn.physiology.org/Downloadedfrom)
where \( t \) is time (s), \( C_{NO}(t) \) is the concentration of NO at \( t \) (M), \( C_D(t) \) is the concentration of donor at \( t \) (M), \( e_{NO} \) is the moles of NO released per mole of donor, \( O_2 \) is the concentration of oxygen (M), \( k_1 \) is the rate constant for donor decomposition (s\(^{-1}\)), and \( k_2 \) is the rate constant for the oxidation of NO (M\(^{-2}\) s\(^{-1}\)). For DEA in aqueous buffer equilibrated with room air at 25°C and pH 7.4, we used the following previously reported values (Schmidt et al. 1997): \( e_{NO} = 1.4 \times 10^{-3} \) M s\(^{-1}\), \( k_1 = 9.2 \times 10^6 \) M\(^{-2}\) s\(^{-1}\), and \( O_2 = 2.1 \times 10^{-4} \) M. The peak NO concentrations generated by 1, 5, 10, and 20 μM DEA were calculated to be 0.5, 1.4, 2.3, and 3.2 μM, respectively. These predicted values are similar to the concentrations of NO measured in our tissue bath during exposure to this range of DEA (Fig. 1, A and B).

To relate NO concentration to cellular responsiveness, we made whole cell voltage-clamp recordings (holding potential = −60 mV; in the presence of TTX) from hypoglossal neurons during exposure to this same range of DEA concentrations (Fig. 1C). The DEA-induced change in holding current was normalized and fit to a single-exponential equation to estimate the DEA EC\(_{50}\) of hypoglossal motoneurons (Fig. 1D). For all slice-patch experiments, we used a DEA concentration of 20 μM, which is near the estimated EC\(_{50}\) of ~18 μM and similar to DEA concentrations used to manipulate motoneuron activity (Gonzalez-Forero et al. 2007; Montero et al. 2008).

**Statistical analysis.** Data are means ± SE and were analyzed using the paired t-test or two-way repeated-measures ANOVA followed by Bonferroni’s multiple comparisons test when appropriate. Differences were considered significant at \( P \leq 0.05 \).

**RESULTS**

To determine whether NO functions as an excitatory transmitter in the HMN, we tested the functional consequence of NO signaling in the HMN on GG muscle activity in vivo and the effects of DEA on membrane potential of hypoglossal motoneurons in vitro. Microperfusion of DEA (10 mM) into the HMN in vivo caused a robust increase in GG activity with no change in diaphragm activation, blood pressure (Fig. 2, A and B), or EEG activity (not shown). Note that microdialysis typically requires 10–100 times higher drug concentrations than used in vitro because of reduced tissue access and limited drug diffusion across the probe membrane (Steenland et al. 2008). Despite this potential limitation, the effects of DEA described here seem specific to the HMN given that there were no changes in blood pressure, EEG, or diaphragm activity. However, higher concentrations of DEA (100 mM) increased respiratory rate (Fig. 2A), suggesting spillover onto neighboring structures. In the brain slice preparation, exposure to 20 μM DEA, which corresponds with a bath NO concentration of 5.1 ± 0.4 nM (Fig. 1, C and D), 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 medium) of hypoglossal motoneurons to depolarizing current injections (Fig. 2, C–E) but with no parallel change in input resistance. We did not observe a change in action potential threshold; however, this is not unexpected considering DEA did not affect input resistance. Decomposed DEA that is no longer able to release NO had no effect (not shown), indicating that effects of DEA on hypoglossal motoneurons are mediated by NO rather than potential reactive by-products. These results are consistent with reported effects of NO on intrinsic excitability of hypoglossal motoneurons (Gonzalez-Forero et al. 2007; Montero et al. 2008) and suggest that NO sensitivity is mediated by offsetting effects on inward and outward currents.

There are several candidate NO-sensitive ion channels in hypoglossal motoneurons, including background K\(^+\) channels (e.g., TASK) and HCN channels (Rekling et al. 2000; Sirois et al. 2002). To gain insight into the potential involvement of these channels in NO sensitivity of hypoglossal motoneurons, we made current- and voltage-clamp recordings (holding potential of −60 mV; 0.1 μM TTX) from these cells during exposure to DEA in the presence of Ba\(^{2+}\) (2 mM) to block contributions of background K\(^+\) channels (Goldstein et al.
with no corresponding increase in depolarizing sag by preferentially increasing $I_{\text{inst}}$ relative to $I_{\text{h}}$. We explore mechanisms underlying NO activation of hypoglossal motoneurons further in the experiments described below.

In voltage clamp, exposure to $\text{Ba}^{2+}$ decreased outward current and conductance as expected for inhibition of a resting $\text{K}^+$ conductance. In the continued presence of $\text{Ba}^{2+}$, exposure to DEA (20 $\mu$M) decreased holding current by $24.8 \pm 3.0$ pA in conjunction with an increase in conductance of $1.25 \pm 0.21$ nS ($n = 10$) (Fig. 4, A and C), suggesting that NO activates an inward current possibly mediated by HCN channels. In a second set of experiments, we replaced $\text{Ba}^{2+}$ with $\text{Cs}^+$ (2 mM) to block HCN channels. Under these conditions, exposure to DEA decreased holding current by $35.8 \pm 2.4$ pA and decreased conductance by $0.95 \pm 0.06$ nS ($n = 25$) (Fig. 4, A and C), suggesting that NO may inhibit a $\text{K}^+$ conductance. In addition, in the combined presence of $\text{Ba}^{2+}$ and $\text{Cs}^+$ or ZD-7288 [50 $\mu$M, a potent and selective HCN channel blocker in the low micromolar range, e.g., 10 $\mu$M ZD-7288 completely blocked $I_h$ in facial motoneurons (Larkman and Kelly 2001)], exposure to DEA had no effect on holding current or conductance (Fig. 4, B and C). Together, these results indicate that mechanisms underlying NO sensitivity of hypoglossal motoneurons involve inhibition of one or more $\text{Ba}^{2+}$-sensitive $\text{K}^+$ channels and activation of a $\text{Cs}^+$-sensitive inward current reminiscent of $I_h$.

Fig. 3. Effects of NO on input resistance and depolarizing sag. A: voltage trajectories in response to hyperpolarizing current steps in $\text{Cs}^+$ alone and $\text{Cs}^+$ plus DEA show that DEA increased input resistance (i.e., decreased conductance). Summary data ($n = 6$) plotted as input resistance in response to $-100$-pA current steps show that when a hyperpolarization-activated inward current ($I_h$) was blocked with Cs$^+$, DEA increased input resistance, suggesting that NO can inhibit a resting $\text{K}^+$ conductance. $^*P < 0.05$. B: voltage trajectories in response to hyperpolarizing current steps in $\text{Ba}^{2+}$ alone and $\text{Ba}^{2+}$ plus DEA show that under these conditions, exposure to DEA decreased input resistance (i.e., increased conductance). Summary data ($n = 4$) plotted as input resistance in response to $-200$-pA current steps show that when background $\text{K}^+$ channels were blocked with $\text{Ba}^{2+}$, exposure to DEA decreased input resistance. $^*P < 0.05$. Insets in A and B show the protocols delivered from a potential of $-60$ mV. Note that in both sets of experiments, input resistance was measured shortly after the capacitance artifact. C: superimposed membrane potential responses to hyperpolarizing current injection ($-120$ pA) in $\text{Ba}^{2+}$ alone and in $\text{Ba}^{2+}$ plus DEA show that DEA caused a decrease in the amplitude of depolarizing sag (measured as the voltage difference shortly after the capacitance artifact and the voltage near the end of the step).

2005) or in the presence of $\text{Cs}^+$ (2 mM) to block HCN channels (Biel et al. 2009). In current clamp, application of DEA (20 $\mu$M) in the presence of $\text{Cs}^+$ depolarized membrane potential $2.3 \pm 0.4$ mV ($n = 6$) (not shown) and increased input resistance from $134 \pm 12$ to $157 \pm 12$ MΩ (Fig. 3A), suggesting that DEA inhibited a resting $\text{K}^+$ conductance. In the presence of $\text{Ba}^{2+}$, application of DEA depolarized membrane potential $6.3 \pm 0.5$ mV ($n = 4$) (not shown) and decreased input resistance (measured just after the hyperpolarizing step) from $152 \pm 6$ to $128 \pm 9$ MΩ (Fig. 3B). To compare effects of DEA on amplitude of depolarizing sag in membrane potential, we used larger current steps in DEA to match peak hyperpolarizing voltage responses. We found that DEA did not change amplitude of the depolarizing sag during hyperpolarizing current steps (Fig. 3C). It is well established that depolarizing sag is determined by the time-dependent activation of $I_h$ (Bayliss et al. 1994). However, it is possible to increase $I_h$ but

Fig. 4. NO modulation of hypoglossal motoneurons involves activation of an inward $\text{Cs}^+$-sensitive current and inhibition of an outward $\text{Ba}^{2+}$-sensitive current. A: traces of holding current ($I_{\text{holding}}$) and conductance ($G$) show that $\text{Ba}^{2+}$ (2 mM) decreased holding current and conductance. In the continued presence of $\text{Ba}^{2+}$, exposure to DEA (20 $\mu$M) decreased holding current and increased conductance. A second exposure to DEA, this time in $\text{Cs}^+$ (2 mM), decreased holding current and conductance. G. conductance. B: in the presence of $\text{Ba}^{2+}$ to block background $\text{K}^+$ channels and ZD-7288 (ZD; 50 $\mu$M) to block HCN channels, exposure to DEA had no effect on holding current or conductance. C: summary data showing DEA-induced conductance ($\Delta G$) in $\text{Ba}^{2+}$ ($n = 10$), $\text{Cs}^+$ ($n = 25$), and $\text{Ba}^{2+}$ plus $\text{Cs}^+$ or ZD ($n = 8$). These results suggest that effects of NO on motoneurons involve inhibition of a background $\text{K}^+$ channel and activation of a $\text{Cs}^+$-sensitive $I_h$-like current.
Evidence suggests that long-term exposure to NO inhibits a TASK-like conductance in hypoglossal motoneurons (Gonzalez-Forero et al. 2007); therefore, we considered the possibility that TASK channels also contribute to acute effects of NO. To test this possibility, we characterized effects of K⁺ channel blockers on the K⁺ component of the DEA-sensitive current. Inhibition of voltage-gated K⁺ channels with tetraethylammonium (TEA; 10 mM) or 4-aminopyridine (4-AP; 50 μM) had no effect on the DEA-sensitive current in Cs⁺ (Fig. 5, A–C). Under these conditions, the I–V relationship of the NO-sensitive current is similar to TASK, i.e., relatively voltage-independent, active at resting membrane potential and reversed near the equilibrium potential for K⁺ (Fig. 5B). 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 the effect of DEA on holding current by 40% and virtually eliminated the residual NO-sensitive current in Cs⁺ (Fig. 5, D–F). In addition, we found in Cs⁺ that bath application of the selective sGC blocker 1H-(1,2,4)oxadiazole(4,3-α)quinoxaline-1-one (ODQ, 10 μM) decreased effects of DEA on holding current by 62 ± 4% (Fig. 6A), suggesting that the signaling pathway responsible for NO modulation of TASK channels in hypoglossal motoneurons is cGMP dependent. Furthermore, in the presence of Cs⁺, bath application of the selective PKG blocker KT-5823 (1 μM) also decreased effects of DEA on holding current by 98 ± 5% (Fig. 6B). These results are consistent with long-term effects of NO/cGMP on TASK channels in hypoglossal motoneurons (Gonzalez-Forero et al. 2007) and suggest that TASK-1 and/or TASK-3 channels are important determinants of NO sensitivity in these cells.

Our evidence also indicates that NO activates an inward current with properties similar to ₊ (i.e., strongly inhibited by Cs⁺ and ZD-7288 but resistant to Ba²⁺). Therefore, to study NO modulation of native HCN channels in relative isolation, we used Ba²⁺ (2 mM) to block background K⁺ channels with minimal effect on HCN channels (Biel et al. 2009). Under these conditions, exposure to DEA (20 μM) decreased holding current by 60.3 ± 3.1 pA and increased conductance by 1.32 ± 0.1 nS (n = 20), indicating that DEA activated an inward current (Fig. 7A). Furthermore, the effects of Cs⁺ on holding current and conductance were 94.7 ± 22% and 75.4 ± 8.3% (n = 9; Fig. 7A) larger in DEA plus Ba²⁺ compared with Ba²⁺ alone, indicating that DEA activates a Cs⁺-sensitive inward current similar to ᵣ. Interestingly, the DEA difference current (Fig. 7B), generated by subtracting current responses to hyperpolarizing voltage steps recorded in DEA plus Ba²⁺ (Fig. 7B2) from those recorded in Ba²⁺ alone (Fig. 7B1), shows that at more negative potentials, DEA increased ᵣ to a greater extent than ᵣ. This can also be seen in superimposed current trajectories in response to a −150-mV step in Ba²⁺ alone and DEA plus Ba²⁺ and in the summary data plotted as amplitude

Fig. 5. NO inhibits a TASK-like current in hypoglossal motoneurons. A: traces of holding current and conductance show that in Cs⁺ (2 mM) to block hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and tetraethylammonium (TEA; 10 mM) and 4-aminopyridine (4-AP; 50 μM) to block voltage-gated K⁺ channels, exposure to DEA (20 μM) decreased outward current and conductance. B: 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⁺. C: summary data (n = 6) show DEA-induced changes in holding current in Cs⁺ alone and in Cs⁺ with TEA and 4-AP. D: traces of holding current and conductance show that in Cs⁺, exposure to DEA decreased outward current and conductance. However, responsiveness to a second DEA exposure was reduced by methanandamide (Met; 10 μM), a TASK channel blocker. E1 and E2: average I–V relationships in Cs⁺ alone and during exposure to DEA in Cs⁺ with and without Met. F: summary data (n = 6) show that in the continued presence of Cs⁺, subsequent inhibition of TASK channels with Met decreased the DEA-induced change in holding current. *P < 0.05.

J Neurophysiol • doi:10.1152/jn.00827.2011 • www.jn.org
of total $I_h$ at $-150$ mV (Fig. 7C). We determined the effects of DEA on the voltage dependency of $I_h$ activation by measuring tail currents at a fixed potential of $-80$ mV after activating $I_h$ with a series of hyperpolarizing voltage steps. Normalized $I_h$ tail currents were plotted as a function of membrane potential during the initial hyperpolarizing steps and fitted with a Boltzmann function to determine the half-activation voltage ($V_{1/2}$) (Serios et al. 2002). The $V_{1/2}$ for $I_h$ activation averaged $-103.6 \pm 3.3$ mV in control conditions and $-100.3 \pm 3.0$ mV in DEA; thus DEA caused a small depolarizing shift of $-3$ mV ($P < 0.015$; $n = 5$) (Fig. 7D). In addition, the I-V relationships of normalized DEA difference currents measured after the capacitance artifact ($I_{inst}$) and at the end of the voltage step ($I_h$) show that DEA increased $I_{inst}$ but blunted inward rectification of $I_h$ to the extent that amplitude of $I_{inst}$ at $-150$ mV was greater than that of $I_h$ (Fig. 7E). To confirm that DEA activates the instantaneous component of $I_h$, we used established protocols to estimate the reversal potential of $I_{inst}$ (Bayliss et al. 1994; Mayer and Westbrook 1983) under control conditions and in the presence of DEA. The $I_{inst}$ was measured immediately following the capacitive transient and plotted against membrane potential at the same time point and fitted with linear regression. The point of intersection between these regression lines represents the reversal potential of the instantaneous current activated by DEA. As shown in Fig. 7F, DEA increased slope conductance, and the extrapolated intersection is near the predicted reversal potential for $I_h$ ($-39$ mV). These results suggest that the instantaneous current activated by DEA is $I_h$.

The observed effects of DEA on HCN channels differ from the well-known effects of cyclic nucleotides on $I_h$ (DiFrancesco and Tortora 1991); suggesting this novel form of HCN channel modulation is conferred by S-nitrosylation. Consistent with this possibility, we found that effects of DEA (in Ba$^{2+}$) on holding current and conductance were fully retained when activity of sGC was blocked with ODQ (20 $\mu$M) (Fig. 8A). There are no selective blockers of S-nitrosylation signaling; however, this is a reduction-oxidation reaction that requires the thiol group of cysteine residues to be in the reduced state. Therefore, we used the cysteine-specific oxidant N-ethylmaleimide (NEM; 300 $\mu$M) to oxidize cysteine residues and thus occlude subsequent S-nitrosylation. Exposure to NEM decreased holding current and conductance by 82.77 $\pm 14.6$ pA ($n = 6$) and 2.63 $\pm 0.57$ nS ($n = 6$), respectively. In NEM, the DEA-induced inward current was reduced by 92.7 $\pm 11.0$% (Fig. 8B), suggesting that NO modulates HCN channels by an S-nitrosylation-dependent mechanism.

**DISCUSSION**

Nitric oxide regulates numerous physiological processes, including rhythmic activity of respiratory motoneurons. However, molecular mechanism(s) underlying NO sensitivity of respiratory motoneurons is not known. Here, we have shown that NO targeted two different types of ion channels in hypoglossal motoneurons (background TASK channels and HCN channels) and modulated them in opposite directions by two distinct signaling pathways: NO inhibited TASK channels by a cGMP-dependent mechanism and activated HCN channels by what appears to be an S-nitrosylation-dependent mechanism. In contrast to the well-known effects of cyclic nucleotides on HCN channels (DiFrancesco and Tortora 1991), we found that modulation by S-nitrosylation increased $I_{inst}$ to a greater extent than $I_h$ at more hyperpolarized potentials, suggesting that $I_{inst}$ can be differentially regulated by NO. In addition, when TASK and HCN channels were blocked, exposure to NO had no effect on holding current or conductance. These results identify TASK and HCN channels as likely molecular substrates responsible for NO modulation of hypoglossal motoneurons.

It is well established that NO can modulate synaptic activity (Prast and Philippu 2001); therefore, we performed all voltage-clamp experiments in the presence of TTX to block action potentials and thus Ca$^{2+}$-dependent transmitter release. However, it is possible that NO may increase spontaneous release of neurotransmitters. For example, NO has been shown to increase Ca$^{2+}$-independent synaptic release (Meffert et al. 1994), and conceivably this effect would be retained in the presence of TTX. However, we did not observe an increase in miniature postsynaptic potentials during DEA application, and our results are consistent with previous evidence that NO modulates

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Fig. 6. NO modulation of TASK channels in hypoglossal motoneurons involves cGMP and PKG. A: traces of holding current and conductance show that when HCN channels were blocked with Cs$^+$ (2 mM), exposure to DEA decreased holding current and conductance by $-25$ pA and $-0.8$ nS, respectively. In the continued presence of Cs$^+$, a second exposure to DEA, this time in the presence of 1H-(1,2,4)oxadiazole(4,3-c)quinoline-1-one (ODQ; 20 $\mu$M), decreased holding current and conductance by only $-13$ pA and $-0.3$ nS, respectively. The asterisks designate a 10-min time break. Right, summary data ($n = 13$) show that ODQ decreased DEAsensitivity of the TASK-like conductance. *$P < 0.05$. B: traces of holding current and conductance show another example where in Cs$^+$, exposure to DEA decreased holding current and conductance by $-30$ pA and $-2$ nS, respectively. A second exposure to DEA in Cs$^+$, this time after $-4$ min of incubation in KT-5823 (1 $\mu$M), had negligible effects on holding current or conductance. Right, summary data ($n = 4$) show that KT-5823 decreased DEA sensitivity of the TASK-like conductance. *$P < 0.05$.  

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**DISCUSSION**

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intrinsic excitability of hypoglossal motoneurons even in the presence of a cocktail of synaptic blockers (Gonzalez-Forero et al. 2007; Montero et al. 2008). Therefore, these results suggest that NO effects are, at least in part, independent of synaptic transmission. Another limitation of our experimental approach is that exogenous application of 20 μM DEA does not exactly mimic endogenous production of NO by NOS1-expressing terminals in the HMN. Nevertheless, the concentration of DEA used here is near the EC50 for hypoglossal motoneurons and similar to those required to mimic endogenous NO effects on intrinsic excitability of hypoglossal motoneurons (Gonzalez-Forero et al. 2007), and so we consider it a reasonable approximation of physiological NO concentration.

**NO/cGMP signaling inhibits a TASK-like current in hypoglossal motoneurons.** Evidence indicates that acute NO exposure depolarized membrane potential of brain stem motoneurons without corresponding changes in input resistance (Abudara et al. 2002; Gonzalez-Forero et al. 2007), suggesting that NO affects multiple channels with offsetting effects on net membrane depolarization and increased excitability. However, it should be noted that bath application of Cs+ can also block inward-rectifying K+ (Kir) channels (Hagiwara et al. 1976); therefore, it remains possible that Kir channels in addition to TASK may also contribute to NO modulation of hypoglossal motoneurons. In addition to TASK, several other members of the KCNK family of background K+ channels are modulated by NO, including TREK-1 (Koh et al. 2001) and TALK-1 and TASK-3 channel subunits appear to be expressed by hypoglossal motoneurons (Gonzalez-Forero et al. 2007).}

![Image](http://jn.physiology.org/)

**Fig. 7. NO increases the instantaneous (Iinst) and time-dependent (Iss) components of I_\text{K} \text{(Kir)} channels.** The traces of holding current and conductance show that in Ba2+ (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 a–e (scale bars are 500 ms and 500 pA). B1–B3: current responses to hyperpolarizing voltage steps recorded in Ba2+ alone (B1), Ba2+ plus DEA (B2), and the corresponding difference current (B3) show that DEA increased both I_{\text{inst}} and I_{\text{ss}} but at more hyperpolarized potentials NO increased I_{\text{inst}} to a greater extent than I_{\text{ss}}. This can also be seen in superimposed current responses to a −150-mV step in Ba2+ alone and Ba2+ plus DEA (B4). C: average I_{\text{inst}} amplitude plotted as I_{\text{inst}} and I_{\text{ss}} evoked by a −150-mV step in Ba2+ alone (n = 8) and Ba2+ plus DEA (n = 8). *P < 0.05; **P < 0.001. D: I_{\text{inst}} activation curves in Ba2+ alone and in Ba2+ plus DEA. Inset: DEA caused a small (3 mV) depolarizing shift in I_{\text{ss}} activation. E: average (n = 8) I-V relationships of the NO-sensitive I_{\text{inst}} and I_{\text{ss}} currents. *P < 0.05. F: I-V relationships of I_{\text{inst}} in Ba2+ alone and in Ba2+ plus DEA. The data were fitted with linear regression, and the extrapolated intersection of the regression lines is the reversal potential (E_{\text{rev}}) for I_{\text{ss}}.

**Detected an NO-sensitive K+ conductance that was voltage independent, active at resting membrane potential, and resistant to TEA or 4-AP but inhibited by methanandamide.** These properties are most consistent with TASK channels. Therefore, we used Cs+ or ZD-7288 to block HCN channels. Under these conditions, we detected an NO-sensitive K+ conductance that was voltage independent, active at resting membrane potential, and resistant to TEA or 4-AP but inhibited by methanandamide. These properties are most consistent with TASK channels.
Fig. 8. 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+} (2 mM) activated an inward current with properties similar to I_{h} (Figs. 3 and 5). Inhibition of soluble guanylyl cyclase with ODQ (10 μM) did not affect NO modulation of I_{h}. Bar graphs at right show summary data (n = 6) plotted as DEA-induced changes in holding current and conductance in Ba^{2+} alone and in 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 incubation in the cysteine-specific oxidant N-ethylmaleimide (NEM; 300 μM) this time had little effect on holding current or conductance. Bar graphs at right show summary data (n = 5) plotted as DEA-induced changes in holding current and conductance in Ba^{2+} alone and in Ba^{2+} plus NEM. Together, these results strongly support the possibility that NO activates I_{h} by a S-nitrosylation-dependent mechanism.

properties distinct from homomeric TASK-1 channels in basal forebrain cholinergic neurons.

NO activates I_{h} by a mechanism consistent with S-nitrosylation. HCN channels (HCN1–4) are members of the subgroup of cyclic nucleotide-regulated cation channels and function as the molecular correlate of I_{h}. It is well documented that cAMP can bind HCN channels to cause a depolarizing shift in I_{h} (DiFrancesco and Tortora 1991). This cAMP-dependent modulation of I_{h} is crucially important for rhythmic activity of pacemaker cells in the heart and spontaneous activity of certain neurons. However, it is less clear whether NO-mediated increase of cGMP works in a similar fashion to increase I_{h} or, alternatively, whether NO activates I_{h} by a cGMP-independent mechanism involving S-nitrosylation. It has been shown that cGMP can bind HCN channels and cause a depolarizing shift in I_{h} gating; however, the affinity of HCN channels for cGMP is ~30-fold lower than cAMP (Zagotta et al. 2003). Nevertheless, there is some evidence in the brain and heart that NO/cGMP signaling increases I_{h}. For example, studies in trigeminal motoneurons and deep cerebellar neurons showed that NO facilitated an I_{h}-dependent depolarizing sag in the voltage response to hyperpolarizing current injection (Abudara et al. 2002; Wilson and Garthwaite 2010); in cardiac pacemaker cells, NO and a cell-permeable nonhydrolyzable cGMP analog (8-BrcGMP) stimulated heart rate by activation of a Cs^{+}-

sensitive current similar to I_{h} (Musialek et al. 1997); and in thalamocortical neurons, NO activated I_{h}, and this response was mimicked by 8-BrcGMP (Pape and Mager 1992). These results suggest that NO/cGMP can modulate HCN channels; however, this does not exclude the possibility that HCN channels can also be modulated by S-nitrosylation signaling.

To explore NO modulation of HCN channels in hypoglossal motoneurons, which express HCN1 and HCN2 and a prominent I_{h} (Chen et al. 2005, 2009), in relative isolation we blocked background TASK channels with high concentrations of Ba^{2+}. Under these conditions, we found that NO activated I_{h} by a cGMP-independent process, whereas incubation in NEM, which oxidizes sulfhydryl groups, renders them resistant to S-nitrosylation, blocked effects of NO on I_{h}. These results suggest that, in addition to the potential cGMP-dependent effects described above, HCN channels (e.g., HCN1 and/or HCN2) may also be modulated by S-nitrosylation. Considering that HCN2 but not HCN1 channels are strongly activated by cyclic nucleotides, it is possible that the observed cGMP-independent modulation of I_{h} in hypoglossal motoneurons reflects an HCN1-specific effect; however, there are no HCN subunit-specific blockers, so this possibility has yet to be elucidated. Our results provide functional support for the possibility that HCN channels are neural substrates for S-nitrosylation (Jaffrey et al. 2001). These results are also consistent with reported effects of cGMP and S-nitrosylation on structurally related cyclic nucleotide-gated channels (Broillet and Firestein 1996), thus further suggesting that cGMP and S-nitrosylation can function in parallel to independently modulate the same target protein. In addition, we found that NO increased both the instantaneous and time-dependent components of I_{h}; however, surprisingly, at more negative potentials NO preferentially increased I_{inst} compared with I_{ss}. These results are consistent with current-clamp evidence suggesting that when background K^{+} channels were blocked with Ba^{2+} exposure to NO decreased input resistance (i.e., increased conductance) but with no corresponding change in the amplitude of depolarizing sag. Considering that depolarizing sag reflects the difference between instantaneous and steady-state I_{h}, it is possible that the preferential activation of I_{inst} by DEA offset the contribution of the time-dependent component of I_{h} to sag. This finding differs from a study in thalamocortical neurons where the NO donor SIN1 activated I_{ss} with little effect on I_{inst} (Pape and Mager 1992). However, it should be noted that SIN1 produces superoxide as a by-product, which may oxidize cysteine residues (Thomas et al. 2002) and occlude the potential contribution of S-nitrosylation. These results may represent the first evidence that NO can change HCN channel activity by selectively enhancing instantaneous I_{h}. Activation of I_{h} can depolarize resting membrane potential and increase the firing rate response to depolarizing current injections (Mccaferri and McBain 1996). Therefore, activation of I_{h} likely contributes to the excitatory effects of NO on hypoglossal motoneurons.

To identify potential S-nitrosylation sites in HCN1 and HCN2, we compiled a list of 898 unique S-nitrosylation sites in mouse and used the motif-x and scan-x algorithms (Schwartz and Gygi 2005; Schwartz et al. 2009) to extract common S-nitrosylation motifs and corresponding position weight matrices. These position weight matrices were then used to scan the protein sequences with positions scoring better than 50% of
known mouse S-nitrosylation sites being deemed potential modification sites. The most probable S-nitrosylation site in HCN1 was predicted to be at Cys531 in the COOH-terminal region and in HCN2 at Cys341 in the cytoplasmic loop between S4 and S5. Notably, the putative S-nitrosylation site in HCN2 is near a basic residue (Arg339) that is important for voltage-dependent channel gating; mutations in Arg339 prevented normal channel closure and increased $I_{\text{ins}}$ (Decher et al. 2004). In light of these results and our evidence that S-nitrosylation preferentially increased $I_{\text{ins}}$ at more negative potentials, we hypothesize that S-nitrosylation of Cys341 stabilizes the channel’s open state. However, this possibility requires further investigation using recombinant channels expressed in a heterologous expression system.

**Physiological significance.** The HMN controls GG muscle activity and is important for a variety of physiological functions including maintenance of airway patency, mastication, and swallowing. It has been known for more than a decade that the H MN receives nitrergic innervation (Pose et al. 2005; Travers et al. 2005), yet potential involvement of NO in H MN function remains obscure because there is limited evidence describing effects of NO on H MN activity in vivo. A study by Montero et al. (2008) showed that microiontophoretic application of an NOS inhibitor or ODQ decreased inspiratory-related H MN activity, whereas injection of DEA or 8-BrcGMP did the opposite. These results are consistent with our evidence that microperfusion of DEA into the H MN increased GG activity, and together these results indicate that NO can influence activity and function of the H MN in vivo. These results suggest that loss of nitrergic drive to the H MN may decrease H MN activity and result in a loss of airway patency. On the other hand, excessive production of NO, as associated with degenerative disorders or injury, may lead to excitotoxicity and motoneuron degeneration (Gonzalez-Forero et al. 2007; Montero et al. 2010; Moreno-Lopez and Gonzalez-Forero 2006; Moreno-Lopez et al. 2011). Therefore, NO/cGMP and S-nitrosylation may represent new avenues for the treatment of pathological conditions resulting from loss of H MN control.

**ACKNOWLEDGMENTS**

We thank Dr. Daniel Schwartz for help in identifying potential S-nitrosylation sites in HCN channels.

**GRANTS**

This work was supported by funds from National Institutes of Health Grant HL104101 (to D. K. Mulkey) and Canadian Institutes of Health Research Grant MT-15563. R. L. Horner is supported by a Tier 1 Canada Research Chair in Sleep and Respiratory Neurobiology.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


