TITLE: Regulation of electrical activity and neuronal excitability in *Helisoma trivolvis* by carbon monoxide

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Abbreviations

Action potential (AP)

Carbon monoxide (CO)

Carbon monoxide releasing molecule-2 (CORM-2)

Dimethyl sulfoxide (DMSO)

Nitric oxide (NO)

N-methyl-d-glucamine (NMDG)

Persistent sodium current (I_{NaP})

Resting membrane potential (RMP)

Small-conductance calcium-activated potassium (SK)

Voltage-gated calcium channel (VGCC)

Abstract

Carbon monoxide (CO), like other gaseous neuromodulators, has a dual nature as both a toxic gas and a physiologically relevant signaling molecule. In the nervous system, high concentrations of CO can lead to neuronal injury while lower concentrations are found to be neuroprotective. The number of cellular targets affected by physiological concentrations of CO are rapidly growing and include ion channels in various cell types. The modulation of ion channels by CO in neurons, however, and the effect it has on neural activity are incompletely understood. Here, the well-characterized buccal neurons, B5 and B19, of the freshwater snail, *Helisoma trivolvis*, were used to investigate the role that CO plays in regulating spontaneous firing activity and neuronal excitability. Neurons were studied in single cell culture, thereby removing other signals normally present in the intact nervous system and allowing for the optimal characterization of physiological effects of CO. We found that the CO donor molecule, carbon monoxide releasing molecule-2 (CORM-2), hyperpolarized the resting membrane potential of B5 neurons and silenced their spontaneous firing activity. These effects were mediated through the inhibition of a persistent sodium current. CORM-2 also inhibited neuronal excitability. This effect was mediated by the inhibition of voltage gated calcium channels by CO. The general findings of CO acting as a hyperpolarizing signal and an inhibitor of neuronal excitability extended to B19 neurons. Taken together, these findings suggest that CO is a potent modulator of ion channels with broad implications for the modulation of neural activity in a wide range of neuron-types.

Keywords: CO, Neuron, Voltage-gated calcium channel, Persistent sodium current, CORM-2

1 Introduction

Carbon monoxide (CO) is typically perceived as a toxic gas; however, the identification of its endogenous production in numerous species (Sjostrand, 1949, Maines, 1997, Wu and Wang, 2005) has led to its description as an important physiological signaling molecule. Within the nervous system, CO affects mammalian circadian rhythms (Artinian et al., 2001), learning and memory (Cutajar and Edwards, 2007), nociception (Steiner et al., 2001, Carvalho et al., 2011), and olfaction (Ingi and Ronnett, 1995). In non-mammalian models, CO affects the sensitivity of olfactory receptor neurons of the salamander, *Ambystoma tigrinum*, (Zufall and Leinders-Zufall, 1997) and neuronal migration of enteric neurons of the locust, *Locusta migratoria* (Knipp and Bicker, 2009), indicating that CO serves as a neuronal signal across a range of species affecting numerous aspects of the nervous system. In recent years, it has become increasingly clear that one of the major mechanisms by which CO can elicit its physiological effects is through the modulation of ion channels (Wilkinson and Kemp, 2011, Peers et al., 2014), however, the roles of this modulation within the nervous system are not fully understood.

Spontaneously firing neurons utilize a combination of ion channels to bring the resting membrane potential (RMP) to threshold and initiate intrinsically produced action potentials (APs) in the absence of presynaptic input. Inhibition of these channels, including hyperpolarization-activated cyclic nucleotide-gated channels, a persistent sodium channel, and a low voltage-gated calcium channel, have been reported to hyperpolarize the RMP and silence spontaneous firing activity (Ghamari-Langroudi and Bourque, 2000, Chevalier et al., 2006, Chu and Zhen, 2010, Xie et al., 2011, Artinian et al., 2012). Conversely, activation of these currents increases spontaneous firing activity (Nakashima et al., 2013). We have previously shown that the gaseous signaling molecule, nitric oxide (NO), increases neuronal firing activity of

regenerating snail neurons, a condition thought to recapitulate an earlier developmental state when these neurons undergo neurite outgrowth, through the modulation of three ionic conductances: persistent sodium current (I_{NaP}), voltage-gated calcium current (VGCC) and small-conductance calcium-activated potassium channel (SK) current (Artinian et al., 2010, Artinian et al., 2012). Moreover, the effects of NO on growth cone morphology were dependent on the production of APs and their shape (Estes et al., 2014), linking the modulation of firing activity by gaseous signals to their developmental effects. Given that CO, like NO, is produced in nervous systems (Wu and Wang, 2005), is present during development (Raju et al., 1997, Bergeron et al., 1998, Li Volti et al., 2004), and shares similar cellular targets, we here tested whether CO affected neuronal firing activity of neurons as well.

While we have evidence that a CO producing, heme oxygenase-like enzyme (unpublished data) is present in the CNS of the freshwater snail, *Helisoma trivolvis*, signaling roles for CO in the gastropod CNS are presently not known. We therefore started a first investigation into CO as a potential mediator of electrical activity. To do this, experiments were performed on well-characterized *Helisoma* buccal neurons, B5 and B19. Neurons were grown in single cell culture, which provides optimal conditions for the direct study of the effects of CO on firing activity, while excluding potential confounds of signaling molecules produced by other cells. Using whole-cell patch clamp techniques, we identified CO as a potent modulator of both spontaneous firing activity and neuronal excitability. Examination of the ion channels responsible for these effects revealed that CO hyperpolarized the membrane potential in B5 neurons through the inhibition of I_{NaP} , and that CO decreased neuronal excitability through the inhibition of VGCC. The effects of CO on multiple ionic conductances suggested that CO can serve as a potent modulator of electrical activity. Using another neuron from the buccal ganglia, B19, we found

that the general effects of CO on RMP and excitability extended to this type of neuron as well. Collectively, this study implicates CO as a potentially important effector of neuronal function and developmental/regenerative events that are dependent on neuronal electrical activity.

2 Experimental procedures

2.1 Neuronal Culture

Identified neurons, B5 and B19, were dissected from the buccal ganglia of the freshwater snail, *Helisoma trivolvis*, and plated onto poly-L-lysine (hydrobromide; molecular weight of 70,000–150,000; 0.25 mg/ml; Sigma) coated glass coverslips glued to the bottom of 35 mm cell culture dishes (Falcon 1008), as previously described (Estes et al., 2014). In brief, neurons were cultured for 24–48 hours in 2 mL of conditioned medium at room temperature. Neurons displaying neurites that were at least 2 cell body lengths long (approximately 180 μ m) from the soma were used for experiments. Conditioned medium was generated by incubating two *H. trivolvis* cerebral ganglia per 1 mL of Leibowitz L-15 medium (Invitrogen, Carlsbad, CA) for 4 days (Wong et al., 1981). L-15 consisted of the following (mM): 44.6 NaCl, 1.7 KCl, 1.5 MgCl₂, 0.3 MgSO₄, 0.14 KH₂PO₄, 0.4 Na₂HPO₄, 1.6 Na pyruvate, 4.1 CaCl₂, 5 HEPES, 50 μ g mL⁻¹ gentamicin, and 0.15 mg mL⁻¹ glutamate in distilled water, pH 7.4.

2.2 Electrophysiology

Electrical activity of cultured *H. trivolvis* B5 and B19 neurons was recorded in whole-cell current-clamp configuration, as previously described (Artinian et al., 2012, Zhong et al., 2013c, b). Briefly, patch electrodes were pulled from borosilicate glass tubes (OD 1.5 mm; ID 0.86 mm; Sutter Instruments) using a Sutter Instruments micropipette puller (P-87) and heat polished (Micro Forge MF-830; Narishige) with a resistance of 5–10 M Ω . To obtain whole-cell patch, petri dishes containing cultured neurons were placed on a fixed stage that was centered below the objectives of an upright microscope (BX51W1F, Olympus, Japan). Electrical signals from neurons were recorded using an Axopatch 700B amplifier (Molecular Devices, Union City, CA)

and analog-to-digital converter (Digidata 1440). Acquisition and analysis of data were conducted using pClamp software version 10 (Molecular Devices). Current-clamp mode was used to evaluate membrane potential, spontaneous and evoked firing properties. Voltage-clamp mode (holding voltage = -60 mV) was used to evaluate the whole-cell configuration parameters as well as to generate voltage-current protocols for measuring I_{NaP} and calcium currents. All voltage and current clamp recordings were conducted using Leibowitz L-15 medium as the extracellular recording solution and an intracellular solution in the recording pipette consisting of (mM): 54.4 K-aspartate, 2 $MgCl_2$, 5 HEPES, 5 Dextrose, 5 ATP, and 0.1 EGTA (127 mOsm), except for calcium channel protocols. For recording of calcium channel activity, L-15 was replaced with an extracellular calcium solution (Hui and Feng, 2008) consisting of (mM): 10 $CaCl_2$, 45.7 TEA-Cl, 1 $MgCl_2$, 10 HEPES, and 2 4-AP, pH adjusted to 7.4 using TEA-OH. In addition, the intracellular solution for the calcium channel recording consisted of (mM): 29 CsCl, 2.3 $CaCl_2$, 2 MgATP, 0.1 GTP-Tris, 11 EGTA, 10 HEPES, pH adjusted to 7.4 by CsOH. The liquid junction potential for each recording paradigm was calculated using Clampex software (v10; Molecular Devices) and found to be approximately -15.7 mV for the standard recording solutions and -8.6 mV for calcium recording solutions. Reported voltages were corrected for the liquid junction potential post hoc, as shown previously (Carter et al., 2012).

2.2.1 Measurement of resting membrane potential (RMP) and excitability

The resting membrane potential of spontaneously firing neurons was determined in current clamp by measuring the voltage at the plateau of the depolarization phase before the membrane potential reached threshold, as previously described (Zhong et al., 2015). The maximum change in membrane potential was measured as the difference in baseline voltage

before and after drug treatment. In both spontaneously firing and silent neurons, the excitability of a neuron was measured as the number of spikes produced by the injection of depolarizing current (0.1, 0.5, and 1.0 nA) for 1 s. Spikes that were less than a third of the initial AP amplitude were omitted from the spike count.

2.2.2 Measurement of persistent sodium channel current (I_{NaP})

Characterization of the I_{NaP} was conducted as previously described (Harvey et al., 2006, Artinian et al., 2012). In brief, I_{NaP} was measured in voltage-clamp mode by applying a slow voltage ramp from -95.7 to -45.7 mV by an increment of 5 mV/s. Using the linear current from the passive leak conductance, measured between -95.7 and -85.7 mV, we extrapolated the fit to obtain the peak current amplitude. Currents were analyzed by normalizing the peak inward current for each cell to the cell capacitance (pA/pF).

2.2.3 Measurement of voltage-gated calcium currents

To study total calcium currents, membrane potential was held at -68.6 mV and stepped from -68.6 mV to +68.6 mV for 500 ms by increments of +10 mV, as previously shown (Artinian et al., 2012, Zhong et al., 2013a). To characterize high VGCC currents, the membrane potential was first stepped to -28.6 mV for 200 ms to fully inactivate the low VGCC currents, followed by stepping the membrane potential from -68.6 to +68.6 mV for 500 ms. Low VGCC currents were calculated by subtraction of high VGCC currents from total calcium currents (Haydon and Man-Son-Hing, 1988). Currents were analyzed by normalizing the peak inward current for each cell to the cell capacitance (pA/pF).

2.3 Pharmacological Agents

The carbon monoxide donor, carbon monoxide releasing molecule-2 (CORM-2; Tricarbonyldichlororuthenium(II) dimer; Sigma-Aldrich, catalogue # 288144) was dissolved in dimethyl sulfoxide (DMSO) to make a stock of 50 mM. CORM-2 was mixed with L-15 10 minutes following the generation of stock solution allowing for saturation of CO at final concentrations of 5, 50, and 100 μ M. Riluzole (Sigma-Aldrich) was dissolved in DMSO at a stock concentration of 40mM and diluted in L-15 to a final concentration of 20 μ M. To control for potential effects of the stable byproducts of CORM-2 once CO was liberated, we generated an inactive form of CORM-2 (iCORM-2)(Sun et al., 2008). Briefly, fresh CORM-2 stock was degassed overnight at 37°C. To remove residual CO left in the solution, nitrogen was bubbled through the solution for 1 hour and then left to off-gas any of the remaining nitrogen for an additional 10 minutes. The final solution was filtered to remove any potential contamination, separated into aliquots, and stored at -20°C. All drugs were mixed with L-15 and bath applied to obtain final concentrations.

2.4 Statistics

All values described were expressed as mean $\pm$ SEM, unless otherwise stated. Data plots were designed using ORIGIN DATA ANALYSIS AND GRAPHING software (version 9.1, OriginLab, Northampton, MA). The significance of effects between control and treatment was evaluated using the Mann-Whitney U test and the Kruskal-Wallis test for comparison of effects over time (SPSS statistical software, SPSS, Chicago, IL). Comparison of the effects before and after treatment were evaluated with the Wilcoxon-Signed-Ranks Test. Significant differences are indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3 Results

3.1 Carbon monoxide hyperpolarizes V_m and silences spontaneous firing activity

We showed previously that the gaseous signaling molecule, nitric oxide (NO), is capable of increasing the spontaneous firing activity and excitability of B5 neurons regenerating in vitro (Artinian et al., 2010). CO, like NO, is produced in the nervous system (Wu and Wang, 2005), however, we lack a clear understanding of the potential roles of CO in the snail nervous system. Therefore, we wanted to test whether CO also affected neuronal firing activity of developing neurons and evaluate its potential role as a signaling molecule in the snail CNS. To characterize the effects of CO on electrical activity, we used the CO-donor, carbon monoxide releasing molecule-2 (CORM-2), which is known to have physiologically relevant signaling effects at concentrations ranging from 20 to 210 μ M (Motterlini et al., 2002, Knipp and Bicker, 2009). CORM-2 was bath applied to isolated B5 neurons while their electrical activity was recorded using a whole-cell current clamp configuration. Prior to CO treatment, B5 neurons had resting membrane potentials (RMP) of -45.1 ± 0.8 mV ($n = 25$) and were spontaneously active producing a range of firing frequencies from 0.4 to 1.6 Hz, similar to our previous findings (Artinian et al., 2010, Zhong et al., 2013a). Following treatment with CORM-2 (5, 50, or 100 μ M), all neurons showed hyperpolarization of the RMP and a decrease in firing frequency [Fig. 1 (A)]. At 5 μ M, CORM-2 hyperpolarized the RMP by an average of -9.2 ± 0.6 mV ($n = 5$) [Fig. 1 (Ai and B)], which was statistically significant when compared with the solvent control DMSO (-0.27 ± 1.7 mV, $n = 7$, MWU, $U = 0$, $p = 0.004$). In 2 out of the 5 neurons, 5 μ M CORM-2 also resulted in the silencing the spontaneous firing activity. Increasing the CORM-2 concentration to 50 μ M [Fig. 1(Aii)] and 100 μ M [Fig. 1(Aiii)] also led to a significant hyperpolarization of the RMP compared to the solvent control (50 μ M: -9.1 ± 1.4 mV, $n = 10$, MWU, $U = 4$, $p = 0.002$; 100 μ M: -11.0 ± 3.4 mV, $n = 5$, MWU, $U = 5$, $p = 0.042$) [Fig. 1 (B)] but did not significantly

change the RMP beyond what was observed with 5 μ M CORM-2 (MWU, 50 μ M: $U = 24$, $p = 0.903$; 100 μ M: $U = 8$, $p = 0.347$). However, at higher CORM-2 concentrations, the spontaneous firing activity of all cells was silenced within 4 ± 1 min ($n = 10$) and 4 ± 1 min ($n = 5$) at 50 and 100 μ M, respectively, suggesting that CO can serve as a potent modulator of spontaneous firing activity. To verify that the effects of CORM-2 were due to CO, and not to a byproduct of CORM decomposition, we degassed CORM-2 generating inactive CORM-2 (i-CORM-2). Application of iCORM-2 did not significantly affect the RMP (MWU, $U = 22$, $p = 0.749$, $n = 7$) [Fig. 1 (B)], suggesting that it was indeed CO that effected spontaneous activity. Given that 50 μ M concentration of CORM-2 produced a significant hyperpolarization of the RMP within a few minutes and resulted in a consistent silencing of spontaneous activity, we utilized this concentration from here on to further characterize the effects of CO on electrical activity. We next wanted to identify which ionic conductance was modulated by CO leading to the hyperpolarization of the RMP.

3.2 CO inhibits a persistent sodium current to hyperpolarize the membrane potential

In previous studies, we found that B5 neurons contain a persistent sodium current (I_{NaP}) that is active at hyperpolarized membrane potentials and contributes to the RMP as well as to spontaneous firing activity (Artinian et al., 2012); therefore, we tested first whether the CO-induced hyperpolarization of the RMP involved modulation of I_{NaP} . To determine the effects of CO on I_{NaP} , we used a slow-voltage ramp protocol from -95.7 to -45.7 mV, which inactivates fast-inactivating voltage-gated ion channels allowing for the characterization of I_{NaP} (Harvey et al., 2006). We observed an inward current that developed around -75.7 mV and peaked at -45.7 mV [Fig. 2 (A)], which was similar to our previous findings where we characterized I_{NaP} using this protocol in these neurons (Artinian et al., 2012). Bath application of CORM-2 inhibited I_{NaP}

within 5 minutes by -32.3 ± 6.5 % of baseline ($n = 7$). This inhibition was found to be significant when compared with the solvent control (MWU, $U = 2$, $p = 0.033$, $n = 4$) and was sustained over time [Fig. 2 (B)]. To verify whether the overall inhibition of I_{NaP} by CORM-2 was due to CO, we tested for the effects of iCORM-2 and found that it did not significantly inhibit I_{NaP} after 30 minutes of incubation compared with the solvent control (I_{NaP} : DMSO: -9.2 ± 9.6 %, iCORM-2: -14.1 ± 7.1 %; MWU, $U = 7$, $p = 0.773$, $n = 4$).

Given that the timing of I_{NaP} inhibition and the silencing of spontaneous firing activity both occurred within 5 minutes of CORM-2 treatment, we next wanted to test for the possibility that CO hyperpolarized the RMP and silenced spontaneous firing activity through the inhibition of I_{NaP} . We first inhibited the I_{NaP} with riluzole ($20 \mu\text{M}$) and measured the change in RMP. Riluzole treatment significantly hyperpolarized the membrane potential from baseline by an average of 25.7 ± 4.8 % from -44.2 ± 0.6 mV to -55.7 ± 2.6 mV (Wilcoxon Signed Ranks Test, $p = 0.012$, $n = 8$) and silenced spontaneous firing activity. These findings were similar to what we previously reported in B5 neurons (Artinian et al., 2012). Next, we added CORM-2 following riluzole treatment to determine whether CO could hyperpolarize the RMP further when I_{NaP} was already inhibited [Fig. 2 (C)]. CORM-2 did not add to the hyperpolarization when compared to riluzole treatment alone (MWU, $U = 27.5$, $p = 0.636$, $n = 8$) [Fig. 2(D)], suggesting that the effect of CO on the RMP can be explained by its inhibition of I_{NaP} . Given that both CORM-2 and riluzole were dissolved in DMSO, we next determined whether additional DMSO might have an effect on the membrane potential. Treatment with DMSO following riluzole treatment did not affect the RMP compared with riluzole treatment alone (MWU, $U = 10$, $p = 0.683$, $n = 3$), suggesting that the increased solvent concentration had no effect on the RMP.

3.3 CO inhibits neuronal excitability

To test whether CO merely caused a hyperpolarization of RMP or also affected the ability of B5 neurons to generate action potentials (APs), we next injected depolarizing current into the neuron to evaluate evoked APs for potential effects of CO on neuronal excitability.

Depolarization of the membrane potential through the injection of current resulted in evoked spiking that increased with the amount of injected current (0.1, 0.5, or 1.0 nA). CORM-2 (50 μ M) treatment inhibited this evoked firing activity over time [Fig. 3(A)]. For the 0.1 nA current injection, CORM-2 treatment led to a rapid nominal decrease in the percentage of evoked APs that became statistically significant 30 minutes post CORM-2 treatment when compared with the solvent control (MWU, $U = 1$, $p = 0.024$) [Fig. 3(Bi)]. Both the 0.5 nA and 1.0 nA current injection showed significant decreases in evoked firing activity within 10 minutes of CORM-2 treatment compared with solvent controls (MWU, 0.5 nA: $U = 2$, $p = 0.021$; 1.0 nA: $U = 0$, $p = 0.009$) that continued to decrease over time [Fig. 3(Bii and Biii)]. iCORM-2 did not significantly affect evoked firing activity induced by 0.1, 0.5 or 1.0 nA current (MWU, 0.1 nA: $U \geq 6$, $p \geq 0.242$; 0.5 nA: $U \geq 4$, $p \geq 0.121$; 1.0 nA: $U \geq 4$, $p \geq 0.123$), suggesting that the effects of CORM-2 on evoked firing activity were due to CO. With CO silencing spontaneous firing activity within 5 minutes [Fig. 1] and evoked firing activity being inhibited 10 minutes following CO treatment, we next investigated which ionic conductance(s) were responsible for the inhibition of evoked APs.

3.4 CO inhibits neuronal excitability through the inhibition of VGCCs.

Previously, we reported that the AP in B5 neurons is calcium-dependent (Artinian et al., 2010) and, therefore, hypothesized that the decrease in neuronal excitability mediated by CO

might involve the modulation of VGCC activity. Using voltage clamp, we applied voltage steps from -68.7 to +51.4 mV to characterize the total calcium current in B5 neurons [Fig. 4(A)]. We observed two inward calcium currents upon stimulation: a low voltage current produced at hyperpolarized voltages with a peak conductance around -30 to -20 mV and a high voltage current produced at relatively more depolarized voltages with a peak conductance around 0 to +10 mV [Fig. 4(B)]. The addition of CORM-2 inhibited both low and high VGCCs over time, with a shift in peak activation voltage for the high VGCCs by 12.5 ± 2.5 mV ($n = 4$). To quantify the effects of CO on high and low VGCC currents, we isolated the contribution of the high VGCC by using a pre-depolarization step to -28.7 mV to inactivate the low VGCC current, followed by voltage steps from -68.7 to +51.4 mV. The contribution of the low VGCC current was calculated by subtracting the high VGCC current from the total current (see Methods). CO reduced both low and high VGCC currents by 76.5 ± 3.8 % and 72.0 ± 16.2 % ($n = 4$), respectively, over a 30 minute time course, with initial reductions in current occurring as early as 5 minutes after CO addition [Fig. 5 (C)]. The inhibition of high VGCC and low VGCC was significant when compared to solvent controls (DMSO: $n = 3$; MWU: High VGCC: $U = 0$, $p = 0.034$; Low VGCC: $U = 0$, $p = 0.034$) [Fig. 5 (C)], suggesting that CO is a potent inhibitor of VGCCs.

3.5 CO similarly modulates neuronal firing activity of B19 neurons

With CO decreasing spontaneous firing activity and neuronal excitability of B5 neurons, we next tested whether these effects extended to another neuron type, buccal neuron, B19. B19 neurons are often silent, and NO has been shown to increase firing activity and neuronal excitability in these neurons (Zhong et al., 2013c). Using two day old B19 neurons in culture, 6 out of 8 neurons were silent, while two B19 neurons were firing spontaneously. Bath application

of CORM-2 (50 μ M) significantly hyperpolarized the RMP by -5.6 ± 2.5 mV ($n = 8$) when compared with the solvent control (DMSO: -0.7 ± 0.6 mV, $n = 5$; MWU, $U = 5$, $p = 0.028$) [Fig. 5(A and B)], and silenced spiking in the two spontaneously firing neurons, suggesting that CO may act as a general inhibitor of spontaneous firing activity. Bath application of iCORM-2 had no significant effect on the RMP of B19 neurons when compared with the solvent control (iCORM-2: -0.1 ± 1.1 mV, $n = 8$; MWU, $U = 19$, $p = 0.884$), suggesting that the hyperpolarization of the RMP by CORM-2 was due to CO.

We next determined whether the effects of CO on the RMP affected the ability of B19 neurons to produce evoked APs. Whereas B19 neurons produced robust spiking activity following 1 nA current injection [Fig. 5(C)], the number of evoked APs was significantly inhibited from baseline recordings by -57.3 ± 13.9 % ($n = 7$) after 30 minutes of CORM-2 treatment when compared to controls (DMSO: -4.0 ± 2.7 %, $n = 5$; MWU, $U = 0$, $p = 0.004$) [Fig. 5(C and D)]. iCORM-2 was not found to significantly affect evoked firing activity (iCORM-2: -11.7 ± 6.8 %, $n = 4$; MWU, $U = 7$, $p = 0.421$), suggesting that CO was responsible for the inhibition of evoked APs. Taken together, these data suggest that CO may act as a general inhibitory signal that hyperpolarizes the RMP of a neuron and decreases neuronal excitability.

4 Discussion

Here, we assessed the role of CO in the modulation of neuronal firing activity using the CO-donor, CORM-2, and found that CO affected firing activity in two ways: 1) It hyperpolarized the RMP, which led to the silencing of spontaneous firing activity and 2) it decreased neuronal excitability [Fig. 6]. These effects appeared to be time dependent and were primarily mediated

through the inhibition of two types of conductances, I_{NaP} and VGCC currents, respectively. We also found that the general effects of CO on the RMP and excitability of B5 neurons extended to another neuron-type, B19. Collectively, our findings suggest that CO can serve as an important modulator of electrical activity in neurons.

4.1 I_{NaP} regulation of the resting membrane potential

Depolarizing currents that are active at hyperpolarized membrane potentials are critical for the generation of spontaneous action potentials. I_{NaP} is a depolarizing current essential for producing spontaneous and rhythmic firing activity in a number of neurons, including hypoglossal neurons (Lamanauskas and Nistri, 2008), spinal neurons (Harvey et al., 2006, Kuo et al., 2006), suprachiasmatic nucleus neurons (Kononenko et al., 2004) and inspiratory neurons (Del Negro et al., 2005). Previously we showed that the replacement of extracellular sodium (Na^+) with N-methyl-d-glucamine (NMDG) hyperpolarized the RMP and silenced spontaneous firing activity in B5 neurons (Artinian et al., 2010), suggesting Na^+ regulates the RMP in B5 neurons. In a subsequent study, we inhibited I_{NaP} with riluzole, and observed similar effects on the RMP and firing activity as with NMDG treatment (Artinian et al., 2012), suggesting that I_{NaP} is responsible for the Na^+ -driven regulation of the RMP. Here, we found that CO inhibited I_{NaP} within 5 minutes of exposure, which was similar in time course to the hyperpolarization of the RMP and silencing of spontaneous firing activity in response to CO. The inhibition of I_{NaP} prior to stimulation with CO prevented additional hyperpolarization of the RMP, indicating that the effects of CO on the RMP and thus the spontaneous firing activity were likely mediated through I_{NaP} , although the possibility that CO could also have acted on another current that was not active or smaller at the hyperpolarized membrane potential upon riluzole treatment cannot be ruled out. While we show that CO inhibits I_{NaP} in neurons, in mammalian tissues CO shows both inhibition

and activation of an epithelial Na^+ current (Althaus et al., 2009, Wang et al., 2009) as well as activation of a sustained sodium current in cardiomyocytes (Dallas et al., 2012), suggesting that the actions of CO on Na^+ currents are cell- and species-specific. It is likely that the underlying mechanisms that control the gating of these channels differ, and while the aim of this study was not to determine the exact mechanism for how CO regulates I_{NaP} , the variable effects of CO on Na^+ currents stress the importance of understanding the molecular identity of these channels and their gating kinetics in future studies.

4.2 VGCC regulation of neuronal excitability

In addition to the silencing of spontaneous firing activity, CORM-2 treatment was found to decrease neuronal excitability. Moreover, these effects were likely due to CO, given that iCORM-2 treatment did not affect the number of evoked APs. While CO was found to modulate I_{NaP} , we showed in a previous study characterizing I_{NaP} using riluzole that there was no discernable effect of I_{NaP} inhibition on evoked firing activity (Artinian et al., 2012). In addition, the replacement of extracellular Na^+ with NMDG showed little impact on the evoked activity of B5 neurons as well (Artinian et al., 2010). Instead, the inhibition of VGCCs with LaCl_3 and CdCl_2 completely blocked evoked APs (Artinian et al., 2010), indicating that the primary conductance for the generation of APs in B5 neurons was through VGCCs. In directly testing for the effects of CO on VGCCs, we found that CORM-2 treatment inhibited VGCCs, which is similar to what has been reported in rat cardiomyocytes (Scragg et al., 2008) and suggests that CO inhibited neuronal excitability through the inhibition of VGCCs. B19 neurons, which have a greater dependency on Na^+ for AP generation (Zhong et al., 2015), also showed decreases in evoked APs following CO exposure. While B5 and B19 neurons showed an overall inhibition of evoked AP by CO, individual differences in ion channel expression may account for cell-specific

modification by CO. We are currently working to investigate the exact molecular identity of ion channels in B5 and B19 neurons to determine how these cell-specific differences may arise.

4.3 Implications of CO in CNS function and development

The endogenous production of CO is primarily mediated by the enzyme heme oxygenase (HO), which is thought to be expressed ubiquitously across phyla and tissue types (Wilks, 2002, Li and Stocker, 2009), however, the amount and location of CO varies based on whether the inducible (HO-1) or constitutive form (HO-2) of the enzyme is present (Maines, 1997). The increased expression of HO-1 following cellular stress or injury has led to the description of CO as an important cytoprotectant (Bilban et al., 2008). Injury to the CNS can result in the hyperexcitability of neurons (Reeves et al., 1995, Kobayashi and Mori, 1998, Gwak and Hulsebosch, 2011), which can lead to aberrant firing activity and neuronal cell death. CO treatment before and up to 3 hours after ischemic injury is neuroprotective by modulating apoptotic and inflammatory signaling pathways (Queiroga et al., 2012, Schallner et al., 2012). Additional reports show that CO can function as a neuroprotectant for a range of CNS pathologies including stroke, pain, and neurodegenerative diseases (Queiroga et al., 2014). With a number of ion channels playing a role in the excitability of a neuron (Armstrong and Hille, 1998), the inhibition of ion channel activity by CO may be another way in which CO can provide neuroprotection. Moreover, the predominance of HO-2 throughout the CNS (Maines, 1997) coupled with growing evidence for CO as an ion channel regulator suggests that CO can provide tonic modulation of neural activity in normal physiological conditions. The exact role that CO plays in modulating neural activity will likely be varied and depend on the synaptic connectivity of the neuron that CO is modulating. For example, while the localized silencing of an excitatory neuron by CO can lead to a decrease in neuronal activity in the downstream synaptic partners of

that neuron, the silencing of inhibitory neurons will have the opposite effect and could result in increased spiking. Therefore, the physiological production of CO in the brain is likely to be well controlled to prevent adverse effects on neuronal electrical properties effecting overall brain activity.

The presence of HO-1 and HO-2 during development (Maines, 1997) suggests that in addition to modulating the activity of a neural circuit, CO can modulate the electrical activity of developing neurons and affect how a neural circuit is formed. Spontaneous and patterned electrical activity generated during neuronal development are vital for axogenesis (Neely and Nicholls, 1995, Yamamoto and Lopez-Bendito, 2012). Disruption of this activity can result in the misguidance of neuronal projections and the formation of inappropriate synapses (Catalano and Shatz, 1998, Hanson and Landmesser, 2004). Electrical activity varies over the course of development for any given neuron and is likely important for establishing distinct neural networks. The growing list of ion channels that are targets of CO modulation coupled with the presence of CO during development (Bergeron et al., 1998, Li Volti et al., 2004), suggests that it may play an important role in the establishment of synaptic connections. For early stages of development, when cell bodies extend neurites, we have shown that the electrical activity in B5 neurons regulates the calcium dynamics of growth cones, the guidance structure of developing axons (Estes et al., 2014). The concentration of calcium within a growth cone is critical for determining an array of guidance behaviors important for the navigation of an axon (Zheng and Poo, 2007). While physiological signaling roles for CO in the gastropod CNS are presently unknown, our findings that CO can inhibit the firing activity and decreases excitability, particularly through the inhibition of VGCCs, serve as an important first step in validating CO as a modulator of neuronal function.

5 Acknowledgement

This work was supported by NSF award # 0843173 to VR and Brains and Behavior Fellowships to SE and LRZ.

6 Figure Legends

Figure 1. CO donor, CORM-2, silences spontaneous firing activity of B5 neurons.

(A) Representative examples of CORM-2 at (i) 5, (ii) 50, and (iii) 100 μ M show hyperpolarization of the resting membrane potential (RMP) from baseline values (indicated by the black dashed line). CORM-2 decreased firing frequency within one minute of exposure (compare firing activity at higher time resolution (black frame rectangles) before (black arrowhead) with firing frequency 60 sec after CORM-2 (gray arrowhead)). Firing frequency continued to decrease resulting in the silencing of spontaneous firing activity in 2 out of 5 recorded cells for 5 μ M CORM-2 and of all cells at 50 and 100 μ M CORM-2. Silencing occurred within 4 minutes at the two higher concentrations of CORM-2. (B) Quantification of the maximum change in RMP across experiments shows that CO significantly hyperpolarized the RMP across experiments and that increasing the CORM-2 concentration above 5 μ M did not have an additional effect on the RMP. Degassed CORM-2 (i-CORM-2) had no effect on RMP.

Figure 2. CO inhibition of I_{NaP} results in the hyperpolarization of the RMP and silencing of spontaneous firing activity.

(A) Recording of I_{NaP} in B5 neurons using a slow-voltage ramp protocol showed a maximal inward current occurring around -45 mV (Control: black trace). CORM-2 treatment (gray trace) almost completely inhibited I_{NaP} after 5 minutes of treatment. Overlay of both traces is shown in the lowest part of the figure for comparison. (B) Time course of the normalized I_{NaP} showed that CO significantly inhibited this current within 5 minutes, and this effect continued for the duration of the recording when compared with the solvent control, DMSO. (C) Treatment with

the inhibitor of I_{NaP} , riluzole, silenced spontaneous firing activity and hyperpolarized the RMP (black dashed line). (D) Quantification of the changes in membrane potential showed that riluzole treatment alone hyperpolarized the RMP and that the addition of either the solvent control (DMSO) or CORM-2 did not significantly hyperpolarize the RMP further.

Figure 3. CO decreases neuronal excitability.

(A) The injection of a depolarizing current (1.0 nA for 1 second) evoked APs in B5 neurons and the CO donor CORM-2 gradually inhibited neuronal excitability (expressed as the number of evoked spikes) over 30 minutes. (B) Quantification of changes in evoked firing activity elicited by depolarizing current injections of 0.1, 0.5 and 1.0 nA (i-iii). Compared with iCORM-2 and DMSO (control), evoked firing activity after treatment with CO decreased over time regardless of stimulation intensity.

Figure 4. CO inhibits voltage gated calcium channels.

(A) Voltage steps from -68.6 to +51.4 mV elicited strong inward total calcium currents that were partially inhibited by CORM-2 (50 μ M, 10 minutes post treatment). (B) Measurements of the time course of CO effects on the total calcium current identified two distinct currents: a low voltage gated calcium channel (VGCC) current opening at around -30 mV and a high VGCC current with maximal activation at approximately 0 mV. CO exposure inhibited both calcium currents over time and shifted the maximal activation of the current to more depolarized

voltages. (C) Change in both low and high VGCC currents from baseline showed that each current became progressively inhibited over time.

Figure 5. CO hyperpolarizes RMP and decreases neuronal excitability in B19 neurons.

(A) Example of a recording trace of a B19 neuron that was silent prior to CORM-2 treatment. CORM-2 treatment (spikes show addition artifact of CORM-2) hyperpolarized the RMP (gray dashed line) from baseline (black dashed line). The break in the recording indicates the transition between two recording files lasting 5 s. Maximum hyperpolarization was reached within approximately 10 minutes of CO addition. (B) Quantification of the change in membrane potential showed that CORM-2 significantly hyperpolarized the RMP, whereas DMSO and iCORM-2 had no effect. (C) Current injection (1 nA for 1 second) evoked firing activity in B19 neurons (i) that was inhibited by CORM-2 (ii, 30 minutes post treatment). (D) Quantification of the change in evoked APs from baseline shows that CORM-2 significantly inhibited evoked firing activity compared to DMSO and iCORM.

Figure 6. Summary for CO modulation of electrical activity

Proposed model for the modulation of electrical activity by CO in *Helisoma trivolvis* B5 neurons. (Left) Under control conditions, B5 neurons produce spontaneous action potentials utilizing the persistent sodium current (I_{NaP}) to depolarize the resting membrane potential to threshold. As the membrane potential approaches threshold voltage-gated calcium channels (VGCCs) provide the primary driving force for the generation of an action potential. The

injection of depolarizing current into B5 neurons produces sustained evoked firing activity.

(Right) Upon CO exposure, I_{NaP} is inhibited leading to hyperpolarization of the resting membrane potential and silencing of spontaneous firing activity. Over time, the inhibition of VGCCs by CO leads to a decrease in neuronal excitability.

7 References

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FIGURE 1

FIGURE 2

(A)

(B)

(i) 0.1 nA

(ii) 0.5 nA

(i) 1.0 nA

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6