Intermittent Hypoxia Enhances Functional Connectivity of Midcervical Spinal Interneurons

Kristi A. Streeter,^{1,5*} Michael D. Sunshine,^{1,5*} Shreya Patel,¹ Elisa J. Gonzalez-Rothi,^{1,5} Paul J. Reier,^{2,4,5}
 David M. Baekey,^{3,5} and David D. Fuller^{1,4,5}

¹Departments of Physical Therapy, ²Neuroscience, and ³Physiological Sciences, ⁴McKnight Brain Institute, and ⁵Center for Respiratory Research and Rehabilitation, University of Florida, Gainesville, Florida 32610

Brief, intermittent oxygen reductions [acute intermittent hypoxia (AIH)] evokes spinal plasticity. Models of AIH-induced neuroplasticity have focused on motoneurons; however, most midcervical interneurons (C-INs) also respond to hypoxia. We hypothesized that AIH would alter the functional connectivity between C-INs and induce persistent changes in discharge. Bilateral phrenic nerve activity was recorded in anesthetized and ventilated adult male rats and a multielectrode array was used to record C4/5 spinal discharge before [baseline (BL)], during, and 15 min after three 5 min hypoxic episodes (11% O₂, H1–H3). Most C-INs (94%) responded to hypoxia by either increasing or decreasing firing rate. Functional connectivity was examined by cross-correlating C-IN discharge. Correlograms with a peak or trough were taken as evidence for excitatory or inhibitory connectivity between C-IN pairs. A subset of C-IN pairs had increased excitatory cross-correlations during hypoxic episodes (34%) compared with BL (19%; $p < 0.0001$). Another subset had a similar response following each episode (40%) compared with BL (19%; $p < 0.0001$). In the latter group, connectivity remained elevated 15 min post-AIH (30%; $p = 0.0002$). Inhibitory C-IN connectivity increased during H1–H3 (4.5%; $p = 0.0160$), but was reduced 15 min post-AIH (0.5%; $p = 0.0439$). Spike-triggered averaging indicated that a subset of C-INs is synaptically coupled to phrenic motoneurons and excitatory inputs to these “pre-phrenic” cells increased during AIH. We conclude that AIH alters connectivity of the midcervical spinal network. To our knowledge, this is the first demonstration that AIH induces plasticity within the propriospinal network.

Key words: hypoxia; interneurons; network plasticity; neuroplasticity; respiratory; spinal cord

Significance Statement

Acute intermittent hypoxia (AIH) can trigger spinal plasticity associated with sustained increases in respiratory, somatic, and/or autonomic motor output. The impact of AIH on cervical spinal interneuron (C-IN) discharge and connectivity is unknown. Our results demonstrate that AIH recruits excitatory C-INs into the spinal respiratory (phrenic) network. AIH also enhances excitatory and reduces inhibitory connections among the C-IN network. We conclude that C-INs are part of the respiratory, somatic, and/or autonomic response to AIH, and that propriospinal plasticity may contribute to sustained increases in motor output after AIH.

Introduction

Spinal interneurons can relay and modulate descending synaptic drive to motoneurons (Jankowska and Hammar, 2002), facilitate motoneuron recruitment (Renshaw, 1941; Hodson-Tole and

Wakeling, 2009), and coordinate discharge between spinal motor pools (Lanuza et al., 2004). In the cervical cord, propriospinal neurons are synaptically coupled to respiratory motoneurons (Lane et al., 2008), limb motoneurons (Stepien et al., 2010), and sympathetic preganglionic neurons (Poree and Schramm, 1992). Many cervical interneurons (C-INs) respond to a brief period of reduced arterial oxygen (i.e., acute hypoxia) by altering discharge frequency (Sandhu et al., 2015; Streeter et al., 2017). The response of C-INs to repeated bouts of hypoxia [acute intermittent hypoxia (AIH)] is of interest because AIH can trigger spinal neuroplasticity and sustained increases in respiratory, autonomic, and/or somatic motor output (Dick et al., 2007; Lovett-Barr et al., 2012; Fuller and Mitchell, 2017). For these reasons, AIH is being actively explored as a therapeutic modality to promote motor recovery following neurologic disorders including spinal cord

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*K.A.S. and M.D.S. contributed equally to this work.

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Correspondence should be addressed to Dr. David Fuller, University of Florida, Department of Physical Therapy, P.O. Box 100154, Gainesville, FL 32610. E-mail: ddf@php.ufl.edu.

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injury (Trumbower et al., 2012; Hayes et al., 2014; Tester et al., 2014; Gonzalez-Rothi et al., 2015) and amyotrophic lateral sclerosis (ALS; Nichols et al., 2013, 2015).

To date, studies of AIH-induced neuroplasticity have primarily used nerve or muscle recordings and mechanistic cellular models have focused on motoneurons (Gonzalez-Rothi et al., 2015). This is in part because the dogma in the field of respiratory neural control is that diaphragm activation on a breath-by-breath basis is driven by monosynaptic bulbospinal inputs to phrenic motoneurons (Lee and Fuller, 2011). However, the robust C-IN response to acute hypoxia (Sandhu et al., 2015; Streeter et al., 2017), and anatomical evidence that C-INs can be synaptically connected to phrenic motoneurons (Lane et al., 2008; Lane, 2011) raises the possibility that C-INs are part of a propriospinal network which can impact phrenic motor output. Further support for this idea comes from experiments showing that monosynaptic activation of phrenic motoneurons can rapidly switch to polysynaptic activation following serotonin release (Mitchell et al., 1992; Ling et al., 1994).

Here we used a multielectrode recording array to simultaneously monitor multiple C-INs during and following AIH. Our *a priori* hypothesis focused on network connectivity within the midcervical (C4/5) spinal cord. This was evaluated using cross-correlation analyses to examine the temporal relationship between discharging C-INs. We hypothesized that AIH would alter propriospinal connectivity as shown by an increase in the incidence of C-INs with temporally related discharge patterns. Testing this hypothesis also enabled us to determine how AIH altered the firing properties (e.g., discharge rate and pattern) of individual C-INs after AIH. Initial evaluation of our data revealed pre-phrenic C-INs which were synaptically coupled to phrenic motoneurons. Using *post hoc* analyses we therefore tested the additional hypothesis that AIH would alter the degree of functional connectivity between pre-phrenic C-INs and other C-INs. Collectively, the data provide the most comprehensive evaluation of C-IN discharge and functional connectivity to date. Our results indicate that AIH alters excitatory and inhibitory connectivity between C-INs and thus we conclude AIH induces plasticity in the cervical spinal network.

Materials and Methods

Animals. All neurophysiologic experiments were conducted with adult male ($n = 12$; 383 ± 8 g) Sprague-Dawley rats (Colony 217, ENVIGO Laboratories). Rats were housed in pairs in a controlled environment (12 h light/dark cycles) with food and water *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida.

Neurophysiologic preparation. Rats were anesthetized with 3% isoflurane (in 100% O_2) and transferred to a heated surgical station where core body temperature was maintained at $37 \pm 0.5^\circ\text{C}$ (model 700 TC-1000, CWE). The trachea was cannulated and rats were pump-ventilated (Rodent Ventilator 683, Harvard Apparatus) and a bilateral vagotomy was performed. Inspired CO_2 was added to maintain end-tidal CO_2 between ~ 45 and 50 mmHg (Capnogard, Respironics). Tracheal pressure was continuously monitored and lungs were periodically hyperinflated via single breath occlusions (~ 1 h). A tail and femoral vein catheter were placed for intravenous delivery of urethane and fluids. Rats were slowly converted (6 ml/h; Harvard Apparatus syringe pump) to urethane anesthesia (1.7 g/kg, *i.v.*; 0.17 g/ml in distilled water) and isoflurane was withdrawn. A femoral arterial catheter was placed to monitor blood pressure and sample blood gases (i-STAT1 Analyzer, Abbot Laboratories). Using a dorsal approach, the left and right phrenic nerves were isolated, cut distally, and desheathed. A midline incision was made to expose spinal vertebrae C3–T2. A suture tied around the T2 spinous process was used to elevate and level the spinal cord. A laminectomy was performed from C3–C6 and the dura and arachnoid/pia over C4/5 were removed to

prevent pillowing of the spinal cord as electrodes were inserted. Before neuronal recording, a pneumothorax was performed to decrease motion artifact associated with chest wall movement and positive end-expiratory pressure of 1–2 cm H_2O was applied to prevent atelectasis. Adequate depth of anesthesia was monitored by assessing arterial blood pressure responses to toe-pinch; urethane supplements (e.g., 0.2 ml bolus) were given if arterial pressure changed when the pinch was applied. Animals received the neuromuscular paralytic pancuronium bromide (2.5 mg/kg, *i.v.*, Hospira) to prevent respiratory muscle contraction during the recording procedures. A continuous infusion (1–3 ml/h) of a 1:4 solution (8.4% sodium bicarbonate/lactated Ringer's solution, *i.v.*) was maintained during the experiment. A subset of rats ($n = 4$), received the nonsteroidal anti-inflammatory drug ketoprofen [(S)-(+)-ketoprofen, 12.5 mg/kg in 50% ETOH, *i.p.*; Sigma-Aldrich]. We did not detect any apparent differences between groups and therefore all data were combined.

Recordings. Bilateral phrenic nerve output was recorded using custom-made bipolar suction electrodes filled with 0.9% saline. Compound action potentials were amplified ($\times 20$ k, Grass Instruments, P511), analog bandpass filtered (3 Hz–3 kHz), digitized [16 bit, 25 k samples/s/channel; Power1401, Cambridge Electronic Design, (CED)], and integrated (time constant: 20 ms) with Spike2 software (CED). A custom-made multielectrode recording array containing 16 tungsten microelectrodes (impedance: $10 \pm 1\text{ M}\Omega$; shank diameter 125 μm ; tip diameter $\leq 1\text{ }\mu\text{m}$; Fred Haer) as previously described (Streeter et al., 2017) was used to record midcervical spinal discharge. Spinal recordings were performed using a unilateral ($n = 8$) or bilateral recording approach ($n = 4$) and data were pooled for analyses. The unilateral recording arrangement consisted of two staggered rows of eight microelectrodes spaced 300 μm apart and therefore extended ~ 2.5 mm. The bilateral recording arrangement had two sets of eight electrodes each consisting of two staggered rows of four. The inner two rows were separated by 1 mm and remaining electrodes were separated by 300 μm . In both arrangements, electrode tips were maintained in a “fixed matrix” in the medial-lateral and rostral-caudal dimension, by an array guide. The array was mounted on a stereotaxic frame and electrodes were inserted above the dorsal root entry zone at C4/5. Microelectrodes were advanced individually using micromotors until action potentials could be discriminated from background activity using an audio monitor. Recordings were amplified ($\times 5$ k), analog bandpass filtered (3 Hz–3 KHz), digitized (Power 1401, CED), and recorded (Spike2 software, CED). After recording stable baseline (BL) phrenic nerve activity and cervical spinal discharge (e.g., at least 10 min), animals were exposed to three 5 min episodes of hypoxia (FiO_2 : 0.11; H1, H2, H3) separated by 5 min of hyperoxia (FiO_2 : 0.50; PH1, PH2, PH3). A baseline arterial blood gas was obtained in 11/12 rats and verified that animals were well oxygenated (e.g., $PaO_2 > 95$ mmHg) and normocapnic (e.g., 40–50 mmHg). In 9/12 animals an arterial blood sample was obtained during H1 and at 15 min posthypoxia.

Data and statistical analyses. All data were collected using Spike2.v8 software (CED). Action potentials were sorted into individual neurons and converted to waveforms using off-line spike-sorting analyses. Spikes were determined to represent a “single unit” based on (1) at least 80% template matching, (2) assessment of inter spike interval, and (3) principle component analysis. The “sorted spike” waveforms and phrenic nerve output were analyzed with custom MATLAB software (MathWorks, R2015a). All data were averaged over the stable portion (center 50 neural breaths) of each experimental time point. Statistical analyses were performed in GraphPad Prism 7 and MATLAB (see Table 1). For all comparisons using ANOVA, values during hypoxic episodes were compared with BL (e.g., BL, H1, H2, H3) and a separate ANOVA was used to examine non-hypoxic time points (e.g., BL, PH1, PH2, PH3, 15). The significance level was set to 0.05, except one instance in which it was set to 0.02 (discussed below).

Blood gas variables (e.g., PaO_2 and $PaCO_2$) were analyzed using a one-way ANOVA and individual comparisons were made using Fisher's least significant difference (LSD) *post hoc* test. Mean arterial pressure was compared using a one-way ANOVA and individual time point comparisons were determined using Fisher's LSD *post hoc* test.

Integrated phrenic amplitude was reported as amplitude (μV) and phrenic burst frequency was expressed as neural breaths per minute.

Figure 1. Phrenic nerve output before, during, and following acute intermittent hypoxia **A**, Representative compressed raw and integrated left phrenic neurogram (μV), instantaneous burst frequency (per minute) before, during, and 15 min post-AIH. **B**, **C**, Average left phrenic burst amplitude (μV ; **B**), and frequency (neural breaths per minute; **C**) during the center 50 breaths of each experimental time point. *Significantly different from BL. Scale bar, 5 min.

Differences in the left and right phrenic nerve burst amplitudes were indistinguishable at all time points ($p = 0.6470$); therefore, analysis of peak bursting focused on the left phrenic nerve. Using the integrated phrenic nerve output, the respiratory cycle was divided into inspiratory and expiratory phases. The beginning of the inspiratory phase and expiratory phase were identified as a departure of phrenic nerve activity ≥ 15 SD above the average activity during the expiratory phase. A two-way repeated-measure ANOVA was used to compare left and right phrenic nerve amplitude. For each phrenic nerve variable (i.e., amplitude and frequency) a one-way ANOVA with repeated-measures design was used for statistical comparisons and individual time point comparisons were determined by Fisher's LSD *post hoc* test.

Spike-triggered averaging (STA) of the phrenic nerve activity in relation to spinal neuron discharge was used to examine the temporal relationship of neuronal spikes and phrenic motor output (Lipski et al., 1983). Phrenic nerve activity was rectified and filtered with a two-pole Butterworth filter (250–3000 Hz) for all subsequent analyses. Left and right phrenic nerves were averaged separately using the sorted spikes (waveforms) of each recorded neuron as trigger events. Short-latency (e.g., <1.0 ms) peaks >5 SD from the average background (calculated over 6 ms before the trigger) were taken as evidence that the recorded neuron was a phrenic motoneuron (Mitchell et al., 1992; Sandhu et al., 2015). Identified phrenic motoneurons were excluded for all subsequent analyses.

If a short-latency feature was not detected in the STA, the recorded cell was classified as a C-IN. C-IN discharge during inspiration and expiration was compared using a one-way ANOVA. For each respiratory phase variable, a one-way ANOVA was used for statistical comparisons and individual time point comparisons were determined with Fisher's LSD *post hoc* test. Baseline frequency of activated versus inhibited C-INS was analyzed using a two-tailed unpaired t test.

To evaluate functional connectivity, cross-correlation histograms were constructed for all possible pairs of simultaneously recorded neu-

rons using a bin width of 0.2 ms (Moore et al., 1970). The detectability index (DI; Aertsen and Gerstein, 1985) was calculated for each correlogram as the peak (or trough) relative to average background activity (calculated over 12 ms before the trigger), divided by the SD. Features were considered significant if the DI was >3 (Melssen and Epping, 1987), and only significant features in the positive direction were counted. Peaks or troughs are consistent with functional excitation or inhibition between the trigger and target neurons, respectively (Kirkwood, 1979; Aertsen and Gerstein, 1985). Summary graphs were expressed as the number of positive cross-correlogram features (CCs) expressed as a percentage of the total number of possible features (i.e., based on the total number of recorded cells at that time point). For statistical comparisons, the proportions of CCs were averaged during hypoxia episodes and during posthypoxia episodes. Using these values, separate χ^2 tests with Yates' correction (Hazra and Gogtay, 2016) were used to analyze differences in the proportion of CCs from BL.

Features in the phrenic nerve STA occurring with a lag time >1.0 ms were taken as evidence that the recorded cell was synaptically antecedent to the phrenic motoneuron pool (i.e., pre-phrenic C-IN; Lane et al., 2008). A 10 Hz high-pass filter was applied to the rectified phrenic signals used to construct pre-phrenic STAs. Features were considered significant if: (1) the STA was 2 SD from the mean activity before the trigger period (calculated in the interval -6 to 0 ms) and (2) the feature remained at this level for at least 0.75 ms. Offset STA features with positive deflections (peaks) were

taken as evidence of excitatory pre-phrenic C-INS, whereas offset STA features with negative deflections (troughs) indicated inhibitory pre-phrenic C-INS. A two-tailed unpaired t test was used to analyze differences in STA parameters (e.g., time to onset and time to peak) between excitatory and inhibitory STA features. In subsequent analyses, cross-correlation histograms were constructed between identified pre-phrenic interneurons and other C-INS. For statistical comparisons, the proportions of CCs were averaged during hypoxia episodes and during posthypoxia episodes. Using these values, separate Fisher's exact tests were used to analyze differences in the proportion of CCs from BL. Linear regression analysis was used to examine the relationship between the proportion of second-order excitatory neurons to excitatory pre-phrenic C-INS during AIH.

Cycle-triggered histograms (Lindsey et al., 1992) were used to classify C-IN firing patterns relative to the respiratory cycle. Cycle-triggered histograms were constructed for each neuron by dividing each respiratory period into 20 bins. Neuronal spikes were then counted within each bin and summated over the respective bin across 50 consecutive breaths. The resulting histogram was overlaid with the averaged integrated phrenic waveform from the same 50 consecutive breaths, providing a visualization of neural discharge in relation to the respiratory cycle. To determine whether neurons were respiratory modulated, the cycle-triggered histograms were separated into inspiration and expiration and the Wilcoxon signed-rank test was used to test the null hypothesis (i.e., no difference between inspiration and expiration). Upon visual inspection of the data, the significance level was adjusted to 0.02 to reflect a physiologically meaningful modulation of neuronal firing with regard to the respiratory cycle. Neurons which were identified as respiratory modulated at BL were then categorized as inspiratory or expiratory modulated according to the phase of the respiratory cycle in which the cell was most active. Neurons not respiratory modulated (i.e., no significant difference if activity between inspiration and expiration) were classified as tonic. Those which were not active during BL were classified as recruited.

Table 1. Output of all statistical tests

	Comparison	Test	Test output	<i>p</i>
Fig. 1	L vs R phrenic amp	Two-way RM ANOVA	$F_{(1, 22)} = 0.2156$	0.647
	L phrenic amp: hypoxia	One-way RM ANOVA	$F_{(1, 205, 12.25)} = 28.34$	0.0001
	L phrenic amp: posthypoxia	One-way RM ANOVA	$F_{(1, 555, 17.11)} = 7.218$	0.0082
	Phrenic frequency: hypoxia	One-way RM ANOVA	$F_{(1, 154, 12.69)} = 5.438$	0.0330
	Phrenic frequency: posthypoxia	One-way RM ANOVA	$F_{(1, 514, 16.65)} = 20.08$	<0.0001
Fig. 3	Activated vs inhibited C-INS BL firing rate inspiration	Unpaired <i>t</i> test (two-tailed)	$t_{(72)} = 2.46$	0.0163
	Activated vs inhibited C-INS BL firing rate expiration	Unpaired <i>t</i> test (two-tailed)	$t_{(70)} = 0.9673$	0.3367
	Activated C-INS inspiration: hypoxia	One-way ANOVA	$F_{(3, 226)} = 4.184$	0.0066
	Activated C-INS inspiration: posthypoxia	One-way ANOVA	$F_{(4, 228)} = 0.3296$	0.8579
	Activated C-INS expiration: hypoxia	One-way ANOVA	$F_{(3, 221)} = 2.799$	0.0409
	Activated C-INS expiration: posthypoxia	One-way ANOVA	$F_{(4, 227)} = 0.4648$	0.7615
	Inhibited C-INS inspiration: hypoxia	One-way ANOVA	$F_{(3, 75)} = 3.061$	0.0333
	Inhibited C-INS inspiration: posthypoxia	One-way ANOVA	$F_{(4, 80)} = 4.623$	0.0021
	Inhibited C-INS expiration: hypoxia	One-way ANOVA	$F_{(3, 68)} = 2.726$	0.0508
	Inhibited C-INS expiration: posthypoxia	One-way ANOVA	$F_{(4, 71)} = 3.781$	0.0076
Fig. 4	All excitatory CCs: BL vs hypoxia	χ^2 with Yates' correction	$\chi^2_{(1, N=696)} = 0.2061$	0.6498
	All excitatory CCs: BL vs posthypoxia	χ^2 with Yates' correction	$\chi^2_{(1, N=673)} = 5.621$	0.0177
	All excitatory CCs: BL vs 15 min	χ^2 with Yates' correction	$\chi^2_{(1, N=534)} = 3.142$	0.0763
	≤1 hypoxia Excitatory CCs: BL vs hypoxia	χ^2 with Yates' correction	$\chi^2_{(1, N=696)} = 17.56$	<0.0001
	≤1 hypoxia Excitatory CCs: BL vs posthypoxia	χ^2 with Yates' correction	$\chi^2_{(1, N=673)} = 3.637$	0.0565
	≤1 hypoxia Excitatory CCs: BL vs 15 min	χ^2 with Yates' correction	$\chi^2_{(1, N=534)} = 2.14$	0.1435
	≤1 posthypoxia Excitatory CCs: BL vs hypoxia	χ^2 with Yates' correction	$\chi^2_{(1, N=696)} = 0.5925$	0.4415
	≤1 posthypoxia Excitatory CCs: BL vs posthypoxia	χ^2 with Yates' correction	$\chi^2_{(1, N=673)} = 36.05$	<0.0001
	≤1 posthypoxia Excitatory CCs: BL vs 15 min	χ^2 with Yates' correction	$\chi^2_{(1, N=534)} = 13.52$	0.0002
Fig. 5	All inhibitory CCs: BL vs hypoxia	χ^2 with Yates' correction	$\chi^2_{(1, N=696)} = 0.2108$	0.6462
	All Inhibitory CCs: BL vs posthypoxia	χ^2 with Yates' correction	$\chi^2_{(1, N=673)} = 3.467$	0.0626
	All Inhibitory CCs: BL vs 15 min	χ^2 with Yates' correction	$\chi^2_{(1, N=534)} = 4.062$	0.0439
	≤1 hypoxia Inhibitory CCs: BL vs hypoxia	χ^2 with Yates' correction	$\chi^2_{(1, N=696)} = 5.801$	0.0160
	≤1 hypoxia Inhibitory CCs: BL vs posthypoxia	χ^2 with Yates' correction	$\chi^2_{(1, N=673)} = 0.2163$	0.6419
	≤1 hypoxia Inhibitory CCs: BL vs 15 min	χ^2 with Yates' correction	$\chi^2_{(1, N=534)} = 1.142$	0.2852
	≤1 posthypoxia Inhibitory CCs: BL vs hypoxia	χ^2 with Yates' correction	$\chi^2_{(1, N=696)} = 0.0150$	0.9023
	≤1 posthypoxia Inhibitory CCs: BL vs posthypoxia	χ^2 with Yates' correction	$\chi^2_{(1, N=673)} = 0.1263$	0.7223
	≤1 posthypoxia Inhibitory CCs: BL vs 15 min	χ^2 with Yates' correction	$\chi^2_{(1, N=534)} = 0.1522$	0.6965
Fig. 6	Excitatory vs inhibitory pre-phrenic onset STA	Unpaired <i>t</i> test (two-tailed)	$t_{(6)} = 0.4851$	0.6448
	Excitatory vs inhibitory pre-phrenic STA peak	Unpaired <i>t</i> test (two-tailed)	$t_{(6)} = 0.8584$	0.4236
Fig. 7	2nd order C-INS to excitatory pre-phrenic C-INS: BL vs hypoxia	Fisher's exact test	$n = 44$	0.4803
	2nd order C-INS to excitatory pre-phrenic C-INS: BL vs posthypoxia	Fisher's exact test	$n = 40$	0.0896
	2nd order C-INS to excitatory pre-phrenic C-INS: BL vs 15 min	Fisher's exact test	$n = 35$	>0.9999
	2nd order C-INS to excitatory pre-phrenic C-INS: hypoxia	Linear Regression	$F_{(1, 2)} = 108.9$	0.0091
	2nd order C-INS to excitatory pre-phrenic C-INS: posthypoxia	Linear Regression	$F_{(1, 3)} = 0.1538$	0.7211
Table 2	PaO ₂	One-way ANOVA	$F_{(2, 26)} = 120.6$	<0.0001
	PaCO ₂	One-way ANOVA	$F_{(2, 26)} = 0.6328$	0.5391
	MAP: hypoxia	One-way ANOVA	$F_{(3, 37)} = 3.266$	0.0320
	MAP: posthypoxia	One-way ANOVA	$F_{(4, 46)} = 0.1974$	0.9385

MAP, Mean arterial pressure.

Table 2. Average arterial PO₂, PCO₂, and MAP before, during, and 15 min after AIH

	BL	H1	15	PH1	H2	PH2	H3	PH3
PaO ₂	228 ± 7	40 ± 2*	188 ± 14*					
PaCO ₂	46.6 ± 1.3	44.8 ± 1.9	47.2 ± 1.3					
MAP	128 ± 9	95 ± 12*	89 ± 11	121 ± 11	88 ± 11*	121 ± 11	87 ± 11*	123 ± 11

*Significantly different from BL. MAP, Mean arterial pressure.

Results of all statistical analyses are provided in Table 1. In the Results section, relevant *p* values from *post hoc* tests are included to illustrate significant differences between individual time points.

Results

Blood gases and arterial pressure

Arterial blood gas data and blood pressure are summarized in Table 2. Baseline arterial blood samples confirmed rats were well oxygenated and normocapnic (Table 2). As expected, PaO₂ was decreased

during H1 ($p < 0.0001$). Rats remained well oxygenated at 15 min post-AIH, but this value was reduced compared with BL ($p = 0.0034$). PaCO₂ values were not different from BL during H1 or 15 min after AIH ($p = 0.5391$). As previously reported in this preparation (Bavis and Mitchell, 2003), the mean arterial blood pressure was decreased during each bout of hypoxia relative to BL (H1: $p = 0.0369$; H2: $p = 0.0121$; H3: $p = 0.0116$), but was not different from BL at any time point posthypoxia (all $p > 0.9385$).

Figure 2. C-IN activity before, during, and following acute intermittent hypoxia. Representative compressed firing rate traces (pulses per second), corresponding locations of C-INs, and integrated phrenic neurogram from one experiment before, during, and 15 min post-AIH.

Phrenic nerve activity during AIH

Representative phrenic neurogram and instantaneous burst frequency are shown in Figure 1A. Average rectified, integrated burst amplitude before, during, and 15 min post-AIH is shown in Figure 1B. As expected, amplitude increased during each bout of hypoxia (H1: $p < 0.0001$; H2: $p = 0.0002$; H3: $p = 0.0004$) compared with BL. Burst amplitude remained elevated during each posthypoxic period (PH1: $p = 0.0039$; PH2: $p = 0.0126$; PH3: $p = 0.0288$), but was not different from BL at 15 min posthypoxia ($p = 0.0951$). Average burst frequency before, during, and 15 min post-AIH is shown in Figure 1C. Phrenic burst frequency tended to increase during H1 and H2, and was significantly increased from BL during H3 ($p = 0.0176$). A decrease in burst frequency occurred following each bout of hypoxia (PH1: $p = 0.0025$; PH2: $p = 0.0031$; PH3: $p = 0.0144$); however at 15 min post-AIH, frequency was not different from BL ($p = 0.3695$).

C-IN discharge during AIH

Representative C-IN firing rate histograms and phrenic neurogram from one animal are shown in Figure 2. To characterize the response to AIH, neurons were separated into two groups based on their firing rate during the first episode of hypoxia (e.g., H1). C-INs increasing firing rate during H1 were classified as “hypoxia activated” and cells which decreased firing rate were considered “hypoxia inhibited”. During the first episode of hypoxia, 73% of C-INs increased firing rate, and 21% decreased firing rate during the inspiratory phase. A similar proportion of neurons increased (71%) and decreased (19%) firing rate during the expiratory phase. The average increase in firing rate (from BL) for hypoxia-activated C-INs was 10.6 ± 1.0 pps, whereas hypoxia-inhibited cells decreased by 8.6 ± 2.1 pps.

Heat maps illustrating the firing rate of individual C-INs as well as average rate during the inspiratory and expiratory phases are shown in Figure 3, A and B, respectively. At BL hypoxia-activated neurons had a significantly lower firing rate during the inspiratory phase (9.5 ± 1.3 pps) compared with hypoxia-inhibited neurons (15.8 ± 2.3 pps; $p = 0.0163$; Fig. 3A). During the inspiratory phase, hypoxia-activated C-INs increased firing rate by an average of $6.7 \pm 2.2\%$ during H1 ($p = 0.0028$), $7.1 \pm 2.3\%$ during H2 ($p = 0.0020$), and $5.8 \pm 2.3\%$ during H3 ($p = 0.0091$). However, the firing rate of these cells was not persistently altered following AIH, and returned to BL values after 15 min ($p = 0.9059$). Hypoxia-inhibited C-INs decreased firing rate during the inspiratory phase by an average of $5.9 \pm 2.8\%$ during H1 ($p = 0.0435$), $6.2 \pm 2.8\%$ during H2 ($p = 0.0337$), and $8.1 \pm 2.8\%$ during H3 ($p = 0.0062$). Cells classified as hypoxia inhibited had a decreased firing rate following all hypoxia episodes (PH1: $p = 0.0108$; PH2: $p = 0.0021$; PH3: $p = 0.0004$), which remained below BL 15 min post-AIH ($p = 0.0034$). Similar changes in discharge frequency during the expiratory phase were noted for both hypoxia-activated and -inhibited C-INs (Fig. 3B). Together, these results indicate that: (1) the majority of C-INs (>90%) alter firing rate in response to hypoxia, (2) hypoxia-activated C-INs had a lower BL firing rate compared with hypoxia-inhibited C-INs, and (3) hypoxia-inhibited C-INs showed a persistent inhibition with discharge rates remaining below BL at 15 min post-AIH.

Functional connectivity of C-INs during AIH

Cross-correlation analysis was used to evaluate short time scale (i.e., 0–10 ms) discharge synchrony among C-INs before, during, and after AIH. Significant features (i.e., peaks and troughs) were

Figure 3. C-IN activity before, during, and following acute intermittent hypoxia. **A, B.** Firing rate heat maps summarizing firing rate of individual neurons and average firing rate (pulses per second) before, during, and 15 min post-AIH for hypoxia-activated and -inhibited neurons during inspiration (**A**, open bars) and expiration (**B**, shaded bars). For all heat maps, each row represents the firing rate of one C-IN and is organized according to BL firing rate. *Significantly different from BL. #Significantly different from hypoxia-activated BL.

identified as departures in the cross-correlation histogram >3 SD above the background (Aertsen and Gerstein, 1985; Melssen and Epping, 1987; Aertsen et al., 1989). Positive (Fig. 4A) and negative (Fig. 5A) correlogram features were taken as evidence for excitatory or inhibitory connectivity, respectively (Kirkwood, 1979; Aertsen and Gerstein, 1985). Heat maps derived from the cross-correlation analysis illustrate connectivity among C-IN pairs. For all heat maps, each row represents a pair of neurons. When no correlogram feature was detected the position in the matrix was colored dark purple; when a feature was present, the position was colored according to the proportion of C-INs that showed a feature at that time point. Thus, the color of each column illustrates the proportion of functional connections observed at each time point. The proportion of positive correlogram features was expressed relative to the total number of possible connections (i.e., based on the total number of recorded cells at that time point).

The average proportion of excitatory connections is shown in Figure 4. During BL, excitatory connections were detected among 32% of C-IN pairs (Fig. 4B). When all C-INs with an excitatory connection were examined, we detected an increase in the average number of positive correlograms following hypoxia (PH1, PH2, PH3) compared with BL (41%, $p = 0.0177$). Excitatory connectivity was slightly elevated 15 min posthypoxia, but this did not reach the threshold for statistical significance ($p = 0.0763$ vs BL). Neuronal pairs for which a correlogram peak was present during one or more hypoxia or posthypoxia periods are summarized in Figure 4, C and D, respectively. In C-INs with positive correlogram features during hypoxia, the average num-

ber of excitatory connections was 19% at BL, and this was increased to 34% during hypoxia (Fig. 4C; $p < 0.0001$). In those neuronal pairs which showed positive correlogram features following hypoxia, the average number of excitatory connections was increased during the initial posthypoxia period (41%; $p < 0.0001$ vs BL) and remained increased 15 min post-AIH ($p = 0.0002$). These data show for the first time that AIH enhances excitatory connectivity among C-INs.

In contrast to the number of excitatory connections, comparatively few inhibitory connections (4%) were detected at BL (Fig. 5). Overall, the number of inhibitory connections did not change during hypoxia ($p = 0.6462$) or following hypoxia ($p = 0.0626$), but was significantly reduced at 15 min post-AIH ($p = 0.0439$). In C-INs functionally connected during hypoxia, the average number of inhibitory connections during hypoxia was increased to 34% compared with 19% at BL (Fig. 4C; $p = 0.0160$). No significant changes were detected in the subset of neurons that were functionally connected during at least one posthypoxia (Fig. 4D). These results indicate that inhibitory connections were enhanced in a subset of C-INs during hypoxia exposure, and reduced 15 min following the AIH paradigm. Together, these data show that AIH alters excitatory and inhibitory connectivity, leading to a more excited proprioceptive network.

“Pre-phrenic” C-INs during AIH

In 8% of recordings, STA of the rectified phrenic nerve activity in relation to neuronal discharge produced a broad feature with a delay >1 ms (Fig. 6). This delay suggests that the cell was synap-

Figure 4. Excitatory connectivity of C-INs before, during, and following acute intermittent hypoxia. **A**, Representative example of integrated phrenic output and activity from a pair of C-INs and resulting excitatory CC. **B–D**, Using cross-correlation analysis, heat maps for excitatory correlograms were constructed before, during, and for 15 min post-AIH for all C-IN pairs. For all heat maps, each row represents a neuronal pair. When no correlogram feature was detected the position in the matrix was colored dark purple and when a feature was present, the position was colored according to the proportion of C-INs that showed a feature at that time point. The color of each column represents the proportion of positive CCs (as a percentage of possible connections) during each time point. Neuronal pairs with a connection during at least one hypoxia (**C**), and neuronal pairs with a connection during at least one posthypoxia (**D**). The proportion of excitatory connections (as a percentage of possible connections) during each time point is shown below each heat map. *Significantly different from BL.

tically coupled to the phrenic motoneuron pool, as the latency is greater than synaptic delay (e.g., 1.0 ms; Borst et al., 1995); these neurons were classified as pre-phrenic C-INs (Lane et al., 2008). Offset STA peaks were taken as evidence of excitatory pre-phrenic C-INs (Fig. 6A), whereas offset troughs indicated inhibitory pre-phrenic C-INs (Fig. 6B). With this approach, a total of five excitatory and two inhibitory pre-phrenic interneurons were identified (Table 3). Of these seven cells, three were ipsilateral to the phrenic nerve in which the STA was detected. In addition, two excitatory and one inhibitory pre-phrenic interneuron produced an STA in the phrenic nerve contralateral to the recording location, consistent with a commissural connection. One inhibitory pre-phrenic interneuron was active during expiration produced an STA for both the left and right phrenic nerve, indicating it was functionally coupled to both the left and right phrenic motor pools. All excitatory pre-phrenic C-INs fired with either a tonic or inspiratory pattern (Fig. 6C), whereas both inhibitory pre-

phrenic C-INs had an expiratory pattern (Fig. 6D). The average time to STA onset, and STA peak for excitatory and inhibitory pre-phrenic C-INs are shown in Figure 6, E and F, and was no different between groups (onset: $p = 0.6448$; peak: $p = 0.4236$).

We next identified C-INs that were functionally connected to pre-phrenic C-INs; these neurons were operationally defined as “second order” (Fig. 7). The relationship between the number of excitatory connections from second-order C-INs to either excitatory pre-phrenic neurons or inhibitory pre-phrenic neurons was quantified (Fig. 7C,D), and examined using linear regression (Fig. 7E,F). A strong, positive relationship was present between the number of excitatory connections ($n = 24$) from second-order neurons to excitatory pre-phrenic interneurons ($n = 5$) during ($R^2 = 0.982$; $p = 0.0091$), but not after hypoxia ($R^2 = 0.048$; $p = 0.7211$; Fig. 7E). We did not detect a sufficient number of inhibitory pre-phrenic interneurons ($n = 2$) to enable a similar statistical test, but data are shown in Figure 7F. These results

Figure 5. Inhibitory connectivity of C-INs before, during, and following acute intermittent hypoxia. **A**, Representative example of integrated phrenic output and activity from a pair of C-INs and resulting inhibitory CC. **B–D**, Using cross-correlation analysis, heat maps for inhibitory correlograms were constructed before, during, and 15 min post-AIH for all C-IN pairs. For all heat maps, each row represents a neuronal pair. When no correlogram feature was detected the position in the matrix was colored dark purple and when a feature was present, the position was colored according to the proportion of C-INs that showed a feature at that time point. The color of each column represents the proportion of positive CCs (as a percentage of possible connections) during each time point. Neuronal pairs with a connection during at least one hypoxia (**C**), and neuronal pairs with a connection during at least one posthypoxia (**D**). The proportion of inhibitory connections (as a percentage of possible connections) during each time point is shown below each heat map. *Significantly different from BL.

provide evidence that the number of excitatory connections between C-INs and excitatory pre-phrenic interneurons is enhanced during hypoxia. Thus we conclude that C-INs are an integral part of the neural network that controls the diaphragm, and that the strength of synaptic connections in this network may be altered during and after AIH. A schematic summarizing the connections from second-order C-INs to excitatory and inhibitory pre-phrenic C-INs is shown in Figure 7, *G* and *H*.

Stratification of C-IN firing patterns according to the respiratory cycle

For a final evaluation, we phenotypically stratified C-INs as tonic (T), inspiratory (I), expiratory (E), or recruited (R) based on their BL discharge pattern (Fig. 8). Recruited neurons were defined as cells which were “off” (e.g., did not fire) during BL, but began firing at a later time point. In addition, a portion of C-INs was active during BL and turned off at a subsequent time point. Representative examples of the firing rate and cycle triggered histograms for phenotypically defined C-IN (e.g., T, I, E, and R) are shown in Figure 8, *A* and *B*.

We next examined whether bursting patterns are altered during or after AIH. Heat maps of C-IN firing patterns are shown in

Figure 8C. For all heat maps, each row represented one neuron and its bursting pattern as defined by color. As illustrated by this figure, C-INs switch which respiratory phase they fire during in response to AIH defined as phase switching. In addition, some C-INs adopted a respiratory-related firing pattern (e.g., inspiratory/expiratory) in response to hypoxia. A summary of the number of C-INs across the experimental time points stratified by firing pattern is presented in Figure 8C. At each time point, the majority of recorded C-INs (54 ± 2) fired tonically through the respiratory cycle. The average number of C-INs with an inspiratory firing pattern was similar during hypoxia (12 ± 2) compared with posthypoxia (13 ± 1). In contrast, a greater number of expiratory neurons were active during each bout of hypoxia (10 ± 2) compared with posthypoxia (5 ± 0). On average, more C-INs (80 ± 2) were active during hypoxia than posthypoxia (72 ± 3), indicating C-INs were recruited during hypoxic episodes.

The approximate anatomical location of each recording was determined using the micromotor coordinates of each electrode. These data were used to create a map of the locations of each cell in respect to the firing patterns across the experiment (Fig. 9). These images show the recording locations spanned the gray mat-

Figure 6. Pre-phrenic C-INs synaptically coupled to phrenic motoneurons. **A, B**, Representative STA of the rectified phrenic nerve activity in relation to neuronal discharge depicting a broad peak (**A**) and trough (**B**) with a latency >1 ms and $>2SD$ from the mean activity (solid line). Open red circle denotes STA onset, open blue circle denotes STA peak. **C, D**, Raw pre-phrenic C-IN and sorted spikes and raw and integrated phrenic nerve activity from an excitatory (**C**) and inhibitory (**D**) pre-phrenic C-IN used to construct STAs in **A** and **B**, respectively. **E, F**, Average time between pre-phrenic discharge and STA onset (ms) and STA peak (ms) for excitatory (**E**) and inhibitory (**F**) pre-phrenic C-INs. Scale bar, 1 s.

Table 3. Summary of excitatory and inhibitory pre-phrenic C-INs bursting pattern, time point in which the neuron was active, and presence of a STA of the rectified ipsilateral and/or contralateral phrenic nerve activity in relation to neuronal discharge

	Firing pattern	Time with activity	Ipsilateral STA	Contralateral STA
Excitatory	Tonic	All	X	
	Tonic	All		X
	Tonic	H1		X
	Inspiratory	All	X	
	Inspiratory	PH1, PH2, PH3, 15 min	X	
Inhibitory	Expiratory	All		X
	Expiratory	All	X	X

ter and show little stratification with regard to C-IN bursting pattern. Of note, tonic C-INs showed the greatest dorsal ventral distribution and phrenic motoneurons were located ventral to C-INs.

Discussion

Here we demonstrate that the incidence of excitatory and inhibitory connectivity among C-INs was persistently altered by AIH, indicating AIH induces plasticity in the proprio-spinal network. Specifically, following AIH we detected a sustained decrease in inhibitory connections as well as increase in excitatory connectivity between C-INs. Successive bouts of hypoxia also caused a progressive increase in the incidence of excitatory connections to C-INs which were synaptically coupled to phrenic motoneurons (i.e., pre-phrenic C-INs). Collectively, the comprehensive evaluation of C-IN bursting presented herein leads us to conclude that the spinal impact of AIH extends beyond motoneurons. AIH-induced alterations in C-IN discharge and associated changes in proprio-spinal network connectivity may contribute to respiratory, somatic, or autonomic responses and to neuroplastic changes in these motor outputs.

C-INs and hypoxia

In the midcervical spinal cord, C-INs show both inspiratory- and expiratory-related discharge (Palissès et al., 1989; Bellingham and Lipski, 1990; Iscoe and Duffin, 1996). We observed respiratory-related C-IN discharge, but also a high prevalence of tonic firing C-INs, some of which were synaptically coupled to phrenic motoneurons. In regards to hypoxia, we previously reported changes in both the rate and pattern of C-IN discharge. Specifically, following a single hypoxic episode, 40% of the recorded C-INs continued to fire at rates above prehypoxia values (Sandhu et al., 2015) and 29% altered their discharge pattern during or following hypoxia (Streeter et al., 2017). Here we observed that $>90\%$ of recorded C-INs had altered firing rates during or following hypoxia. We also provide the following new information regarding C-IN phenotype and the response to hypoxia. First, baseline C-IN firing rate was significantly different between cells which were activated versus inhibited during hypoxia, indicating the initial discharge pattern may predict the response to hypoxia exposure. Second, following AIH cells which were activated by hypoxia rapidly returned to BL firing rate, whereas C-INs which were inhibited by hypoxia had persistent decreases in firing rate, suggesting AIH has a differential impact on populations of C-INs. Last, we noted that C-INs which were initially active across the entire respiratory cycle (i.e., tonic) can rapidly switch to a respiratory-related firing pattern (e.g., inspiratory/expiratory) upon exposure to hypoxia. This provides further evidence that the cervical proprio-spinal network is responding to hypoxia in a manner that is coordinated with the respiratory network. Overall, the data indicate that hypoxia responsiveness is a general property of C-INs, but that this can manifest in numerous ways.

The mechanisms which cause C-INs to alter their firing rate and pattern in response to hypoxia are unknown, but several potential explanations are available. C-INs receive synaptic input from brainstem nuclei associated with both respiratory (Fedorko et al., 1983; Davies et al., 1985; Hayashi et al., 2003; Lane et al., 2008) and sympathetic control (Guyenet, 2000). These brainstem

Figure 7. C-INs temporally linked to pre-phrenic neurons before, during, and following acute intermittent hypoxia. **A, B**, Schematic of second-order excitatory C-IN projecting to excitatory pre-phrenic (**A**) and inhibitory pre-phrenic C-IN (**B**). **C, D**, Excitatory C-INs (as a percentage of possible connections) temporally linked to excitatory pre-phrenic C-INs (**C**) and inhibitory (**D**) pre-phrenic C-INs. **E, F**, Linear regression analysis of the proportion of second-order excitatory connections to excitatory (**E**) and inhibitory (**F**) pre-phrenic C-INs. **G, H**, Schematic summarizing second-order excitatory C-INs projecting to excitatory (**G**) and inhibitory (**H**) pre-phrenic interneurons at BL (brown) and recruited (orange) during intermittent hypoxia.

Figure 8. Characterization of C-IN firing patterns before, during, and following acute intermittent hypoxia. **A**, Representative examples of the firing rate (pulses per second) during inspiration (open circles) and expiration (filled circles) of phenotypically stratified C-INs (e.g., T, I, E, and off) before, during, and 15 min post-AIH. **B**, Representative examples of C-INs firing patterns depicting sorted spikes (top trace), integrated phrenic nerve output (middle trace), and cycle-triggered histogram (CTH; bottom trace) during 50 consecutive breaths overlaid with the average integrated phrenic waveform. Open circles denote the beginning of the inspiratory phase; closed circles denote the end of the inspiratory phase/beginning of the expiratory phase. Open bars in CTH depict activity during the inspiratory phase and shaded bars depict activity during the expiratory phase. **C**, Heat maps summarizing firing patterns before, during, and 15 min post-AIH. For all heat maps, each row represents one neuron and are organized according to BL cell type (T, E, I, off). Stacked bar graph depicts the number of C-INs with each firing pattern before, during, and 15 min post-AIH and posthypoxia.

regions are robustly activated by hypoxia (Hirooka et al., 1997), and may therefore drive the C-IN response via bulbospinal synaptic inputs. The observed switch from tonic to phasic respiratory bursting during hypoxia indicates that at least a subset of C-INs are receiving synaptic inputs related to the endogenous respiratory cycle. An alternative possibility is that C-INs directly sense oxygen. Indeed, oxygen-sensing neurons are found in various brain regions (Neubauer and Sunderram, 2004), and recent evidence suggests a subpopulation of C-INs sense changes in oxygen in lieu of brainstem input (Wilson et al., 2015).

Pre-phrenic C-INs

Several laboratories have suggested that C-INs are functionally connected to phrenic motoneurons (Palisses et al., 1989; Bellingham and Lipski, 1990; Douse and Duffin, 1993; Sandhu et al., 2009). Neuroanatomical evidence indicates that pre-phrenic C-INs have the potential to relay information from medullary neurons to phrenic motoneurons (Lane et al., 2008), or serve as spinal commissural connections (Lois et al., 2009). Our data are consistent with both suggestions because we show pre-phrenic C-INs can modulate ipsilateral and contralateral phrenic output.

We add to our current understanding of pre-phrenic C-INs by showing both excitatory and inhibitory inputs to phrenic motoneurons. Whereas excitatory pre-phrenic C-INs fired with either a tonic pattern, or burst in phase with inspiration, inhibitory pre-phrenic C-INs had an expiratory firing pattern. In the medulla, pre-inspiratory/inspiratory neurons (i.e., those that start before and continue firing into inspiration) are critical for respiratory rhythm generation (Smith et al., 2013). Whether inspiratory pre-phrenic C-INs have rhythmogenic properties is unknown, but there is evidence suggesting that spinal networks can produce oscillations in phrenic output (Viala and Fretton, 1983). The pre-phrenic C-IN data presented here support the hypothesis that the cervical proprio-spinal network is capable of modulating the output of the phrenic motor nucleus on a breath-by-breath basis.

Our data also show that AIH alters the effective connections between second-order excitatory C-INs and pre-phrenic C-INs. Thus, AIH may trigger *de novo* recruitment of C-INs into proprio-spinal network which is coupled to the phrenic motor nucleus. Due to the short time scale in which this occurs, this likely reflects activation of existing, but previously silent synaptic connections.

Figure 9. Locations of recorded neurons stratified by firing pattern. **A–D**, Summary of locations of recorded C-INs and phrenic motoneurons (MN) during BL (**A**), hypoxia 1 (**B**), posthypoxia 1 (**C**), and 15 min post-AIH (**D**).

tions. One established mechanism underlying this phenomenon is serotonin release (Li and Zhuo, 1998). Several prior studies show that serotonin can rapidly activate polysynaptic spinal pathways to phrenic motoneurons (Mitchell et al., 1992; Ling et al., 1994). Because serotonin is released during hypoxia (Kinkead et al., 2001), we hypothesize that serotonin may be involved in shaping the response of the cervical propriospinal network to AIH.

AIH, C-INs, and spinal cord plasticity

AIH induces plasticity in sympathetic (Dick et al., 2007), somatic (Lovett-Barr et al., 2012; Trumbower et al., 2012), and respiratory motor output (Baker et al., 2001). To date, studies of AIH-induced spinal plasticity have primarily used recordings of nerve and/or muscle activity as outcome measures, but provide little to no insight regarding propriospinal networks. Data from Mitchell and colleagues suggest that AIH-induced phrenic motor plasticity

requires mechanisms within phrenic motoneurons. Specifically, delivery of relevant siRNA molecules (e.g., TrkB or PKC θ) to the intrapleural space and subsequent knockdown of TrkB or PKC θ in phrenic motoneurons attenuates AIH-induced plasticity (Devinney et al., 2015; Dale et al., 2017). These observations indicate that motoneuron plasticity is a fundamental component of the response to AIH, but do not preclude a role for C-INs in the expression of phrenic motor plasticity. Our data confirm that the cervical propriospinal network is responding to acute hypoxia, and show persistent changes in network excitability post-AIH. C-INs may therefore be a fundamental part of the neural substrate driving AIH-induced motor plasticity in respiratory and other systems. There is precedent that chemoafferent stimulation can alter network connectivity in brainstem structures. Specifically, Morris et al. (2000) showed that carotid chemoreceptor stimulation (i.e., mimicking hypoxia) can alter synaptic connec-

tions in medullary neural networks. However, our data are the first to show that changes in excitatory and inhibitory connectivity among C-INs are evoked by AIH. Determining the functional impact of these changes is challenging due to the complexity of propriospinal networks (Jankowska, 2001), and likely will require genetic and/or viral approaches to selectively inhibit or activate specific populations of C-INs. However, based on the current data showing rapid and sustained changes in propriospinal connectivity, we propose that C-INs are an integral part of the neuronal substrate orchestrating the respiratory, somatic, and autonomic neuroplastic responses to AIH.

Significance

It is well established that propriospinal neurons play a critical role in shaping motor output along the spinal neuraxis (Cherniak et al., 2017). In regards to the cervical spinal cord and breathing, prior work establishes that some C-INs are synaptically coupled to spinal respiratory motoneurons (Lane, 2011), and that C-INs can show respiratory-related discharge patterns. However, the functional impact of the cervical propriospinal network on phrenic (diaphragm) motor output is less clear. In this context, the current data establish a few significant points. First, AIH can cause persistent changes in connectivity within the overall propriospinal network. AIH is firmly established to be a powerful trigger of spinal respiratory neuroplasticity (Fuller and Mitchell, 2017), and these results indicate that C-INs are an important component of the neuroplastic response to AIH. This may be particularly important in the context of using AIH as a “neurorehabilitation modality” (Gonzalez-Rothi et al., 2015), because AIH exposure can promote functional recovery of respiratory as well as nonrespiratory motor output following spinal cord injury in animal models and humans (Trumbower et al., 2012; Hayes et al., 2014; Tester et al., 2014; Gonzalez-Rothi et al., 2015). Indeed, remodeling of propriospinal networks is an important component of motor recovery after incomplete spinal cord injury (Bareyre et al., 2004) and AIH may facilitate this process. Second, these data show that C-INs which are pre-phrenic (i.e., synaptically coupled to phrenic motoneurons) have excitatory and inhibitory inputs to phrenic motoneurons, and that excitatory input to excitatory C-INs increases during AIH. Thus, C-INs may have a more important role in controlling the phrenic motor pool and the diaphragm than is currently appreciated. Together, our data indicate that the midcervical spinal cord contains a network that responds dynamically to acute hypoxia, and that AIH alters the overall connectivity of the interneuronal network.

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