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Hypoxia triggers short term potentiation of phrenic motoneuron discharge after chronic cervical spinal cord injury

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Abstract

Repeated exposure to hypoxia can induce spinal neuroplasticity as well as respiratory and somatic motor recovery after spinal cord injury (SCI). The purpose of the present study was to define the capacity for a single bout of hypoxia to trigger short-term plasticity in phrenic output after cervical SCI, and to determine the phrenic motoneuron (PhrMN) bursting and recruitment patterns underlying the response. Hypoxia-induced short term potentiation (STP) of phrenic motor output was quantified in anesthetized rats 11 wks following lateral spinal hemisection at C2 (C2Hx). A 3-min hypoxic episode (12–14% O₂) always triggered STP of inspiratory burst amplitude, the magnitude of which was greater in phrenic bursting ipsilateral vs. contralateral to C2Hx. We next determined if STP could be evoked in recruited (silent) PhrMNs ipsilateral to C2Hx. Individual PhrMN action potentials were recorded during and following hypoxia using a “single fiber” approach. STP of bursting activity did not occur in cells initiating bursting at inspiratory onset, but was robust in recruited PhrMNs as well as previously active cells initiating bursting later in the inspiratory effort. We conclude that following chronic C2Hx, a single bout of hypoxia triggers recruitment of PhrMNs in the ipsilateral spinal cord with bursting that persists beyond the hypoxic exposure. The results provide further support for the use of short bouts of hypoxia as a neurorehabilitative training modality following SCI.

Keywords

phrenic; spinal cord injury; hypoxia; neuroplasticity; recruitment

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Introduction

Hemileision of the spinal cord at the second cervical segment (C2Hx) results in paralysis of the ipsilateral diaphragm. However, spontaneous inspiratory phrenic motor activity resumes over a period of weeks to months post-injury (Fuller et al., 2008; Lee et al., 2014a; Nantwi et al., 1999). This recovery appears to be mediated by descending mono- and possibly polysynaptic neuronal pathways that cross the spinal midline caudal to C2 to innervate phrenic motoneurons (PhrMNs) (Goshgarian, 2003, 2009; Hoh et al., 2013; Lane et al., 2009). The activation of ipsilateral PhrMNs after C2Hx is termed the crossed phrenic phenomenon (CPP), and makes a small contribution to inspiratory tidal volume during spontaneous breathing (Dougherty et al., 2012b). The relative strength of the CPP, however, can be considerably enhanced with neurorehabilitation strategies (Alilain et al., 2011; Doperalski and Fuller, 2006; Gransee et al., 2013; Lovett-Barr et al., 2012). Controlled exposure to hypoxia holds promise in this regard since appropriate paradigms can trigger robust spinal neuroplasticity (Baker-Herman et al., 2004) and both somatic (Hayes et al., 2014; Trumbower et al., 2012) and respiratory (Tester et al., 2014) motor recovery after SCI in humans. To date, studies involving short-term exposure to hypoxia in SCI models have focused on the persistent increase in phrenic activity that is triggered by repeated exposures. This response, termed phrenic long-term facilitation (LTF), can be evoked following chronic C2Hx in rats, and the response is more robust in the ipsilateral compared to contralateral motor output (Doperalski and Fuller, 2006). A recent report confirms that LTF of ventilation occurs following intermittent hypoxia in humans with SCI (Tester et al., 2014).

Short term hypoxic exposure can also induce a more transient form of phrenic motor plasticity called short-term potentiation (STP). This response is manifest as a progressive enhancement of phrenic activity that follows the initial carotid-body mediated acute hypoxic response followed by a gradual decline to baseline levels after removal of the hypoxic stimulus (Lee et al., 2009; Powell et al., 1998). To our knowledge, no prior studies have specifically evaluated the capacity of the phrenic motor system to express STP after cervical SCI. The fundamental changes in phrenic motoneuron (PhrMN) regulation that occur after cervical SCI (Lee et al., 2013) are likely impact hypoxia induced neuroplasticity (Golder and Mitchell, 2005). For example, in the rat C2Hx model, both serotonin and glutamate receptor expression are altered in PhrMNs, and both receptors are involved in modulation of the phrenic bursting and plasticity (Mantilla et al., 2012). In addition, both the magnitude of the acute phrenic response to hypoxia (Fuller et al., 2008) and the ability to induce LTF are altered (Doperalski and Fuller, 2006). Accordingly, our first purpose was to define the temporal characteristics of hypoxia-induced phrenic STP following chronic C2Hx. Since the contralateral phrenic motor pool shows compensatory increases in output after C2Hx, and this may limit subsequent neuroplasticity (Doperalski and Fuller, 2006), we hypothesized that STP would be more robust in the ipsilateral motor pool. There are two prior publications of PhrMN discharge patterns after SCI (El-Bohy and Goshgarian, 1999; Lee et al., 2013). The first report from El-Bohy and Goshgarian described ipsilateral PhrMN bursting in rats a few hours after a C2Hx lesion. During induction of the crossed-phrenic phenomenon the discharge frequency of PhrMNs ipsilateral to C2Hx was increased, and previously silent PhMNs were recruited during intense respiratory stimulation (El-Bohy and

Goshgarian, 1999). We recently reported that following chronic C2Hx most active PhrMNs in the ipsilateral spinal cord initiate bursting well after the onset of inspiration, and PhrMNs showed a decrease in discharge duration and burst frequency (Lee et al., 2013). To our knowledge, there is currently no information on how PhrMNs respond to hypoxia following chronic SCI, and PhrMN burst patterns during any form hypoxia-induced phrenic motor plasticity remains an almost completely unexplored (Lee and Fuller, 2011). As mentioned above, several groups are investigating hypoxia as a neurorehabilitative tool following chronic SCI (Hayes et al., 2014; Tester et al., 2014; Trumbower et al., 2012). Accordingly, the second purpose of our investigation was to determine the impact of an acute hypoxic exposure on PhrMN bursting ipsilateral to chronic C2Hx. The majority of PhrMNs in the ipsilateral spinal cord are expected to be inactive following C2Hx (Lee et al., 2013), and accordingly we predicted that hypoxia would cause recruitment of PhrMNs, but more importantly, that the discharge of these recruited cells would persist upon restoration of arterial oxygen levels to baseline values. In other words, the second goal of this work was to describe the capacity for short term hypoxia-induced plasticity in PhrMN bursting following chronic cervical SCI.

Materials and Methods

Animals

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida. Male Sprague-Dawley rats (N=23) were obtained from Harlan Inc. (Indianapolis, IN, USA). The experimental design did not require uninjured animals since our *a priori* purpose and hypotheses focused on whether or not a phrenic motor response (i.e., hypoxia-induced STP), which has been well documented in spinal-intact animals (Lee et al., 2009), could also be evoked after SCI. Thus, in accordance with the recommendations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the University of Florida IACUC we studied only C2Hx animals, and this served to reduce the number of experimental animals needed to complete this study. The C2Hx lesion was surgically induced at 3 months of age (93 ± 1 day), and all terminal neurophysiology procedures were done approximately 3 months post-injury (11 ± 1 wks).

Spinal cord injury

These procedures were adapted from our prior reports (Dougherty et al., 2012a; Dougherty et al., 2012b). Animals were anesthetized by xylazine (10 mg/kg, s.c.) and ketamine (140 mg/kg, i.p., Fort Dodge Animal Health, USA), and then placed in the prone position. A dorsal cervical incision was made from the base of the skull to the C3 spinal segment followed by laminectomy and durotomy at C2. A C2Hx lesion was induced on the left side using a micro-scalpel followed by aspiration. The dura and overlying muscles were sutured with 9-0 suture (Ethicon, USA) and 4-0 polyglycolic acid suture (Webster Veterinary, USA), respectively. The skin was then closed with stainless steel wound clips (Stoelting, USA). Following surgery, yohimbine (1.2 mg/kg, s.c., Lloyd, USA) was given to reverse the effect of xylazine. Animals were then given sterile lactated Ringers solution (5 ml s.c.) and an analgesic (buprenorphine, 0.03 mg/kg, s.c., Hospira, USA). The post-surgical care

protocol included daily injection of lactated Ringers solution (5 ml, s.c.) and a high calorie oral supplement (Nutri-cal, 1–3 ml, Webster Veterinary, USA) until adequate volitional drinking and eating resumed.

Neurophysiology preparation

Isoflurane anesthesia (3–4%) was induced in a closed chamber and then maintained via nose cone (2–3%). Rats were tracheotomized and mechanically ventilated (model 683; Harvard Apparatus, Inc., USA) with a hyperoxic gas mixture (50–60% O₂, balance N₂). The tidal volume was set at 7 ml/kg and frequency maintained at 60–70 per minute. Rectal temperature was monitored by an electrical thermometer and maintained at 37.5±1°C by a servo-controlled heating pad (model TC-1000, CWE Inc., USA). The femoral vein was catheterized (PE-50) to enable conversion to urethane anesthesia (1.6 g/kg, i.v., Sigma, USA) and delivery of a neuromuscular blocking agent (pancuronium bromide, 2.5 mg/kg, i.v., Hospira, Inc., Lake Forest, USA). Another catheter was inserted into the femoral artery for blood pressure measurement (Statham P-10EZ pressure transducer, CP122 AC/DC strain gauge amplifier, Grass Instruments, USA). The vagus nerves were isolated in the mid-cervical region and sectioned to prevent entrainment of phrenic bursting with the rate of the ventilator. The vagotomy procedure has been used extensively in studies of respiratory neuroplasticity and also crossed phrenic activity after C2Hx (Doperalski and Fuller, 2006; Lee et al., 2010a). This procedure helps to ensure standardized conditions across animals and within each experiment, which is an important consideration for basic studies of hypoxia-induced plasticity. However, activation of vagal afferent neurons has an inhibitory influence on ipsilateral phrenic motor output after C2Hx (Lee et al., 2010a), and accordingly the vagotomy is likely to have increased the relative activity of ipsilateral PhrMNs. The end-tidal CO₂ partial pressure (P_{ET}CO₂) was monitored throughout the experiment using a Capnogard neonatal CO₂ monitor placed on the expired line of the ventilator circuit (Novamatrix Medical Systems, Wallingford, USA).

The phrenic nerves were isolated in the cervical region via a ventral approach and sectioned distally (Lee et al., 2009; Lee et al., 2010a). Activity of both phrenic nerves were recorded by silver hook electrodes, and then amplified (1000x, Model 1700, A-M Systems, Carlsborg, WA, USA), band-pass filtered (0.3–10 kHz), full-wave rectified and integrated (time constant 100 ms; model MA-1000; CWE Inc., USA). In some of the experiments, the phrenic nerve ipsilateral to C2Hx (i.e. left side) was stripped of connective tissue, desheathed and then separated into small filaments to enable extracellular recording of PhrMN action potentials (Lee et al., 2013; Lee et al., 2009). All signals were digitized using Cambridge Electronic Design (CED) Power 1401 data acquisition interface and a sampling frequency of 10 KHz for the raw neural signals and 100 Hz for the blood pressure and integrated neural signals. These data were recorded on a PC and analyzed using Spike 2 software (CED Limited, UK).

Experimental protocols

In the first protocol, bilateral phrenic nerve activity was recorded to investigate hypoxia-induced phrenic STP following chronic C2Hx (N=12). After establishing stable phrenic nerve recordings, P_{ET}CO₂ was gradually reduced by increasing the rate of the mechanical

ventilator until rhythmic bursting in the right phrenic nerve (i.e. contralateral to C2Hx) ceased for at least 2 minutes. The ventilator rate was then gradually decreased until inspiratory phrenic bursting reappeared, and this point was designated as the $P_{ET}CO_2$ recruitment threshold. $P_{ET}CO_2$ was then maintained at 2–3 mmHg above the recruitment threshold during all subsequent experimental procedures. After a stable baseline recording was maintained for at least 10 minutes, the animal was exposed to a three minute bout of hypoxia (12–14% O_2 , balance N_2). Following hypoxia, both inspired O_2 and $P_{ET}CO_2$ levels were regulated at the baseline levels.

A second experimental protocol was used to study the discharge patterns of PhrMNs ipsilateral to C2Hx during hypoxia-induced STP. In these experiments, the right phrenic nerve and a left “phrenic filament” were recorded in 23 rats. Twelve of these rats were also studied as part of the first experimental protocol. Identification of PhrMNs which were inactive (i.e., silent) at baseline was accomplished by maintaining $P_{ET}CO_2$ 10 mmHg above the recruitment threshold during the initial action potential recordings. After PhrMN recordings were established, the $P_{ET}CO_2$ was returned to 2–3 mmHg above the recruitment threshold. A three minute bout of hypoxia (12–14 % O_2 , balance N_2) was then used to induce STP.

All rats were exposed to identical experimental conditions, but arterial blood gases and pH were measured in a subset of animals (N=13) from 0.2 ml arterial blood samples (i-Stat, Heska, USA) during baseline and the final minute of hypoxia.

Previous authors have defined hypoxia-induced STP as the progressive augmentation of respiratory motor output that typically follows the acute hypoxic response (i.e., an “onset phase”), and the short-lasting increase in phrenic activity that persists upon removal of the hypoxic stimulus (an “offset phase”) (Lee et al., 2009; Powell et al., 1998). In this study, we focused on the post-hypoxia phrenic response, and therefore defined STP as an increase in phrenic nerve burst amplitude or PhrMN discharge rate (i.e., above pre-hypoxia baseline values) that was present during the period of 2:30–3:00 (minutes:seconds) following the hypoxic exposure.

Spinal cord histology

At the end of the neurophysiology protocol, animals were systemically perfused with saline followed by 4% paraformaldehyde (Sigma, USA). The cervical spinal cord was removed, cryoprotected and sectioned at 40 μ m using a vibratome. Spinal cord tissue sections were mounted on glass slides (Fisher Scientific, USA) and stained with the cresyl violet. The tissue sections were evaluated by light microscopy, and a C2Hx lesion was confirmed in each case by the absence of apparently healthy white and grey matter in the left C2 spinal cord (Lee et al., 2010a).

Data analyses

Inspiratory (T_I) and expiratory duration (T_E) were calculated from the phrenic neurograms recorded contralateral to the lesion as previously described (Lee et al., 2013; Lee et al., 2009; Lee et al., 2010a). Respiratory frequency (bursts* min^{-1}) was calculated as $60/(T_I + T_E)$. Phrenic burst amplitude was defined as the difference between the maximum and

minimum value of the integrated phrenic neurogram during a respiratory cycle. Burst amplitude data were expressed as arbitrary units (a.u.) and also normalized relative to baseline activity (% baseline) and relative to the maximal response recorded during the protocol (% max). All normalization procedures were done in comparison to the same neurogram (i.e., ipsilateral normalized to ipsilateral).

Some PhrMNs were inactive during the normocapnic baseline condition but were recruited during hypoxia. These motoneurons were classified as “silent”. Cells which discharged throughout the whole respiratory cycle during the baseline were classified as “tonic”. Parameters which were analyzed included the PhrMN discharge onset, frequency, duration and spike number. The burst onset time of PhrMNs was calculated using the right phrenic neurogram as an index of the respiratory cycle (e.g., Fig. 1). Discharge frequency was calculated by dividing the total spike number per inspiratory effort by the total discharge duration (i.e., the period between the first and last spike). Spike number was measured as the total number of spikes per inspiratory effort. These values were calculated during the 30 seconds immediately preceding hypoxia (i.e., the baseline period), the initial and final 30 seconds of hypoxia exposure, and for the final 30 seconds of the three minute post-hypoxic period.

The impact of hypoxia on phrenic nerve bursting was examined using a one-way repeated measurement analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc test. Two way repeated measures ANOVA was used to compare the hypoxic response of the contralateral vs. ipsilateral phrenic nerves and silent PhrMNs showing post-hypoxia STP vs. those that did not. Changes in PhrMN firing were analyzed using one-way repeated measures ANOVA. All data are presented as the mean $\pm$ 1 standard error. A P-value less than 0.05 was considered statistically significant.

Results

Arterial blood gases, mean arterial pressure, and heart rate

Compared to baseline conditions, reductions in inspired O₂ caused the expected decrease in PaO₂ (Table 1). The hypoxic stimulus was isocapnic with no significant changes in PaCO₂, and arterial pH was also similar between the baseline and hypoxic conditions (Table 1). Mean arterial blood pressure was reduced during hypoxia (Table 2) but returned to the baseline value by 3 min post-hypoxia. Heart rate tended to increase during hypoxia, but the response was variable and not statistically significant (Table 2).

Phrenic nerve activity

A representative example of extracellularly recorded phrenic nerve activity is provided in Fig. 1. Note that clear, rhythmic inspiratory bursting was recorded from both phrenic nerves during the baseline condition. Inspiratory phrenic nerve activity ipsilateral to C2Hx (i.e., the left side) was present in all animals; however, the burst amplitude was substantially reduced when compared to the contralateral side (Fig. 2A, $F_{1,11}=84.1$, $P < 0.001$).

Hypoxia caused an increase in the inspiratory burst amplitude in both phrenic nerves, as expected (e.g., Fig. 1). To facilitate comparison between the two phrenic recordings, burst

amplitude was evaluated as both %baseline and %max (Fig. 2B–C). Both normalization methods revealed an interaction between time (i.e., baseline, hypoxia) and amplitude (%baseline: $F_{1,11} = 19.3$, $P < 0.001$; %max: $F_{1,11} = 100.3$, $P < 0.001$). Inspection of the data indicates that increases in bursting were greater in the ipsilateral vs. contralateral recording, and were much more pronounced at the end of the hypoxic period (Fig. 2B–C). Thus, the onset of STP was particularly robust in ipsilateral phrenic motor output. Post-hypoxia STP of burst amplitude was present in both phrenic nerve recordings, and again the magnitude was greater in ipsilateral compared to contralateral output (Fig. 2B).

Inspiratory burst frequency ($\text{br} \cdot \text{min}^{-1}$) is reported in Table 3. Frequency showed a rapid increase at the onset of hypoxia due to shortening of both T_I and T_E ($P < 0.05$). A “roll off” in burst frequency occurred over the 3 min hypoxic stimulus, but values remained above baseline ($P < 0.05$). Following removal of the hypoxic stimulus, post-hypoxia frequency depression (Bach et al., 1999) occurred due to a prolongation of T_E ($P < 0.01$).

Classification of PhrMNs based on bursting patterns

Forty-seven PhrMNs were recorded and the distribution of burst onset relative to the inspiratory activity recorded in the contralateral phrenic nerve is shown in Fig. 3. Under baseline conditions, four PhrMNs were recorded which clearly were activated earlier in the inspiratory effort as compared to all other recorded neurons (Fig. 4). The discharge onset of these cells occurred at $11.5 \pm 2.7\%$ T_I . However, the majority of PhrMNs which were active at baseline initiated bursting later in the inspiratory effort (Fig. 5). These cells had a mean discharge onset of $29.1 \pm 1.9\%$ T_I (Fig. 3). Two PhrMNs were recorded which fired throughout the respiratory cycle (i.e., tonic) but with a higher discharge frequency during inspiration (Fig. 6). During baseline conditions, the majority of PhrMNs recorded in our study were not active (i.e., silent PhrMNs, $N=29$, Fig. 7).

The impact of hypoxia on PhrMN firing patterns

Quantification of PhrMN discharge onset, duration and frequency across the four time points (i.e., baseline, hypoxia onset, final 30 sec of hypoxia, and three minutes post-hypoxia) is presented in Fig. 8. Due to the small sample size, early onset PhrMNs are not included in the statistical analyses of the hypoxic response. Upon recruitment, silent PhrMNs were activated late in the inspiratory burst (Fig. 7). During and following hypoxia, the onset of PhrMN discharge was relatively stable with the following exception. By the end of hypoxia, the onset of bursting for the “late-inspiratory” cells occurred earlier in the inspiratory effort (Fig. 8A).

Discharge frequency during hypoxia had a different temporal profile in cells active at baseline vs. silent cells (Fig. 8B). For cells active at baseline, frequency was increased immediately at the onset of hypoxia, and the increased frequency was maintained throughout the hypoxic stimulus and the 3 min post-hypoxia period (Fig. 8B). Silent PhrMNs were recruited during hypoxia and their burst frequency increased approximately fourfold from the onset to the end of hypoxia. In addition, at three minutes post-hypoxia, approximately 50% of the recorded silent cells remained active. Thus, on average, silent PhrMN burst

frequency remained elevated following hypoxia (but see below - *Stratification of silent PhrMNs based on presence of STP*).

The duration of discharge (ms) was considerably different between recruited (silent) PhrMNs and those cells which were active prior to hypoxia (Fig. 8C). Previously active PhrMNs had stable discharge duration with no significant changes either during or following hypoxia. In contrast, the recruited silent cells showed a progressive increase in the discharge duration over the course of hypoxia (Fig. 8C).

Cells which burst throughout the respiratory cycle at baseline (i.e., tonic PhrMNs) became “phasic” during hypoxia. More specifically, by the end of hypoxia, firing during the late expiratory phase was eliminated and these cells discharged only during the inspiratory and post-inspiratory phase (Fig. 6). PhrMNs which burst tonically were not included in the analyses shown in Fig. 8 because it was not possible to discern their onset and offset times at baseline.

Stratification of silent PhrMNs based on presence of STP

Following the hypoxic stimulus, approximately one half of the recorded silent PhrMNs remained active (15/29) while the remainder immediately ceased bursting (14/29). To determine if bursting patterns *during* hypoxia could predict whether or not a silent PhrMN would demonstrate post-hypoxia STP, we compared discharge properties between these two subpopulations (Fig. 9). The discharge onset (% TI) was similar, but discharge frequency (Hz), duration (ms) and spikes per inspiratory cycle were all different between silent PhrMNs showing post-hypoxia STP vs. those that did not ($P < 0.05$). Thus, during the onset of hypoxia, the PhrMNs which showed STP burst at a higher frequency (Fig. 9B), and also had greater discharge duration throughout the hypoxic stimulus (Fig. 9C).

Discussion

This is the third study to evaluate PhrMN discharge patterns following cervical SCI (El-Bohy and Goshgarian, 1999; Lee et al., 2013), and the first to explore discharge patterns during hypoxia-induced neuroplasticity. A short bout of hypoxia was sufficient to induce STP of phrenic motor output after chronic C2Hx, and the response was more robust ipsilateral to the spinal cord lesion. Comprehensive analysis of discharge patterns indicated that STP of discharge frequency occurred in approximately 50% of PhrMNs which were silent at baseline but initiated bursting during hypoxia. Thus, a single brief hypoxic episode was sufficient to trigger recruitment of a population of silent PhrMNs which continued to burst beyond the period of hypoxic stimulation. This result supports the continued exploration of hypoxia as a neurorehabilitation tool in the context of SCI (Hayes et al., 2014; Tester et al., 2014; Trumbower et al., 2012).

STP of phrenic motor output

STP of respiratory motor output has been described in humans (Fregosi, 1991) and animal models (Hayashi et al., 2003; Lee et al., 2009; Mitchell et al., 2001) and can therefore be considered a biologically robust response. The temporal features of STP have been qualitatively consistent across published reports with both an onset (i.e., during hypoxia) and

offset phase (i.e., post-hypoxia) (Powell et al., 1998). Here we confirm that STP of phrenic motor output can be evoked following chronic C2Hx, a condition that deprives ipsilateral PhrMNs of the vast majority of descending synaptic inputs. Thus, despite profound changes in the neural regulation of PhrMNs (Lee et al., 2013), conditions are still appropriate for this particular form of hypoxia-induced neuroplasticity. Indeed, our results indicate that STP is actually enhanced in the denervated phrenic motor pool (i.e., ipsilateral to the spinal lesion). This observation could reflect the greater number of silent PhrMNs after C2Hx (Lee et al., 2013) and therefore a greater neuronal substrate for recruitment and plasticity. An alternate view is that neuroplastic changes in the spinal cord (Alilain and Goshgarian, 2008; Mantilla et al., 2012; Sperry and Goshgarian, 1993) and/or brainstem (Golder et al., 2001; Huang and Goshgarian, 2009; Lee et al., 2010a) create conditions which enhance the ability of ipsilateral PhrMNs to express hypoxia-induced plasticity. We suggest that differences in PhrMN discharge patterns between spinal intact compared to spinally injured rats provide support for this possibility. For example, in a prior study of spinal intact rats (Lee et al., 2009), we found that recruited PhrMNs always stopped bursting immediately upon removal of a hypoxic stimulus (i.e., there was an absence of post-hypoxia STP in silent PhrMNs). In contrast, in the current study, we noted that approximately 50% of recruited PhrMNs continued to burst during the post-hypoxic period. Accordingly, following SCI there appears to be a fundamental change in the manner by which silent PhrMNs respond to hypoxia. We hypothesize that post-C2Hx neuroplastic changes in the neurons and/or networks which control the diaphragm muscle create conditions which favor induction of hypoxia-induced plasticity in PhrMN discharge.

PhrMN discharge patterns

An increased percentage of silent PhrMNs is expected after C2Hx because the ipsilateral motor pool has been deprived of the majority of bulbospinal synaptic inputs. The current results confirm this prediction, and show that after chronic C2Hx the acute hypoxic response of the ipsilateral phrenic motor pool reflects a mixture of PhrMN recruitment and rate coding. In other words, hypoxia caused active PhrMNs to increase discharge rate in parallel with initiation of bursting in previously silent PhrMNs. Following hypoxia, a persistent increase in bursting (i.e., STP) was observed in many PhrMNs. Thus, cells activated during hypoxia are contributing to the post-hypoxia increase in phrenic motor output. Interestingly, all of the PhrMNs which showed STP were activated after the onset of the inspiratory effort, and would be considered to have a “late-inspiratory” phenotype based on previous publications (reviewed in (Lee and Fuller, 2011)). On the other hand, none of the four PhrMNs which were recruited early in the inspiratory effort showed post-hypoxia STP (e.g., Fig. 4). Following C2Hx, the recruitment of PhrMNs is likely to be determined by an interaction between the prevailing strength of synaptic inputs and intrinsic motoneuron properties. Thus, the onset times recorded in this study, while descriptive, provide no direct information regarding the mechanisms driving PhrMN bursting. Nevertheless, the data suggest that cells active early in the inspiratory effort after C2Hx are less likely to show STP after a short bout of hypoxia.

Similar to a few prior reports (Lee et al., 2013; Marchenko et al., 2012), we noted a small number of PhrMNs which burst throughout the respiratory cycle (i.e., tonic bursting).

Rogers and colleagues have reported tonic PhrMN activity in spinal-intact rats (Marchenko et al., 2012), and we have observed the same using an intraspinal recording approach in spinal-intact rats (M.S. Sandhu and D.D. Fuller, unpublished observations). Thus, tonic PhrMN bursting is not unique to conditions created by cervical SCI. In the current study, cells with tonic bursting switched to a more phasic pattern when respiratory drive was increased during hypoxia (e.g., Fig. 6). This observation confirms our prior report in which tonic PhrMN bursting in rats with C2Hx became phasic during hypercapnia (Lee et al., 2013). We are unaware of any other prior reports describing PhrMNS shifting from tonic and phasic bursting as respiratory drive increases, and accordingly this may be a discharge pattern that emerges only after cervical SCI.

We next briefly consider the mechanisms which could underlie this potentially unique feature of PhrMN bursting. The C2Hx lesion is certain to cause a loss of both excitatory and inhibitory synaptic inputs to ipsilateral PhrMNs. Based on the limited number of PhrMNs recruited during the spontaneous crossed phrenic phenomenon (Fuller et al., 2008), many PhrMNs are likely to receive little, if any, excitatory synaptic input during inspiration after C2Hx. Under these conditions, mechanisms intrinsic to the motoneuron may drive tonic bursting. For example, Murray and colleagues (Murray et al., 2010) recently showed that following chronic SCI, serotonin receptors on motoneurons can become constitutively active (*i.e.* activated without ligand binding). This response occurs in the absence of descending inputs to motoneurons, and is associated with persistent inward currents and tonic bursting. It is not known if a similar mechanism is active in PhrMNs following SCI, but the occurrence of tonic PhrMN bursting during inspiratory apnea after C2Hx (Lee et al., 2013) is consistent with that possibility. Another potential mechanism is a constant, excitatory synaptic input to a subset of PhrMNs that is not inhibited during the expiratory period (Hayashi and Fukuda, 1995). In this scenario, inspiratory bursting would emerge if inhibitory (expiratory) synaptic inputs were activated during respiratory stimulation. Finally, we previously reported that vagotomy enhances ipsilateral phrenic bursting after C2Hx in rats (Lee et al., 2010b). Therefore, removal of vagal inhibition in the mechanically ventilated experimental preparation may have resulted in, or contributed to, the appearance of tonic PhrMN discharge.

Conclusion

These results provide further support for the use of short bouts of hypoxia as a neurorehabilitative training modality following SCI (Dale et al., 2014; Hayes et al., 2014; Lee et al., 2014b; Tester et al., 2014). Recent work from Trumbower's laboratory has shown a remarkable rehabilitative impact of hypoxia training on motor function following SCI in humans (Hayes et al., 2014). This work was based on multiple studies in animal models which firmly established that hypoxia based therapies can enhance respiratory motor recovery (reviewed in (Dale et al., 2014)). To date, both the clinical and preclinical work in this area has focused on repeated bouts of hypoxia. Here we show that even a single bout of hypoxia can induce recruitment and sustained bursting of PhrMNs after chronic spinal cord injury. These cells would thus be "primed" for further activity during subsequent exposures to hypoxia (*i.e.*, an intermittent hypoxia paradigm, (Dale et al., 2014)), or locomotor based training (Hayes et al., 2014).

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Abbreviations list

C2Hx	C2 spinal cord hemisection
Early-I	early-inspiratory
Late-I	late-inspiratory
P_{ET}CO₂	end-tidal CO ₂ partial pressure
PhrMNs	phrenic motoneurons
SCI	spinal cord injury
T_I	inspiratory duration
T_E	expiratory duration

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Highlights

- Rats were studied 3 months after lateral spinal cord hemisection at C2 (C2Hx)
- Hypoxia-induced short term potentiation (STP) was robust in ipsilateral phrenic activity
- Hypoxia caused recruitment of silent phrenic motoneurons (PhrMNs) ipsilateral to C2Hx
- STP of PhrMN discharge was evident in recruited cells
- Hypoxia holds promise as a rehabilitative training modality following spinal injury

Fig. 1. Representative data illustrating the impact of hypoxia on phrenic (Phr) nerve activity recorded ipsilateral (IL) and contralateral (CL) to C2Hx

The $\int$ Phr trace represents the moving averaged or “integrated” signal. Panel B shows expanded time scale traces depicting a single neural breath taken from the areas indicated by i-iv in panel A. The horizontal solid line indicates the period of hypoxia exposure. Phrenic motor output recorded ipsilateral to the C2Hx lesion is relatively low during baseline (i) but shows a robust increase during hypoxia (ii and iii). Following hypoxia, the burst amplitude in both phrenic nerve recordings remains elevated above baseline values (iv). BP: Blood pressure

Fig. 2. Mean inspiratory burst amplitude recorded in the ipsilateral (IL) and contralateral (CL) phrenic nerves during and following hypoxia

Data are expressed as arbitrary units (a.u.) in panel A. The data in panel B are normalized relative to baseline activity (% baseline). In panel C, changes in phrenic burst amplitude are expressed relative to the maximal response recorded during the protocol (% max). *: $P < 0.05$; **: $P < 0.01$ compared with the baseline. ##: indicates difference between CL and IL phrenic burst amplitude, $P < 0.01$.

Fig. 3. The distribution of PhrMN discharge onset

The discharge onset of PhrMN bursting is represented as a percentage of inspiratory duration (% T_I). Active PhrMNs were stratified based on discharge during the normocapnic baseline condition. Those PhrMNs classified as silent (dark grey bars) were not active during baseline, but were recruited during hypoxia. As described in the text, several tonically active PhrMNs were recorded in this study, but these cells are not included in this figure since onset time could not be defined.

Fig. 4. Representative example of a PhrMN activated at the onset of inspiration

The contralateral (CL) phrenic neurogram is presented as both raw (Phr) and integrated ($\int$ Phr) signals. PhrMN activity is presented as the instantaneous discharge frequency ($Inst f$) and individual spikes recorded using the “single fiber” method (PhrMN). Panel B shows expanded time scale traces depicting a single neural breath taken from the areas indicated by i–iv in panel A. The bottom traces labeled “Superimposition” show a superimposition of the individual PhrMN spikes, and are provided to demonstrate that the recordings are from the same neuron.

Fig. 5. Representative example of a PhrMN which initiated bursting after the onset of inspiration Hypoxia caused a progressive increase in the neurons discharge frequency (ii and iii). Following hypoxia, discharge frequency remained elevated above the baseline value indicating STP of burst activity (iv). The labels on this figure are the same as described in the Fig. 4 legend.

Fig. 6. Representative example of tonic PhrMN bursting

This PhrMN fired during both the inspiratory and expiratory phases of the respiratory cycle during the baseline (i). However, this neuron burst with a phasic bursting pattern by end of the hypoxic exposure (iii). Following hypoxia, discharge frequency remained elevated above the baseline value indicating STP of burst activity (iv). The labels on this figure are the same as described in the Fig. 4 legend.

Fig. 7. Representative examples of hypoxia-induced recruitment of silent PhrMNs

These traces show recruitment of two previously inactive PhrMNs during exposure to hypoxia. The trace labeled “Marker” represents the individual neuronal spikes. In this example, only one of the recruited PhrMNs (Marker-1) maintained firing at 3 min post-hypoxia. The labels on this figure are the same as described in the Fig. 4 legend. In the top panel (A), there is an interruption in the blood pressure trace just before the period indicated by iii. During this time, an arterial blood sample was withdrawn.

Fig. 8. Analyses of mean PhrMN discharge properties

PhrMNs are grouped as Previously active and Silent. Discharge onset (A), frequency (B) and duration (C) are presented during baseline, hypoxia onset and end, and 3 min post-hypoxia. *: P < 0.05; **: P < 0.01 compared with the baseline value.

Fig. 9. The mean response of Silent (recruited) PhrMNs during and following hypoxia
 Discharge onset (A), frequency (B) and duration (C) are presented during baseline, hypoxia onset and end, and 3 min post-hypoxia. For this analyses, cells were grouped based on whether their bursting immediately returned to baseline (i.e., “no post-hypoxia STP”) or was persistently increased (i.e., “post-hypoxia STP”). Discharge onset and peak hypoxic discharge frequency is similar between these two groups. However, those cells which went on to show post-hypoxia STP showed greater discharge duration during the hypoxic stimulus (panel C). **: $P < 0.01$ compared with the baseline value. #: $P < 0.05$; ##: $P < 0.01$ significant difference between the two groups.

Table 1

Arterial blood gas parameters during baseline and hypoxia.

	Baseline	Hypoxia
PaO ₂ (mmHg)	204 ± 7	39 ± 2 **
PaCO ₂ (mmHg)	39 ± 2	40 ± 2
pH	7.38 ± 0.01	7.36 ± 0.02

Data are means ± SE.

** : P < 0.01 compared with the baseline value. N=13.

Table 2

Mean arterial blood pressure (MAP) and heart rate (HR) during the baseline, hypoxia and 3 min post-hypoxia

	Baseline	Onset	End	3 min
MAP (mmHg)	130 ± 5	125 ± 5	114 ± 8**	137 ± 4
HR (beats•min ⁻¹)	430 ± 9	436 ± 9	442 ± 9	434 ± 12

“Onset” and “End” represent the initial and last 30 seconds of the hypoxic treatment; “3 min” represents the last 30 seconds of 3 minutes after termination of hypoxia.

** : P < 0.01 compared with the baseline value. N=12.

Table 3

Respiratory pattern during baseline, hypoxia and 3 min post-hypoxia

	Baseline	Onset	End	3 min
T _I (sec)	0.35 ± 0.02	0.31 ± 0.01 **	0.26 ± 0.01 **	0.33 ± 0.02
T _E (sec)	0.95 ± 0.05	0.64 ± 0.02 *	0.85 ± 0.05	1.38 ± 0.10 *
Frequency (burst•min ⁻¹)	47 ± 2	64 ± 2 **	55 ± 2 **	37 ± 2 **

Data are means ± SE.

* : P < 0.05;

** : P < 0.01 compared with the baseline value.

The data presented in this table were calculated from the phrenic neurogram recorded contralateral to the C2Hx lesion.