Title: Phrenic motoneuron discharge patterns during hypoxia-induced short term potentiation in rats

Authors: Kun-Ze Lee*, Paul J. Reier², David D. Fuller¹

Addresses:
¹University of Florida, College of Health and Health Professions
McKnight Brain Institute
Department of Physical Therapy
PO Box 100154, 100 Newell Dr, Gainesville, FL 32610

²University of Florida, College of Medicine
McKnight Brain Institute
Department of Neuroscience
PO Box 100244, 100 Newell Dr, Gainesville FL 32610

*, corresponding author
Email: kzlee@ufl.edu
Phone: (352) 392 7873
Fax: (352) 273 6109

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Abstract

Hypoxia-induced short term potentiation (STP) of respiratory motor output is manifested by a progressive increase in activity following the acute hypoxic response and a gradual decrease in activity upon termination of hypoxia. We hypothesized that STP would be differentially expressed between physiologically defined phrenic motoneurons (PhrMN). Phrenic nerve “single fiber” recordings were used to characterize PhrMN discharge in anesthetized, vagotomized and ventilated rats. PhrMNs were classified as early- (Early-I) or late-inspiratory (Late-I) according to burst onset relative to the contralateral phrenic neurogram during normocapnic baseline conditions. During hypoxia (FIO₂=0.12-0.14, 3-min), both Early-I and Late-I PhrMNs abruptly increased discharge frequency. Both cell types also showed a progressive increase in frequency over the remainder of hypoxia. However, Early-I PhrMNs showed reduced overall discharge duration and total spikes/breath during hypoxia whereas Late-I PhrMNs maintained constant discharge duration and therefore increased the number of spikes/breath. A population of previously inactive (i.e. silent) PhrMNs was recruited 48±8 sec after hypoxia onset. These PhrMNs had a Late-I onset and the majority (8/9) ceased bursting promptly upon termination of hypoxia. In contrast, both Early-I and Late-I PhrMNs showed post-hypoxia STP as reflected by greater discharge frequencies and spikes/breath during the post-hypoxic period (P < 0.01 vs. baseline). We conclude that the expression of phrenic STP during hypoxia reflects increased activity in previously active Early-I and Late-I PhrMNs and recruitment of silent PhrMNs. Post-hypoxia STP primarily reflects persistent increases in the discharge of PhrMNs which were active prior to hypoxia.
Introduction

The hypoxic ventilatory response is a complex, time-dependent process reflecting both acute and delayed onset mechanisms (Powell et al. 1998). The acute response includes a rapid increase in respiratory motor output (e.g. increased amplitude of respiratory muscle electromyogram [EMG] bursts or tidal volume) coupled with an increase in breath frequency. The acute response is followed by short-term potentiation (STP) of respiratory output (Powell et al. 1998; Fuller et al. 2005) which is manifest by a progressive increase in respiratory activity during hypoxia (i.e. the “onset” of STP) followed by a gradual return towards pre-hypoxia baseline activity after termination of hypoxia (i.e. the “offset” of STP).

Respiratory-related STP has been described using phrenic neurograms (e.g. compound, extracellular action potentials; Hayashi et al. 1993; Wagner and Eldridge 1991), diaphragm EMG (Mateika and Fregosi 1997) and ventilation measurements (Fregosi 1991; Georgopoulos et al. 1992; Kline et al. 2002). However, the in vivo behavior of respiratory motoneurons, including phrenic motoneurons (PhrMNs), has not been evaluated in the context of hypoxia-induced STP or other forms of respiratory neuroplasticity.

PhrMNs are classified based on their bursting patterns as early-inspiratory (Early-I; neurons which begin bursting at the onset of inspiration) or late-inspiratory (Late-I; cells initiating bursting activity later in the inspiratory effort) (Kong and Berger 1986; St. John and Bartlett 1979). In addition, several studies have described “silent” PhrMNs that are inactive during quiet breathing but can be recruited during chemoreceptor stimulation and/or cough (Milano et al. 1992; St. John and Bartlett 1979). However, it is unknown if the expression of respiratory motor plasticity associated with hypoxia (e.g. STP and/or long-term facilitation, LTF, Baker-Herman et al. 2004) is accompanied by persistently enhanced bursting of previously active Early-
and Late-I PhrMNs, or alternatively, recruitment of previously silent PhrMNs.

The primary purpose of the current study was to investigate PhrMN behavior, including discharge frequency, onset, duration, and total spikes per breath during hypoxia-induced STP of PhrMN output. The segregation of PhrMN responses following electrical activation of descending inputs in the cervical lateral funiculus (Hayashi et al. 2003; McCrimmon et al. 1997) led us to hypothesize that STP would be differentially expressed across physiologically defined groups of PhrMNs.

Materials and Methods

Animals. Male Sprague-Dawley rats (n=17; 397 ± 6 g) purchased from Harlan Inc. (Indianapolis, IN, USA) were used in the present study. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida.

General animal preparation. These procedures were similar to those described in our previous reports (Doperalski and Fuller 2006; Fuller et al. 2009). Animals were placed in a closed chamber and anesthetized by 3 - 4% isoflurane in oxygen. Stable anesthesia (2 - 3%) was then maintained via a nose cone. A short length of PE-240 tubing was then inserted into the trachea below the larynx and rats were mechanically ventilated for the remainder of the experiment (model 683; Harvard Apparatus, Inc, South Natick, MA, USA). A PE-50 catheter was inserted into the femoral artery for blood pressure measurement (Statham P-10EZ pressure transducer, CP122 AC/DC strain gauge amplifier, Grass Instruments, WestWarwick, RI, USA) and withdrawal of blood samples. Another catheter was placed into the femoral vein to enable conversion from the volatile anesthesia (isoflurane) to urethane (1.6 g/kg, i.v., Sigma, St. Louis, MO, USA) and injection of a paralytic drug (pancuronium bromide, 2.5
mg/kg, i.v., Hospira, Inc., Lake Forest, IL, USA). The vagus nerves were sectioned bilaterally in the cervical region to prevent entrainment of respiratory output with the ventilator. End-tidal CO₂ partial pressure (P_{ET}CO₂) was analyzed with a Capnogard neonatal CO₂ monitor placed on the expired line of the ventilator circuit (Novametrix Medical Systems, Wallingford, CT, USA). Arterial blood gases and pH were measured in 10/17 rats from 0.2 ml arterial blood samples (i-Stat, Heska, Fort Collins, CO, USA) obtained at baseline and during the final minute of hypoxia (see below). Rectal temperature was monitored by an electrical thermometer and maintained at 37.5 ± 1 °C using a servo-controlled heating pad (model TC-1000, CWE Inc., Ardmore, PA, USA).

**Phrenic nerve and motoneuron recording.** Both phrenic nerves were exposed in the neck region with a ventral surgical approach and sectioned peripherally. The methods for recording phrenic fibers were similar to our previous reports (Lee et al. 2007a, b, 2008). Briefly, the left phrenic nerve was stripped of connective tissue, desheathed and then separated into small filaments. The isolated filaments were placed across a monopolar silver electrode; the contralateral phrenic nerve was placed on a bipolar silver electrode. Neural signals were amplified (1,000x) and band pass filtered (0.3 - 10 KHz) using differential A/C amplifier (Model 1700, A-M Systems, Carlsborg, WA, USA). Raw signals from the whole phrenic nerve were full-wave rectified and integrated (time constant 100 ms; model MA-1000; CWE Inc., Ardmore, PA, USA). Waveforms recorded from the phrenic fibers were confirmed to represent action potentials from a single PhrMN by tracking the waveform amplitude and shape using Spike 2 software (e.g. Fig. 1B). We recorded a total of 32 PhrMN spikes from 17 rats. All neural signals were digitized using a CED Power 1401 data acquisition interface.

Experimental protocol. After adequate anesthesia and stable phrenic recordings were established, the $P_{ET}\text{CO}_2$ apneic and recruitment threshold for inspiratory bursting in the whole phrenic nerve were determined. Ventilation was gradually increased until phrenic bursting ceased for two minutes. The recruitment threshold was defined by the reappearance of rhythmic phrenic activity when ventilation was gradually reduced. $P_{ET}\text{CO}_2$ was then maintained at 2-3 mmHg above the recruitment threshold during the experimental procedure. After individual PhrMNs were identified in the phrenic fiber recordings, STP was induced by exposing the rat to a three minute bout of hypoxia ($F_i\text{O}_2= 0.12 - 0.14$).

Data analyses. Phrenic nerve signals including whole nerve and phrenic fiber recordings were analyzed using Spike 2 software. The integrated phrenic neurogram ($\int Phr$) was used to calculate respiratory frequency (bursts per minute), inspiratory and expiratory phrenic burst duration ($T_i$ and $T_e$), and peak amplitude. The $T_i$ was defined as the period between inspiratory phrenic onset and the time point when $\int Phr$ amplitude declined by 50% of the peak value (see Fig. 1). The respiratory frequency was calculated as $60/(T_i + T_e)$. The waveforms recorded from phrenic fibers were analyzed for discharge duration (i.e. time from initial to final spike within each neural breath), total number of spikes per respiratory cycle, and frequency (total spike number divided by discharge duration). The discharge frequency of PhrMNs in the representative figures (e.g. Figs. 2, 4 and 6) were calculated in 100 ms bins. The onset time of PhrMN bursting was expressed as a percentage of $T_i$ as calculated from the contralateral $\int Phr$ signal. Phrenic nerve and PhrMN data were averaged over 30
sec periods immediately prior to hypoxia (baseline), at both the onset and end of hypoxia, and 3 min post-hypoxia. A one way repeated measures analysis of variance (ANOVA) and the Student-Newman-Keuls post hoc test (Sigma Stat 2.03, Jandel Scientific, St. Louis, MO) was used to analyze changes in the respiratory cycle, \( \Delta \text{Phr signal} \), blood pressure and heart rate. Firing behaviors of Early-I and Late-I PhrMNs were compared using a two-way repeated measures ANOVA and the Student-Newman-Keuls post hoc test. Blood gas data were compared between baseline and hypoxia using a paired t-test. Linear regression analyses were used to examine the relationship between discharge onset time (\% T\textsubscript{1}) and PhrMN behavior (e.g. discharge duration, spike numbers and discharge frequency) during baseline and hypoxia. A p-value of < 0.05 was considered statistically significant for all analyses. All data are presented as the mean ± standard error.

Results

Arterial blood gases and cardiovascular responses.

The arterial partial pressure of O\textsubscript{2} (PaO\textsubscript{2}) was reduced during hypoxia as expected (Table 1). Hypoxia was accompanied by hypocapnia as the arterial partial pressure of CO\textsubscript{2} (PaCO\textsubscript{2}) dropped by 5.5 ± 1.9 mmHg (Table 1). Thus we were concerned that the change in PaCO\textsubscript{2} could influence phrenic STP. However, prior work indicates that moderate CO\textsubscript{2} reductions during hypoxia do not prevent respiratory STP (Song and Poon 2009), and indeed a robust STP of phrenic activity was observed during and following hypoxia (see below). In addition, the magnitude of the acute hypoxic phrenic response reported here is both quantitatively and qualitatively similar to prior reports in which strict isocapnia was maintained (Fuller 2005, Golder et al. 2005). Finally, we confirmed in additional experiments that the drop in PaCO\textsubscript{2} during hypoxia did not significantly alter the expression of phrenic STP.
Arterial pH dropped by 0.03 ± 0.01 units during hypoxia despite the reduction in PaCO₂. Decreases in arterial pH during hypoxia have been reported previously (Golder and Martinez, 2008; Iizuka and Fregosi, 2008), possibly reflecting an increase in plasma lactate concentration (Romeh and Tannen 1986). Mean arterial blood pressure (MAP) was reduced during hypoxia (P < 0.01, Table 2) as previously reported (Bavis and Mitchell 2003; Fuller 2005; Wilkerson et al. 2008). However, MAP returned to baseline values by 3 min post-hypoxia (Table 2). Heart rate (HR) increased during hypoxia (P < 0.05) but also returned to baseline by 3 min post-hypoxia (Table 2).

Whole phrenic nerve.

An example of phrenic motor output as assessed with whole nerve recordings is shown in Fig. 2. Baseline phrenic Tᵢ and Tₑ were 0.41 ± 0.02 and 1.34 ± 0.14 sec, respectively, and Tᵢ was progressively reduced over the course of hypoxia (P < 0.01, Fig. 3A). In contrast, Tₑ was shortened at hypoxia onset but then returned towards baseline values by the end of the hypoxic exposure (Fig. 3B). At three min post-hypoxia, Tᵢ was comparable to baseline (P > 0.05, Fig. 3A), but Tₑ was significantly prolonged (P < 0.01, Fig 3B). The increased Tₑ was associated with a significant reduction in overall phrenic burst frequency after hypoxia as previously described (i.e. post-hypoxia frequency decline or PHFD, Bach et al. 1999; Coles and Dick 1996). Inspiratory phrenic burst frequency was 39 ± 3 breaths/min at baseline, and this increased to a peak value of 74 ± 2 breaths/min (P<0.01, Fig. 3C) at the onset of hypoxia. As hypoxia progressed, phrenic burst frequency gradually declined to 56 ± 2 breaths/min (Fig. 3C) and further declined to 28 ± 2 breaths/min at 3 min post-hypoxia (Fig. 3C).
Hypoxia caused an initial rapid increase in [Phr burst amplitude (i.e. the acute response) followed by a progressive increase with a slower time course (i.e. STP onset, Poon et al. 1999) (Fig. 3D). After hypoxia was terminated, [Phr amplitude gradually declined but remained above baseline after three min (i.e. STP offset, Poon et al. 1999) (Fig. 3D). Due to concerns that reductions in PaCO₂ during hypoxia could have influenced the magnitude of STP (see above), additional experiments were performed (n=10) in which strict isocapnia was maintained during hypoxia (PaCO₂: baseline = 32 ± 1 mmHg, hypoxia = 32 ± 1 mmHg; P = 0.68) while phrenic neurograms (whole nerve) were recorded. Both the magnitude of the acute hypoxic phrenic response and phrenic STP were virtually identical to the earlier results (2 way repeated measures ANOVA, factor 1: time [baseline, hypoxia onset, etc.], factor 2: treatment [isocapnic hypoxia or hypocapnic hypoxia]; P = 0.72; data not shown). Accordingly, in this preparation, hypocapnia in the range of 5.5 ± 1.9 mmHg PaCO₂ below baseline (32 ± 2 mmHg) does not impact phrenic STP. However, in an additional experiment (n=1) we confirmed the earlier finding of Eldridge (1980) that more substantial reductions in PÉT CO₂ (e.g. >10 mmHg from baseline) substantially reduces the acute phrenic response to hypoxia and virtually eliminates phrenic STP (data not shown).

Phrenic motoneuron discharge during baseline.

Twenty-three active PhrMNs were recorded during the baseline condition (FiO₂ = 0.58 ± 0.01) (see Figs. 2 and 4). The distribution of PhrMN discharge onset relative to inspiratory bursting recorded in the contralateral phrenic nerve is depicted in Fig. 5. Those PhrMNs which initiated bursting during the initial 20% of Ti were classified as “Early-I PhrMNs” (Fig. 2; St John and Bartlett 1979). Of the 15 Early-I PhrMNs recorded, 11 ceased firing at the mid- to end-stage of Ti; the other 4 neurons exhibited
a few spikes during TE (1.0 ± 0.5 spikes/respiratory cycle). The remaining PhrMNs active at baseline (i.e. bursting initiated at > 20% TI, n=8) were classified as “Late-I PhrMNs” (Fig. 4). Baseline discharge frequency was similar between Early-I and Late-I PhrMNs (P > 0.05, Fig. 7B), however, overall discharge duration and total spike number/breath were significantly less in Late-I PhrMNs (P < 0.01, Figs. 7C and 7D).

An additional nine PhrMNs were inactive during baseline conditions, but were recruited during hypoxia (see below).

The relationship between onset of PhrMN bursting (expressed as % Ti) and baseline burst parameters including spike numbers, overall discharge duration and discharge frequency was examined using regression analysis. Both the total number of PhrMN spikes per respiratory cycle ($r^2 = 0.71$) and the PhrMN discharge duration ($r^2 = 0.86$) correlated significantly with discharge onset time (P < 0.01). In other words, PhrMNs recruited earlier in the breath had longer discharge durations and greater spike numbers compared to cells with delayed activation. However, the discharge frequency of PhrMNs was unrelated to the discharge onset time (P > 0.05). Thus, baseline discharge frequency was similar between Early- and Late-I PhrMNs.

Phrenic motoneuron behavior during and following hypoxia.

The discharge frequency of Early-I PhrMNs was progressively enhanced over the duration of hypoxia (P < 0.01, Fig. 7B). In contrast, the overall discharge duration was gradually reduced during hypoxia (P < 0.01, Fig. 7C). As a result of the shorter burst duration, the total number of Early-I spikes per breath was reduced from 12.5 ± 0.9 (baseline) to 10.1 ± 0.4 at the end of hypoxia (P < 0.01, Fig. 7D). At 3 minute post-hypoxia, the overall discharge duration of Early-I PhrMNs had returned to baseline values (P > 0.05, Fig. 7C). In contrast, both the discharge frequency and total spike number/breath remained significantly above baseline values (P < 0.01,
Thus, Early-I PhrMNs showed STP of burst frequency following hypoxia.

During hypoxia, Late-I PhrMNs began bursting earlier in the breath (i.e. reduced onset time, P < 0.05, Fig. 7A). However, the overall Late-I discharge duration was not altered during hypoxia (P > 0.05, Fig. 7C). Similar to Early-I PhrMNs, Late-I cells showed a gradual increase in burst frequency as hypoxia progressed (P < 0.01, Fig. 7B). However, in contrast to the Early-I PhrMN response, the total number of Late-I spikes/breath was increased during hypoxia (P < 0.05, Fig. 7D). Following hypoxia, Late-I PhrMNs also showed evidence of STP. Specifically, Late-I cells showed a significantly higher discharge frequency and total spike number/breath at 3 min post-hypoxia as compared to baseline values (P < 0.01, Figs. 7B and D).

An additional nine PhrMNs were inactive during baseline conditions, but were recruited during hypoxia. These initially silent PhrMNs all exhibited a late onset time (40 ± 4 % Ti) and were designated as “recruited late-I PhrMNs” (Fig. 6 and Table 3). These recruited Late-I PhrMNs exhibited discharge frequency at 37.9 ± 2.8 Hz at end stage of hypoxia, and all but one of these PhrMNs ceased firing upon termination of hypoxia. The remaining “outlier” PhrMN continued to burst following hypoxia (frequency = 32 Hz at 3 min). Thus, recruited PhrMNs do not appear to contribute substantially to post-hypoxia STP.

Regression analyses of the relationship between the onset of PhrMN bursting (i.e. % Ti) and changes in burst behavior (i.e. spike numbers, discharge duration and discharge frequency) during and following hypoxia is shown in Fig. 8. The increase in number of spikes/breath during hypoxia onset showed a robust correlation with onset time (r² = 0.75, Fig. 8A, left panel). In other words, PhrMNs recruited earlier in the breath tended to have little change or even a decline in overall spike number during hypoxia. Conversely, cells recruited later in the breath showed an increase in spike
numbers during hypoxia. This relationship was maintained at the end of hypoxic exposure (Fig. 8A, middle panel, $r^2 = 0.79$), but was less robust (although still significant) during the post-hypoxia period (Fig. 8A, right panel, $r^2 = 0.45$). PhrMN onset time and overall discharge duration showed a similar linear relationship during hypoxia onset, end, and the post-hypoxic period (Fig. 8B). Therefore, the probability of having a greater number spikes during STP is greater for PhrMNs recruited later (Late-I) vs. earlier in the breath (Early-I). In contrast to spike number and duration (Figs. 8A-B), changes in PhrMN discharge frequency showed no correlation with onset time ($P > 0.05$, Fig. 8C). Thus, changes in discharge frequency during and following hypoxia were similar between Early-I and Late-I PhrMNs.

Discussion

The results of this study first show that STP can be evoked in most, if not all, PhrMNs that are active prior to hypoxia. Specifically, both Early-I and Late-I PhrMNs showed STP of discharge frequency during and following hypoxic challenge. Second, previously silent PhrMNs (i.e. recruited cells not active prior to hypoxia) are active during the onset of phrenic STP but do not appear to make a significant contribution to the post-hypoxia phase of phrenic STP. Accordingly, the onset and offset phases of respiratory STP are associated with distinct PhrMN recruitment strategies.

Critique of methodology

We used a traditional "fiber-picking" approach (Kong and Berger 1986; Lee et al. 2007a, b, 2008; St John and Bartlett 1979) to record action potentials from PhrMN axons. Although the overall sample size of 32 PhrMNs is relatively small, we were able to record enough cells to statistically differentiate differences in firing behavior.
between Early-I, Late-I and silent PhrMNs. In addition, the overall distribution of PhrMNs reported here is quite similar to what has been observed in previous reports (Kong and Berger 1986; St. John and Bartlett 1979). It is nevertheless possible that our description of PhrMNs, and in particular silent PhrMNs is not representative of the entire population of these cells. For example, Sieck and Fournier (1989) propose that 10-15% of diaphragm motor units are active during eupneic breathing, and this number rises to only ~30% during chemical challenge (e.g. hypoxia, hypercapnia). Accordingly, a considerable number of silent PhrMNs may become active only during expulsive behaviors such as emesis (Sieck and Fournier, 1989). The population of silent PhrMNs described in the present study is presumably representative of only those cells which can be recruited by hypoxia.

Other potential concerns include the integrity of the vagus nerves and the use of anesthesia (Hwang et al. 1983). Respiratory STP has been observed in both vagal intact (Xi et al. 1993) and vagotomized conditions (Golder et al. 2005; Hayashi et al. 1993; Wagner and Eldridge 1991). However, vagal afferent feedback probably attenuates the expression of phrenic STP. For example, blockade of vagal inputs slows the decay of ventilation following hypoxia in conscious dogs (Xi et al. 1993). Golder and Martinez (2008) reported that the overall increase in phrenic burst amplitude during hypoxia is more robust in vagal intact vs. vagotomized rats. However, to our knowledge the impact of vagotomy on STP has not been evaluated in the rat. Our data revealed a robust phrenic STP in vagotomized rats (e.g. Fig. 2), and based on the data of Xi et al. (1993) we predict that STP would be attenuated in vagal intact rats. The potential impact of urethane anesthesia on STP is not certain. However, respiratory STP occurs in both anesthetized animals (e.g. current data; Hayashi et al. 1993; Golder et al. 2005) and conscious humans (Fregosi 1991) and animals (Kline et al. 2002; McGuire 2008). Urethane was selected as the anesthetic.
in this study because robust hypoxic phrenic responses occur in urethane
anesthetized rats (Bach and Mitchell 1996, Baker-Herman et al. 2004). In summary,
despite some caveats associated with the urethane anesthetized and vagotomized rat,
this preparation provides an opportunity to examine PhrMN behavior during
hypoxia-induced STP under controlled experimental conditions.

**PhrMN discharge during baseline.**

Two populations of PhrMNs (i.e., Early-I and Late-I) were active during
normocapnia as previously described (Kong and Berger 1986; Nail et al. 1972; St
John and Bartlett 1979). The difference in burst onset between Early- and Late-I
PhrMNs probably reflects both intrinsic motoneuron properties as well as differential
regulation via pre-synaptic inputs (Hilaire et al. 1983; St. John and Bartlett 1981).
With regard to the former possibility, intracellular recordings in rats indicate that earlier
onset PhrMNs have both larger membrane resistance and smaller rheobase current
relative to Late-I cells (Hayashi and Fukuda 1995). Thus, in accordance with
Henneman’s size principle (Henneman et al. 1965), Early-I PhrMNs may be more
likely to depolarize for a given synaptic input. Nevertheless, any differences in
intrinsic cellular properties did not seem to impact STP as both Early-I and Late-I
PhrMNs had similar changes in burst frequency during and following hypoxia (see
below). Several studies have provided evidence that differential pre-synaptic inputs
contribute to PhrMN recruitment order (Hilaire et al 1983; Saboisky et al. 2007; St
John and Bartlett 1981). For example, Hilaire et al. (1983) used cross-correlation to
show that common synaptic inputs to Early-I and Late-I PhrMNs appear to be
relatively rare. Accordingly, the pattern of activation of these cell groups may reflect
distinct innervation of Early-I and Late-I neurons vs. exclusively intrinsic neuronal
properties. Similarly, Saboisky et al. (2007) suggest that respiratory motoneuron
bursting in humans is determined primarily by descending inputs (vs. intrinsic motoneuron properties). However, the neuroanatomical substrate underlying any potential differences in pre-synaptic input to PhrMNs is not defined. In this regard, we recently reported that some cervical spinal interneurons are interposed between the rostral ventral respiratory group (rVRG) and PhrMNs in the rat (Lane et al. 2008) as has been reported in other species (Bellingham 1999; Lois et al. 2009; Palisses et al. 1989; Yates et al. 1990). Given that at least some of these pre-phrenic interneurons show respiratory modulation (Hayashi et al. 2003; Lane et al. 2009), it is tempting to speculate that these cells may be involved in shaping the overall pattern of Early-I and Late-I PhrMN recruitment during breathing.

PhrMN discharge during and following hypoxia.

Both Early-I and Late-I PhrMNs responded acutely to hypoxia with a brisk increase in discharge frequency (St. John and Bartlett 1979). Moreover, both PhrMN types showed a progressive increase in discharge frequency as hypoxia progressed. We suggest that this gradual increase in discharge frequency represents activation of phrenic STP mechanisms. However, contrary to our initial hypothesis, the relative increases in discharge frequency were similar between PhrMN sub-types. Accordingly, “frequency STP” was similar between PhrMNs regardless of when the cells were recruited during the inspiratory cycle. Thus, intrinsic differences in excitability between these PhrMN populations (Hayashi and Fukuda 1995) may not be a key factor in determining STP of phrenic motor output.

Similar to prior reports (St John and Bartlett, 1979), hypoxia influenced the onset of bursting in Late-I but not Early-I PhrMNs. More specifically, PhrMNs normally activated later in the inspiratory cycle (e.g. Late-I) began to burst earlier during the hypoxic challenge (St. John and Barlett 1979). In contrast, Early-I PhrMNs had a
similar onset time during both baseline and hypoxic challenge. Early-I PhrMNs also
had a reduction in overall discharge duration during hypoxia. The net result was that
despite the increased discharge frequency, the total number of action potentials
(spikes) per breath was actually reduced for Early-I PhrMNs during STP onset. On
the other hand, Late-I PhrMNs maintained their discharge duration during hypoxia,
and thus the total spikes/breath was increased during the onset of STP. It is thus
conceivable that Late-I cells make a relatively greater functional contribution to STP of
diaphragm activity and ventilation during hypoxia.

In addition to previously active Early- and Late-I PhrMNs, a population of silent
PhrMNs was recruited during hypoxia. Similar quiescent PhrMNs have been
observed in neonatal and adult rats as well as adult cats (Nail et al. 1969; Milano et al.
1992; Hayashi and Fukuda 1995; Su et al. 1997; St John and Bartlett 1979). In our
study, the recruited PhrMNs began bursting after the initial hypoxic response, and
thus were not observed until $48 \pm 8$ sec following the initiation of hypoxia. This
delayed onset suggests that recruitment of these neurons represents an important
component of the onset of STP but not the initial acute hypoxic response. We
suggest that silent PhrMNs represent a pool of cells that can be recruited during
respiratory plasticity including STP, LTF and perhaps the crossed phrenic
phenomenon following high cervical spinal cord hemisection injury (El-Bohy and
Goshgarian, 1999). However, to our knowledge, there have been no previous formal
investigations of the role of silent PhrMNs in the expression of respiratory-related
neuroplasticity. Since only a relatively small portion of the total PhrMN pool is active
during “eupneic” breathing (Sieck and Fournier 1989), recruitment of previously
inactive PhrMNs may represent a common mechanism associated with the
expression of a variety of forms of respiratory neuroplasticity.
It is generally accepted that STP reflects a central neural mechanism because it can be evoked by electrical stimulation of the carotid sinus nerve (Wagner and Eldridge 1991). Thus, although the acute hypoxic response is triggered primarily by peripheral chemoreceptors (Bavis and Mitchell 2003), their continued activation is not necessary for STP expression. Within the CNS, there is evidence that both brainstem (Mifflin 1997; Young et. al. 2003) and spinal cord (McCrimmon et al. 1997; Hayashi et al. 2003) mechanisms contribute to STP. For example, Mifflin (1997) showed that high frequency stimulation of the carotid sinus nerve potentiated subsequent evoked responses in neurons within the nucleus of the solitary tract (NTS). Thus, “integration” of peripheral inputs within the NTS may be enhanced during STP. On the other hand, spinal cord stimulation can evoke STP-like changes in respiratory motor output in spinalized rats (McCrimmon et al. 1997; Hayashi et al. 2003) and turtles (Johnson and Mitchell 2002). Accordingly, the mechanisms underlying phrenic STP may also include changes in PhrMNs and/or spinal circuits (Fuller et al. 2005) including respiratory interneurons (Lane et al 2008). In other words, STP could reflect a (relatively) constant descending respiratory drive but a more responsive motor pool.

It is unknown if similar mechanisms contribute to the onset (i.e. during hypoxia) vs. offset (i.e. after hypoxia) phases of respiratory STP. One hypothesis is that the gradual decrease of respiratory output after hypoxia represents a time-dependent “decay” of the STP mechanism (Powell 1998). However, since the onset and decline of STP generally follow a different time course (Wagner and Eldridge 1991) it may be that distinct mechanisms contribute to each phase (Fuller et al. 2005). We suggest that our PhrMN discharge data are consistent with this latter suggestion. For example, the expression of STP during hypoxia occurred in parallel with increased
discharge frequency of both Early-I and Late-I PhrMNs as well as recruitment of previously silent late-I PhrMNs. Thus, the increased bursting in each of these cell populations contributed to the observed STP of phrenic motor activity. Following hypoxia, however, STP was associated with increased bursting of those Early-I and Late-I PhrMNs which were active prior to hypoxia but the recruited, silent PhrMN population did not make a substantial contribution. The abrupt cessation of silent PhrMN bursting after hypoxia is consistent with a removal of descending drive to these cells. Taken together, the observed PhrMN behavior indicates that the decay of STP does not represent a non-specific or “general” potentiation of all PhrMNs activated during hypoxia. Rather, the STP mechanism is specific between PhrMN types, and is differentially regulated during onset and offset.

Other differences in PhrMN behavior were noted during the onset vs. offset of STP. For instance, the enhanced discharge frequency of Early-I PhrMNs during the onset of STP was accompanied by a reduction in both overall discharge duration and total spike numbers. In contrast, during the recovery phase of STP, the number of Early-I PhrMN spikes per breath was increased without alteration in overall discharge duration. This is consistent with earlier observations that the time course of STP differs between the onset and offset phases (Wagner and Eldridge 1991; Fuller et al. 2005).

Conclusion

The purpose of hypoxia-induced respiratory STP is not precisely known. However, STP may serve as a mechanism to reduce variability in respiratory output and thereby enhance the stability of breathing during and following hypoxia (Fuller et al. 2005). In this regard, it is interesting that STP may be impaired in patients with congestive heart failure and obstructive sleep apnea (Georgopoulus et al. 1992;
Here we report that the initiation of STP involves three types of PhrMNs (i.e. Early-I, Late-I, and silent recruited Late-I). However, post-hypoxia STP primarily reflects discharge of Early-I and Late-I PhrMNs. Thus, the induction of plasticity associated with persistent recruitment of silent PhrMNs may require more robust and/or repeated activation such as would occur with intermittent bouts of hypoxia.

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Figure legends

Fig. 1. Analyses of inspiratory duration (Tt) using the ∫Phr waveform. The top panel shows an example of the raw (Phr) and integrated phrenic burst (∫Phr). The bottom panel demonstrates the impact of measuring Tt at either the peak ∫Phr (a), at the point where ∫Phr declines by 50% of the peak (b), or at the return of ∫Phr to baseline (c). Assessing Tt at peak ∫Phr clearly underestimates Tt, whereas measuring Tt at the return of ∫Phr to baseline results the inclusion of post-inspiratory activity. Accordingly, we assessed Tt as the interval between phrenic burst onset and the point where the ∫Phr signal declined by 50% of the peak value (b).

Fig. 2. Representative phrenic neurogram and Early-I PhrMN bursting during hypoxia. Panel A depicts electrical activity recorded from the whole phrenic nerve and a single fiber before, during and following hypoxia. Whole phrenic activity is presented as both the unprocessed or "raw" signal (Phr) and the moving averaged or "integrated" signal (∫Phr). PhrMN discharge is shown as the raw signal (PhrMN) and the mean burst frequency (mean f) as calculated in 100 ms bins. Panel B presents expanded time scale traces showing a single neural breath from the areas marked i-iv in panel A. Panel Bi indicates that the single fiber recording reflects the bursting of an Early-I PhrMN (i.e. discharge onset occurring early during inspiration). The discharge frequency of this neuron was enhanced during both the onset (Bii) and end...
(Biii) and of hypoxia. Discharge frequency remained above baseline at three min post-hypoxia (Biv). The bottom traces of panel B shows superimposition of the individual spikes from panels Bi-Biv. BP: blood pressure.

**Fig. 3. Effects of hypoxia on Ti (A), Te (B), phrenic burst frequency (C) and ∫Phr burst amplitude (D).** Both Ti and Te were reduced, and burst frequency was enhanced during hypoxia. ∫Phr amplitude showed a progressive increase as hypoxia progressed. Following hypoxia, Te was elongated resulting in a decrease of phrenic burst frequency. ∫Phr amplitude was maintained above baseline at 3 min post-hypoxia.

*: P < 0.05; **: P < 0.01 vs. baseline.

**Fig. 4. Representative phrenic neurogram and Late-I PhrMN bursting during hypoxia.** Both ∫Phr amplitude and Late-I PhrMN discharge frequency were progressively enhanced during hypoxia, and remained above baseline levels at three min post-hypoxia (Panel A). Panel B presents expanded time scale traces showing a single neural breath from the areas indicated by i-iv in panel A. Late-I PhrMN onset time became earlier during hypoxia (e.g. compare Bi vs. Biill). The bottom traces of panel B show superimposed individual spikes from Panels Bi-Biv. Labels are the same as in Fig. 1.
Fig. 5. Distribution of PhrMN discharge onset. PhrMN discharge onset time was expressed as percentage of Ti as measured from the contralateral phrenic neurogram. Early-I (white) and Late-I PhrMNs (black) were active during baseline conditions. The recruited late-I PhrMNs (gray) began bursting during hypoxia.

Fig. 6. Representative example of hypoxia-induced recruitment of a silent Late-I PhrMN. A previously silent PhrMN was recruited after ~45 sec of hypoxia exposure (A). As shown in Panel B, this recruited PhrMN burst with a Late-I pattern during but not following hypoxia. Labels are the same as in Figs. 2 and 5.

Fig. 7. Mean discharge properties of Early-I and Late-I PhrMNs. The discharge onset time (%Ti; A), frequency (Hz; B), overall duration (ms; C) as well as total number of spikes per neural breath (D) were quantified before, during and following hypoxia. *: P < 0.05; **: P < 0.01 vs. baseline. #: P < 0.05; ##: P < 0.01 significant difference between Early-I and Late-I PhrMNs.

Fig. 8. The relationship between discharge onset time and PhrMN burst parameters during and following hypoxia. Onset time was expressed as % Ti
(abscissa) and PhrMN bursting was expressed relative to baseline values (ordinate).

The results of linear regression analyses are presented on the Figure for spike number (A), spike discharge duration (B), and spike discharge frequency (C).
Fig. 1

\[
\text{Phr} \quad \text{Onset} \quad a \quad b \quad c
\]

\[
\text{Phr}
\]

\begin{itemize}
\item[a] Tt assessed at peak |Phr|
\item[b] Tt assessed at 50% decline
\item[c] Tt assessed at baseline
\end{itemize}

0.2 s
Fig. 2

A

BP (mmHg)

Phr

Mean f (Hz)

PhrMN

Hypoxia

20 sec

B

i

ii

iii

iv

0.1 s

0.25 ms
Fig. 3

A

Baseline Onset End 3 min

Hypoxia

B

C

Baseline Onset End 3 min

Hypoxia

D

Baseline Onset End 3 min

Hypoxia

**
Fig. 5

- Onset time (% T₁)
- Number of PhrMN's

Graph showing the number of PhrMN’s over different onset times (% T₁) with categories Early-I, Late-I, and Recruited late-I.
Fig. 7

A

Baseline Onset End 3 min

Discharge frequency (Hz)

B

Baseline Onset End 3 min

Discharge duration (ms)

C

Baseline Onset End 3 min

Onset of PhrMN (% of Ti)

D

Baseline Onset End 3 min

Spike numbers/cycle
Fig. 8

A  Onset of hypoxia  End of hypoxia  3 min post-hypoxia

B

C
Table 1. Arterial blood gases and pH during baseline and hypoxia.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaO₂ (mmHg)</td>
<td>210 ± 16</td>
<td>33 ± 2 **</td>
</tr>
<tr>
<td>PaCO₂ (mmHg)</td>
<td>32 ± 2</td>
<td>27 ± 2 *</td>
</tr>
<tr>
<td>pH</td>
<td>7.37 ± 0.01</td>
<td>7.34 ± 0.01**</td>
</tr>
</tbody>
</table>

Values are means ± SE.  PaCO₂: arterial PCO₂; PaO₂, arterial PO₂. *: P < 0.05; **: P < 0.01 compared with value during baseline.
Table 2. *Mean arterial blood pressure (MAP) and heart rate (HR)*

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Onset</th>
<th>End</th>
<th>3 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>124 ± 6</td>
<td>119 ± 5</td>
<td>94 ± 8**</td>
<td>123 ± 5</td>
</tr>
<tr>
<td>HR (beats•min⁻¹)</td>
<td>440 ± 7</td>
<td>451 ± 8*</td>
<td>458 ± 10**</td>
<td>445 ± 7</td>
</tr>
</tbody>
</table>

Values are means ± SE. Onset and End represent the initial and final 30 seconds of the hypoxic episode; 3 min represents 3 minutes following cessation of hypoxia. *: P < 0.05; **: P < 0.01 compared with value during baseline.
Table 3. *Firing behaviors of recruited late-I PhrMNs during the onset and end of the hypoxic challenge. These PhrMNs were silent during baseline recordings.*

<table>
<thead>
<tr>
<th></th>
<th>Onset</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discharge frequency (Hz)</td>
<td>5.4 ± 4.7</td>
<td>37.9 ± 2.8</td>
</tr>
<tr>
<td>Discharge duration (ms)</td>
<td>16.5 ± 15.1</td>
<td>80.2 ± 13.8</td>
</tr>
<tr>
<td>Spike numbers</td>
<td>0.7 ± 0.6</td>
<td>3.4 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE.