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Determinants of frequency long-term facilitation following acute intermittent hypoxia in vagotomized rats

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Abstract

Acute intermittent (AIH), but not acute sustained hypoxia (ASH) elicits a form of respiratory plasticity known as long-term facilitation (LTF). In anesthetized rats, LTF is expressed as increased respiratory-related nerve burst amplitude, with variable effects on burst frequency. We analyzed a large data set from multiple investigators using the same experimental protocol to determine factors influencing frequency LTF. Our meta-analysis revealed that AIH elicits both phrenic amplitude and frequency LTF in anesthetized and vagotomized rats, but frequency LTF is small in comparison with amplitude LTF (12% *versus* 60%, respectively). ASH elicits a small, but significant frequency and amplitude LTF (8% and 10%, respectively) that is not significantly different than controls. Similar to all published reports, analysis of this large data set confirms that phrenic amplitude LTF following AIH is significantly greater than ASH. Multiple regression analysis revealed a strong correlation between baseline burst frequency and frequency LTF. Variations in baseline burst frequency may contribute to variation in frequency LTF and may underlie the apparent effects of some drug treatments.

Keywords

respiratory plasticity; LTF; phrenic nerve; intermittent hypoxia; sustained hypoxia; rat; respiratory frequency; ventilatory control; meta-analysis

1. Introduction

Central neural plasticity in respiratory control has been a subject of increasing interest in recent years (Feldman et al., 2003; Huey et al., 2003; Mitchell and Johnson, 2003; Mahamed and Mitchell, 2006; Prahbakar et al., 2007; Zimmer et al., 2007). One of the most extensively studied examples of respiratory plasticity is respiratory long-term facilitation (LTF), a prolonged increase in respiratory motor output that cannot be accounted for by changes in chemoreceptor stimuli (Baker et al., 2001; Mitchell et al., 2001a; Feldman et al., 2003; Mahamed and Mitchell, 2006). Millhorn and colleagues were the first to describe LTF, although it was not referred to as LTF at that time (Millhorn et al., 1980a,b; Eldridge and Millhorn, 1986). In their pioneering studies on anesthetized and vagotomized cats, Millhorn

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and colleagues electrically stimulated the carotid sinus nerve in an episodic pattern, and observed that inspiratory phrenic nerve activity remained increased above baseline levels for at least 90 min post-stimulation (Millhorn et al., 1980a). Although this prolonged enhancement of phrenic activity was primarily expressed as increased phrenic burst amplitude (the neural equivalent of tidal volume), lesser increases in phrenic burst frequency were also observed (the neural equivalent of breathing frequency). Similar LTF is also observed following acute intermittent hypoxia (AIH) (Hayashi et al., 1993; Bach and Mitchell, 1996; for review, see: Powell et al., 1998; Mitchell et al., 2001a).

LTF has now been reported in many species under different experimental conditions (for review, see: Fuller et al., 2000; Baker et al., 2001, Mitchell et al., 2001a; Feldman et al., 2003; Mahamed and Mitchell, 2006). In general, studies on anesthetized animals report that LTF is expressed as increased burst amplitude from respiratory-related nerves, such as the phrenic or hypoglossal nerve (for review see Mitchell et al., 2001a; Feldman et al., 2003), with inconsistent changes in burst frequency. By contrast, studies on awake animals generally report that LTF is associated with increased breathing frequency, with small and inconsistent changes in tidal volume (Turner and Mitchell 1997; Mitchell et al., 2001b; Olson et al., 2001; McGuire et al., 2002, 2003, 2004; Kline et al., 2002; McGuire and Ling 2005; Terada et al., 2008). LTF in awake animals also tends to be of smaller magnitude and shorter duration than in anesthetized preparations (Mitchell et al., 2001a). In reduced, but unanesthetized preparations, such as the working heart brainstem preparation (Tadjalli et al., 2007) or the rhythmogenic brainstem slice (Blitz and Ramirez, 2002), LTF is also expressed largely as increased respiration-related nerve burst frequency (but note that *in vitro* LTF induced with neuromodulators and not hypoxia primarily involves amplitude changes; Lovett-Barr et al., 2006, Bocchiaro and Feldman, 2004, Neverova et al., 2007). On the other hand, in documented slow wave sleep, AIH-induced ventilatory LTF is expressed as increased tidal volume (Nakamura et al., 2006; Pierchala et al., 2007; Terada et al., 2008), as well as increased breathing frequency (Nakamura et al., 2006; Terada et al., 2008). The significance of differences in frequency versus amplitude responses under different experimental conditions is unclear, but may suggest that different neural mechanisms underlie prolonged increases in ventilation (or its neural analog) in different experimental conditions.

In order to better understand factors that influence frequency LTF, we performed a meta-analysis on an extensive data set collected in our laboratory by different investigators using similar equipment and experimental protocols on anesthetized and vagotomized male rats, the most extensively used model for studies of cellular/synaptic mechanisms of LTF (Mitchell et al., 2001a; Feldman et al., 2003; Mahamed and Mitchell, 2006). These data were taken largely from published studies (Baker and Mitchell, 2000; Zabka et al., 2001a, 2006; Fuller et al., 2001a,b; Baker-Herman and Mitchell, 2002; Bavis and Mitchell, 2003; Behan et al., 2003; Baker-Herman et al., 2004; Golder and Mitchell 2005; Golder et al., 2008; Wilkerson et al., 2008; MacFarlane and Mitchell, 2007a,b; Mahamed and Mitchell, 2008). All data were collected since our previous meta-analysis in 2000 (Fuller et al., 2000), which focused exclusively on phrenic amplitude LTF without consideration of changes in phrenic burst frequency.

Our meta-analysis confirmed significant AIH-induced phrenic amplitude and frequency LTF in anesthetized, vagotomized rats; however, frequency LTF in this preparation is considerably smaller than phrenic amplitude LTF. We also report a strong correlation of frequency LTF with the initial, pre-hypoxia baseline burst frequency. This relationship may account for considerable variation in reported values of frequency LTF, even by the same investigator using the same experimental protocol, and may underlie the apparent effects of certain drugs. Thus, our analysis reveals that frequency LTF in anesthetized rats is small and variable, and cautions that loss of frequency LTF following certain experimental manipulations must be

interpreted with consideration of this fact. Anesthetized, vagotomized rat preparations are more suited to studies of phrenic amplitude LTF, where the results are robust and repeatable.

2.1. Methods

302 male Sasco/Charles River and Harlan Sprague Dawley rats from 18 different studies were used in our analysis. Animal husbandry and all procedures were approved by the Institutional Animal Care and Use Committee of the School of Veterinary Medicine at the University of Wisconsin, Madison.

2.2. Surgical preparation

All rats used in the analysis were prepared similarly, although there are undoubtedly subtle investigator differences in how the protocols were performed. Rats were anesthetized initially with isoflurane in 50% O_2 (balance N_2) and then placed on a custom-designed heated table to maintain body temperature at 37–38°C. The rats were tracheostomized, vagotomized and pump-ventilated (2.0–2.5 ml, rodent respirator model 683; Harvard Apparatus, South Natick, MA). The femoral artery was cannulated to sample blood gases (ABL-500; Radiometer, Copenhagen, Denmark) and monitor blood pressure, and either the femoral or a tail vein was cannulated to deliver drugs and fluids (5 ml/kg/h, lactated Ringers with 0.8% sodium bicarbonate i.v.). The left phrenic nerve was isolated via a dorsal approach, desheathed, placed on bipolar silver recording electrodes and submerged in mineral oil. End-tidal PCO_2 was measured continuously using a CO_2 monitor (Capnogard; Novametrix Medical Systems, Wallingford, CT) with sufficient response time to measure end-tidal CO_2 in anesthetized rats. Following surgery, the rats were converted to urethane anesthesia (1.6–1.7 g/kg i.v.) while isoflurane was gradually discontinued over a 10–20 min period. Rats received pancuronium bromide (1 mg/kg i.v.) for neuro-muscular paralysis (supplemented as necessary to prevent spontaneous breathing movements).

In some protocols, rats received additional “sham surgeries” that were not considered relevant to the analysis. For example, some rats were prepared for an intrathecal catheter as described in Baker-Herman and Mitchell (2002), but only those receiving vehicle treatments were included in the analysis. Others received sham surgery for gonadectomy (Behan et al., 2003), spinal hemisection (Golder and Mitchell, 2005) or carotid sinus nerve transection (Bavis and Mitchell, 2003). No differences were noted between these experimental groups and those that did not receive sham surgery.

Phrenic nerve activity was amplified (gain, 10,000; A-M Systems, Everett, WA), band-passed filtered (100 Hz to 10 kHz), rectified, and processed with a moving averager (CWE 821 filter; Paynter, Ardmore, PA; time constant 50 ms). The signal was digitized, recorded, and analyzed using the WINDAQ data-acquisition system (DATAQ Instruments, Akron, OH). Blood pressure and phrenic nerve activity were allowed to stabilize for at least 1 hour following urethane administration before the CO_2 recruitment threshold was determined to allow establishment of standardized baseline conditions.

2.3. Experimental protocol

The apneic and recruitment thresholds for CO_2 were measured in each rat. Briefly, end-tidal PCO_2 was decreased by altering ventilator frequency and/or inspired CO_2 until phrenic inspiratory activity ceased, and then slowly increased until phrenic inspiratory activity resumed. The end-tidal PCO_2 in which phrenic inspiratory activity resumed was deemed to be the recruitment threshold. Baseline PCO_2 was set at 2–3 mmHg above this threshold, and then the integrated phrenic neurogram was recorded for 20–30 min to establish baseline values of respiratory activity.

Following establishment of baseline conditions, rats were exposed to one of three protocols: acute intermittent hypoxia (AIH), acute sustained hypoxia (ASH) or no hypoxia (sham/controls). Hypoxia was created by changing the inspired gas mixture from 50% O_2 to 11% O_2 (balance N_2). Intermittent hypoxia consisted of 3 hypoxic episodes separated by a 5 min return to baseline conditions. The duration of the hypoxic episodes varied from 3–5 min, depending on the study. Sustained hypoxia consisted of one 9–25 min episode of hypoxia. Finally, a subgroup of rats were maintained for an equivalent duration at baseline conditions and were not exposed to hypoxia to control for time dependent changes in phrenic activity unrelated to hypoxia (time controls). In all rats, the inspired gas mixture was returned to baseline conditions following the hypoxic exposures (or equivalent duration in time controls) and maintained at this level for at least 60 min.

Arterial blood (0.4 ml) was sampled during baseline conditions immediately before treatment, during the first hypoxic episode, and 15, 30 and 60 min following hypoxia (or equivalent duration in time controls not receiving hypoxia) to ensure that arterial PO_2 and PCO_2 met defined criteria. Briefly, these criteria included: $PO_2 > 120$ mmHg before and after hypoxia, PO_2 between 35–45 mmHg during hypoxic exposures and arterial PCO_2 within 1 mmHg of baseline following hypoxia.

2.4. Analysis

Phrenic burst frequency and amplitude were analyzed in 30 sec bins immediately prior to blood gas samples that met the criteria outlined above. Frequency LTF was determined by calculating the absolute change in phrenic burst frequency 60 min following hypoxia from burst frequency prior to hypoxia (i.e., baseline). Phrenic (burst amplitude) LTF was determined as the percentage change in amplitude 60 min following hypoxia from the pre-hypoxia (baseline) levels.

Two-way ANOVA with a repeated measures design was used to determine significant differences in $PaCO_2$, body temperature and breathing frequency (raw values) before and after AIH, ASH and equivalent duration in time controls. Individual comparisons were made using the Student-Neuman-Keuls *post hoc* test. A t-test was used to determine if the percentage change in phrenic amplitude or burst frequency 60 min post-hypoxia (i.e., phrenic amplitude LTF and frequency LTF, respectively) were significantly different than zero. One-way ANOVA was used to determine significant differences between rats exposed to AIH, ASH and control conditions in frequency LTF and phrenic amplitude LTF (expressed as % changes from baseline).

Multiple regression analysis with backwards selection was used to determine factors significantly correlated with frequency LTF and phrenic amplitude LTF. The factors identified as significant predictors were then subjected to a simple linear regression analysis. Differences were considered significant at $p<0.05$. Reported variances represent +/− 1 standard error.

3. Results

We surveyed published studies concerning AIH-induced respiratory LTF in rats (search terms: “Long-term facilitation AND hypoxia AND rat”), and assessed reported values of frequency versus amplitude/volume LTF. The results from that search are summarized in Table 1, and include studies on both male and female rats, ranging from neonatal to geriatric, and in a variety of states (anesthesia, decerebrate, unanesthetized). In 33 studies on anesthetized rats exposed to AIH, only 15 report statistically significant frequency LTF (8 studies do not present frequency data), whereas 31 studies report significant LTF in phrenic burst amplitude. There was no relationship between the type of anesthetic used and the appearance of frequency LTF. By contrast, of 7 published studies investigating LTF in unanesthetized rats, 6 report significant

frequency LTF, but only 3 report significant tidal volume LTF. Thus, LTF in anesthetized rats is most commonly expressed as increased respiratory-related amplitude (the neural equivalent of tidal volume), whereas LTF in unanesthetized rats is most commonly expressed as increased breathing frequency.

We then pooled data collected in our laboratory from nine different investigators using common experimental procedures. Rats were anesthetized with urethane, paralyzed, ventilated and vagotomized; three different treatment groups were included in the analysis: 1) acute intermittent hypoxia (AIH; n=180), 2) acute sustained hypoxia (ASH; n=47) or 3) time controls (n=75). Figure 1 depicts representative traces from rats exposed to AIH and ASH, illustrating the development of phrenic amplitude LTF following AIH, with lesser effects following ASH.

Since changes in $PaCO_2$ as small as 1–2 mmHg can have a large impact on breathing, we closely monitor arterial PCO_2 levels throughout experiments. Baseline $PaCO_2$ values for AIH (44.5 +/− 0.3 mmHg), ASH (44.4 +/− 0.5 mmHg) and TC (45.0 +/−0.5 mmHg) experiments were not significantly different ($p>0.05$), nor were there differences in $PaCO_2$ values 60 min post-AIH (44.6 +/− 0.3 mmHg), post-ASH (43.9 +/− 0.6 mmHg) or at the equivalent time in control experiments (44.8 +/− 0.5 mmHg, $p>0.05$). $PaCO_2$ 60 min post-hypoxia was not significantly different from baseline in any group ($p>0.05$), confirming that neither phrenic amplitude LTF nor frequency LTF was influenced by uncontrolled changes in $PaCO_2$.

3.1. Intermittent and sustained hypoxia elicit frequency LTF

When we analyzed studies from our laboratory investigating AIH-induced LTF individually, statistically significant frequency LTF was detected in only half of the studies included in our meta-analysis, whereas all had significant phrenic amplitude LTF. When all of the data from individual studies were combined into one large data set, a small, but highly significant increase in burst frequency was noted 60 min post-AIH when compared to baseline values (change from baseline: 4.1 +/− 0.5 bursts/min; $p<0.001$; Fig. 2a). This effect was not specifically pattern-sensitive since a similar small, but statistically significant, increase was observed in burst frequency 60 min post-ASH (2.6 +/− 0.8 bursts/min; $p=0.003$; Fig. 2a), despite no change in burst frequency noted in any of the individual studies investigating ASH. By contrast, control rats subjected to the same surgical preparation, but not exposed to hypoxia, did not exhibit significant changes in burst frequency at the equivalent time (1.1 +/− 0.6 bursts/min; $p=0.07$; Fig. 2a), although there was an apparent trend towards an increase.

Figure 2b illustrates the average burst frequency and phrenic amplitude LTF 60 min following AIH and ASH, and at an equivalent time in control rats for all of the rats included in our meta-analysis. Frequency and phrenic amplitude LTF are both expressed as a percentage change from baseline for ease of comparison. AIH elicited a small, but highly significant frequency LTF (11.6 +/− 1.2% baseline; $p<0.001$), but larger phrenic amplitude LTF (58.7 +/− 3.5% baseline; $p<0.001$). Both values were significantly greater than responses in time control rats (frequency LTF: 3.1 +/− 1.3% baseline; phrenic amplitude LTF: 3.9 +/− 3% baseline; both $p<0.001$ versus AIH). ASH also induced statistically significant frequency and phrenic amplitude LTF (frequency LTF: 7.5 +/−2.3% baseline; phrenic LTF: 10 +/− 4.1% baseline; both $p<0.02$ relative to no change). However, neither response was significantly different from control rats (both $p > 0.08$). Similar to all previous reports, phrenic amplitude LTF was significantly greater following AIH versus ASH ($p < 0.001$), although there were no differences in frequency LTF between treatment protocols ($p > 0.05$).

3.3. Multiple regression analysis of frequency LTF

The analysis above demonstrates that, although small, AIH induces significant frequency LTF in anesthetized and vagotomized rats. In order to better understand variables that influence the

expression of frequency LTF (and that may help explain variation among studies), we performed stepwise multiple linear regression analysis with backwards selection using the following independent variables: baseline burst frequency, change in burst frequency during hypoxia, change in phrenic burst amplitude during hypoxia, change in $PaCO_2$ from baseline at 60 min post-AIH, PaO_2 during hypoxia, rat temperature, change in temperature from baseline to 60 minutes post-AIH, and the magnitude of phrenic LTF at 60 min post-AIH. For this regression analysis, frequency LTF was expressed as the absolute change in burst frequency from baseline at 60 min post-AIH.

Stepwise regression analysis revealed that baseline burst frequency (multivariate coefficient = −0.39; $p < 0.001$), the change in burst frequency during hypoxia (multivariate coefficient = 0.16; $p < 0.001$), and phrenic amplitude LTF (multivariate coefficient = 0.024; $p < 0.002$) were all highly significant predictors of frequency LTF. The change in $PaCO_2$ from baseline to 60 min post-AIH was a marginally significant predictor of frequency LTF ($p = 0.06$), but the exclusion of this variable did not alter the overall regression significantly ($R^2 = 0.509$ with $PaCO_2$; R2 = 0.501 without $PaCO_2$). Other factors tested were not significantly correlated with frequency LTF in this data set, and did not improve the overall value of R^2 when included in the regression. The final regression equation was ($R^2 = 0.501$, $p<0.001$):

$$\text{Frequency LTF} = 17.9 - (0.39 * \text{baseline frequency}) + (0.16 * \text{change in frequency in hypoxia}) + (0.0237 * \text{phrenic amplitudeLTF})$$

Figure 3a illustrates the relationship between baseline burst frequency and frequency LTF in rats exposed to AIH (without factoring in the other predictive variables). Simple regression analysis on this data set confirmed a negative correlation between baseline burst frequency and frequency LTF, such that lower initial baseline burst frequencies predict greater frequency LTF ($R^2 = 0.389$, intercept = 25.4, $p<0.001$). Our analysis also suggests that the frequency response following AIH may be bidirectional, since high baseline burst frequencies (>50 bursts/min) tended to result in prolonged frequency depression (i.e., a negative frequency LTF).

Some of the correlation between baseline burst frequency and frequency LTF may be a consequence of the experimental preparation since a smaller, but still significant relationship between baseline burst frequency and frequency LTF was observed in control rats without hypoxic exposure ($R^2 = 0.11$, intercept = 10.3; $p = 0.004$, figure 3b). Nevertheless, comparison of the regression slopes reveals that the relationship between baseline burst frequency and frequency LTF is considerably greater in rats exposed to AIH since the slope in AIH-treated rats was significantly greater than in control rats (AIH slope: −0.507; control slope: −0.21, $p<0.001$). Thus, AIH elicits respiratory burst frequency plasticity beyond the normal “drift” intrinsic to our anesthetized experimental preparation.

Since hypoxia is associated with hypothermia (for review, Bicego et al., 2007) and changes in body temperature can have profound effects on respiratory frequency (Grunstein et al., 1973), we analyzed the average body temperature of the rats during baseline and 60 min post-AIH. No significant differences were detected (baseline: 37.5 +/− 0.06; 60 min post-AIH: 37.5 +/− 0.07), which is an expected result since the rats body temperature was actively regulated by adjusting the temperature of a heated surgical/recording table. Thus, time-dependent changes in burst frequency cannot be attributed to hypoxia-induced changes in body temperature.

Our multivariate analysis also indicated that the change in burst frequency during hypoxia and phrenic amplitude LTF were significant predictors of frequency LTF. Figure 4 illustrates the relationship between frequency LTF and these factors (without factoring in the other predictive variables). A simple linear regression analysis confirmed a positive relationship between the magnitude of the frequency response during hypoxia and the subsequent frequency LTF

(Figure 4a; $R^2 = 0.35$, $p<0.001$). This relationship also provided suggestive evidence that the direction of the hypoxic frequency response is related to the sign of frequency LTF (i.e., facilitation versus depression). A weak, positive correlation was observed between frequency LTF and the magnitude of phrenic amplitude LTF (Fig 4b; $R^2 = 0.028$, $p =0.02$). While the multiple regression model described above suggested a slight (but not significant) relationship between frequency LTF and variations in $PaCO_2$ during the experimental protocol, a simple linear regression provided no evidence for such a relationship (not shown; $R^2=0.0004$, $p=0.79$), again demonstrating that inadequate control of blood gas composition is not related to the expression of frequency LTF.

3.4. Multiple regression analysis of phrenic LTF (a follow up)

Phrenic burst amplitude LTF data were analyzed using stepwise regression analysis with backwards selection to determine if factors influencing frequency LTF also influence phrenic amplitude LTF. Variables analyzed were: baseline burst frequency, the change in burst frequency during hypoxia, frequency LTF, phrenic burst amplitude during hypoxia and PaO_2 during hypoxia. The first three variables were not included in our earlier meta-analysis (Fuller et al., 2000), and thus serve to expand our original conclusions. Our analysis revealed that baseline burst frequency (multivariate coefficient = 1.8; $p=0.001$), frequency LTF (multivariate coefficient = 2.3; $p=0.001$) and phrenic amplitude during hypoxia (multivariate coefficient = 0.3; $p<0.001$) were all significant predictors of phrenic amplitude LTF, with burst amplitude during hypoxia exhibiting the strongest relationship. The following regression equation was obtained ($R^2=0.25$, $p<0.001$):

$$\text{Phrenic LTF} = -61.595 + (1.8 * \text{baseline frequency}) + (2.27 * \text{frequency LTF}) + (0.31 * \text{amplitude in hypoxia})$$

Figure 5 illustrates the relationship between phrenic amplitude LTF and phrenic amplitude response during hypoxia (without factoring in the other predictive variables). As reported previously, phrenic amplitude LTF is positively correlated with phrenic amplitude during hypoxia (figure 5; $R^2 = 0.200$, $p < 0.001$; Fuller et al., 2000). A weaker relationship exists between phrenic LTF and frequency LTF (figure 4b; $R^2 = 0.028$; $p < 0.04$), suggesting a possible common mechanism, or at least reliance on common factors. While the multiple regression analysis suggested a possible relationship between phrenic LTF and baseline burst frequency, a simple linear regression analysis (without factoring in the other factors) revealed no such relationship (not shown; $R^2 = 0.0008$; $p = 0.7$).

4. Discussion

Frequency LTF expression

Collectively, our meta-analysis confirms that LTF of phrenic burst amplitude dominates the manifestation of respiratory LTF following AIH in anesthetized and vagotomized rats. Despite some variability in reports of frequency LTF among published studies, we also confirm the existence of frequency LTF in this preparation. However, the magnitude of frequency LTF is quite limited (12% frequency LTF *versus* 60% phrenic amplitude LTF), and a large data set is necessary to clearly demonstrate statistical significance. Unlike burst amplitude LTF, frequency LTF may not be as strongly pattern sensitive since both acute intermittent and sustained hypoxia induced frequency LTF, although the latter was not significantly different from time control rats.

One surprising finding from this meta-analysis is the observation that baseline burst frequency strongly predicts the magnitude and direction of frequency LTF in anesthetized and vagotomized rats; since frequency LTF and baseline frequency are inversely related, frequency LTF is observed following AIH only in rats with a relatively low baseline burst frequency. High baseline burst frequencies are associated with either no frequency LTF or a frequency

depression following AIH. While the transition from facilitation to depression was similar between control and AIH rats (control: 49.1; AIH: 50.1 breaths/min), the magnitude of the subsequent facilitation or depression was magnified in rats exposed to (intermittent) hypoxia. Other factors influencing the expression of frequency LTF include the change in frequency during hypoxia and (to a lesser extent) the magnitude of phrenic amplitude LTF. It is unknown if similar relationships are found in unanesthetized rats exposed to AIH.

Possible mechanisms linking frequency LTF with the baseline burst frequency are unknown. One possibility is that higher baseline frequencies represent a maximal value in vagotomized rats, and that there is no longer sufficient "dynamic range" to enable the expression of frequency LTF. Supportive evidence for this possibility is provided by the observation that the frequency response during hypoxia is correlated with frequency LTF. In this scenario, rats with low baseline burst frequencies had the capacity to increase breathing frequency in hypoxia and, therefore, exhibit frequency LTF following AIH. By contrast, rats with a high baseline burst frequency may have been restricted by a frequency "maximum" characteristic of this anesthetized and vagotomized preparation, and, therefore, could not further increase breathing frequency during hypoxia or exhibit post-AIH frequency LTF. The constraint of a maximal frequency may not apply in vagi-intact rats, which might be why awake rats exhibit robust frequency LTF. Alternatively (or in addition), neuromodulators that induce post-hypoxia frequency changes may exert complex effects on the respiratory rhythm generator, a likely site for respiratory frequency plasticity. The finding that very high baseline burst frequencies are associated with apparent post-AIH frequency depression is consistent with this speculation. Onimaru et al. (1998) demonstrated that the effects of exogenous serotonin on *in vitro* brainstems from neonatal rats depends on baseline burst frequency; excitatory effects were obtained at low baseline burst frequencies whereas inhibitory effects were observed when baseline burst frequencies were high. Thus, since LTF is a serotonin-dependent mechanism, serotonin released in the vicinity of the respiratory rhythm generator, or neurons that relay primary afferent activity to the rhythm generator, may elicit frequency LTF contingent on the initial baseline frequency. Detailed mechanistic studies on the relationship between frequency LTF and baseline frequency would be studied most effectively in an experimental preparation that exhibits greater frequency LTF since it will be very difficult to establish statistical significance using the anesthetized and vagotomized preparation.

Phrenic amplitude LTF expression

In a previous meta-analysis, we found that phrenic amplitude LTF is correlated with phrenic burst amplitude during hypoxia, but not with hypoxic PaO_2 levels (Fuller et al., 2000). Our current results confirm these findings. While the mechanism giving rise to the relationship between the hypoxic phrenic response and phrenic amplitude LTF are unknown, one possibility is suggested by recent observations that: 1) spinal serotonin receptor activation is necessary for AIH-induced phrenic LTF (Baker-Herman and Mitchell, 2002), and 2) intermittent spinal serotonin application is sufficient to elicit phrenic amplitude LTF (Lovett-Barr et al., 2006; MacFarlane and Mitchell, 2007a). A strong hypoxic ventilatory response may result in greater activation of raphe serotonergic neurons (Morris et al., 1996, 2001), thereby releasing more serotonin in the vicinity of phrenic motor neurons and triggering greater phrenic amplitude LTF (see below). An alternate hypothesis is that the correlation between the hypoxic amplitude response and phrenic LTF may arise from a limited "dynamic range" of phrenic motor output, similar to our argument that frequency LTF and the hypoxic frequency response may be restricted by a similar limit. Thus, if the capacity to increase phrenic burst amplitude is limited during hypoxia, the capacity to exhibit phrenic LTF may be similarly limited. Lastly, it may be that there is no causal relationship between hypoxic phrenic burst amplitude and phrenic amplitude LTF.

We did not consider frequency changes in our previous meta-analysis (Fuller et al., 2000); thus, we extend our previous analysis of factors influencing the expression of phrenic amplitude LTF. We found that phrenic amplitude LTF is marginally correlated with frequency LTF, suggesting that these two forms of plasticity occur at different loci, but may share partial reliance on common factors. Unlike frequency LTF, there was no relationship between phrenic amplitude LTF and baseline burst frequency or burst frequency during hypoxia.

Pattern-sensitivity of LTF

Our meta-analysis supports literature indicating that intermittent hypoxia is fundamentally different than sustained hypoxia in its ability to elicit LTF (Dwinell et al., 1997; Baker and Mitchell, 2000; Baker et al., 2001; Olson et al., 2001; McKay et al., 2004; Tajalli et al., 2007). However, while both phrenic amplitude LTF and frequency LTF following ASH were significantly greater than zero, neither the amplitude nor frequency LTF was statistically greater than in time control rats. Thus, the small, apparent LTF in amplitude and frequency following sustained hypoxia cannot be distinguished from drift in this experimental preparation. The mechanisms giving rise to pattern-sensitivity in phrenic amplitude LTF are not well understood, but may involve differential ROS generation and regulation of protein phosphatases (Wilkerson et al., 2007, 2008).

State-dependence of LTF

Most literature suggests that AIH elicits predominantly frequency LTF in awake or decerebrate preparations, with less (or even no) tidal volume LTF. On the other hand, anesthetized preparations express a prominent amplitude LTF following AIH, with a small and inconsistent frequency LTF. Recent data suggest that sleeping animals may behave similarly to anesthetized animals in terms of their LTF manifestation. For example, unanesthetized rats (Nakamura et al., 2006), mice (Terada et al., 2008) and humans (Pierchala et al., 2007) in documented slow wave sleep exhibit tidal volume LTF, and, at least in rodents, frequency LTF. Mechanisms giving rise to state-dependent effects on LTF are unknown. Vagal feedback may influence the expression of frequency or amplitude LTF. Indeed, awake, spontaneously breathing preparations have an intact vagus nerve, whereas anesthetized preparations have been most frequently pump ventilated and vagotomized to prevent entrainment of respiratory motor output with the ventilator. However, the few available studies indicate that vagi-intact animals have the capacity to express volume/amplitude LTF since anesthetized animals with intact vagi express LTF in tidal volume (Mateika and Fregosi, 1997) or phrenic nerve burst amplitude (F. Golder, personal communications), whereas unanesthetized, sleeping animals with intact vagi exhibit tidal volume LTF (Nakamura et al., 2006; Pierchala et al., 2007; Terada et al., 2008). Differential $PaCO_2$ regulation in anesthetized versus unanesthetized animals may also contribute to differential LTF expression. Investigators generally regulate $PaCO_2$ within tight constraints in protocols using anesthetized animals, whereas unanesthetized animals are most often poikilocapnic, allowing $PaCO_2$ to drop as ventilation increases. Decreased $PaCO_2$ is expected to dampen ventilation via chemofeedback, thereby reducing LTF. Indeed, Olson and colleagues (2001) exposed a group of unanesthetized rats to poikilocapnic or isocapnic AIH, and while both groups exhibited frequency LTF, only rats exposed to poikilocapnic AIH expressed tidal volume LTF. While changes in $PaCO_2$ undoubtedly dampen (and obscure) tidal volume LTF, it is uncertain whether poikilocapnic conditions would differentially affect LTF in sleep *versus* wakefulness. A third possibility to account for differential LTF expression in anesthetized versus unanesthetized experimental models is state-dependent alterations in neuromodulatory inputs to respiratory-related neurons. For example, brainstem respiratory neuron and motoneuron responses to intermittent hypoxia may be impacted by decreased neuromodulatory influences during sleep (Aston-Jones and Bloom, 1981; Veasey et al., 1995; Jacobs and Fornal, 1999; Horner, 2007) or altered neuromodulator release due to

anesthesia, as has been demonstrated in the hypothalamus (Shimokawa et al., 1998; Kushikata et al., 2005; Mukaida et al., 2007).

Mechanism of LTF: phrenic burst amplitude

Mechanisms giving rise to phrenic burst amplitude LTF have been extensively investigated and reviewed in recent years (see Baker et al., 2001; Mitchell et al., 2001a; Feldman et al., 2003; Baker-Herman et al., 2004; Mahamed and Mitchell, 2006; Wilkerson et al., 2007) and will not be discussed extensively here. The bulk of the evidence suggests that respiratory burst amplitude LTF occurs at or near the respective motor neuron pools. For example, AIH-induced phrenic, but not XII LTF requires spinal serotonin receptor activation (Baker-Herman and Mitchell, 2002) and new serotonin-dependent BDNF protein synthesis within the ventral cervical spinal segments containing the phrenic motor nucleus (Baker-Herman et al., 2004). XII LTF is thought to occur via similar mechanisms, but operating in the hypoglossal motor nucleus. Indeed, repetitive serotonin (type 2) or norepinephrine (alpha 1) receptor activation enhances AMPA currents similar to AIH-induced XII LTF in synaptically isolated XII motor neurons in vitro (Bocchiaro and Feldman, 2004; Neverova et al., 2007), demonstrating that XII amplitude LTF can arise from local mechanisms at sites distant from respiratory pre-motor neurons. In summary, amplitude LTF appears to arise largely from neuromodulatory actions on (or near) the target motor neurons, a site that cannot account for changes in burst frequency by any currently postulated model of respiratory rhythm generation. To explain respiratory frequency LTF, it is necessary to consider plasticity at the level of the rhythm generator itself, or in neural pathways from afferent neurons projecting to the rhymogenic network (Feldman et al., 2003).

Mechanism of LTF: burst frequency

There is at least some evidence indicating that frequency LTF arises from brainstem mechanisms distinct from the mechanisms giving rise to phrenic amplitude LTF. For example, when rhythmogenic brainstem slices are exposed to episodic anoxia, long-lasting increases in the burst frequency of pre-Botzinger complex neurons are observed (Blitz and Ramirez, 2002). However, *in vivo* frequency plasticity may also arise from indirect effects on the respiratory rhythm generator. For example, spinal serotonin administration elicits long-lasting synaptic plasticity in sensory neurons of the spinal dorsal horn (see Zhuo et al., 2000). Thus, if AIH-induced serotonin release in the spinal dorsal horn alters synaptic inputs arising from sensory afferents that modulate respiratory rhythm, long-lasting changes in frequency may occur without direct effects on the respiratory rhythm generator. We have in fact observed changes in breathing frequency in unanesthetized goats following spinal application of serotonin receptor antagonists (GS Mitchell, unpublished observations), suggesting that such indirect mechanisms of frequency LTF are possible and should at least be evaluated.

The concept that frequency and amplitude plasticity may arise from distinct neuronal mechanisms has been addressed previously (Powell et al., 1998). Indeed, frequency LTF most likely occurs in brainstem respiratory rhythm generating neurons, whereas amplitude LTF most likely occurs in respiratory motor neurons. While the site of plasticity in these different respiratory outputs may be distinct, similar cellular/synaptic (serotonin-dependent, pattern sensitive) mechanisms may be involved. At this point, we have little knowledge concerning the cellular/synaptic mechanisms of frequency LTF.

Frequency LTF may involve peptides including substance P or orexin since frequency LTF is not observed in tachykinin-1 (Berner et al., 2007) or prepro-orexin knockout mice (Terada et al., 2008). However, the specific role of serotonin in frequency LTF is unclear. Studies from our laboratory concerning serotonergic mechanisms of phrenic amplitude LTF made only secondary conclusions about frequency LTF since similar compounds appeared to block both

manifestations of LTF (Bach and Mitchell, 1996; Baker-Herman and Mitchell, 2002). However, in hindsight, and with the benefit of this meta-analysis, the compounds used to block serotonin receptors often also increased baseline burst frequency; thus there is some doubt as to whether frequency LTF was abolished directly by serotonin receptor antagonists, or indirectly by the change in baseline frequency. The role of serotonin in frequency LTF needs further investigation.

Significance and unanswered questions

This meta-analysis makes several important points. First, respiratory LTF is almost certainly not the result of a single cellular/synaptic mechanism operating at a single site of the CNS; future studies should consider this important issue in their interpretations. The importance of frequency *versus* burst amplitude LTF is far from clear, yet it does appear that they are differentially expressed in a variety of experimental and/or physiological states (*e.g.*, sleep *versus* awake, anesthetized *versus* decerebrate). None of these states should be considered more important than another in assessing the physiological significance of LTF since it is very hard to argue, for example, that control of breathing during sleep is less important than control of breathing during wakefulness. Reduced preparations offer experimental advantages in controlling relevant variables, yet it must be remembered that the preparation itself may alter the mechanism under study. For example, perfused and decerebrate preparations such as the working heart brainstem/spinal cord preparation may offer advantages in the study of frequency (*versus* amplitude) LTF following AIH (Tajalli et al., 2007). On the other hand, anesthetized, vagotomized and ventilated rat may be best suited to study amplitude LTF (i.e., more similar to slow wave sleep). Although the urethane anesthetized and vagotomized rats analyzed in this meta-analysis express a marginal degree of frequency LTF, it is a poor model for studies of its underlying mechanism since frequency LTF magnitude is small and inconsistent; large sample sizes will be necessary to make statistical inferences using this experimental model.

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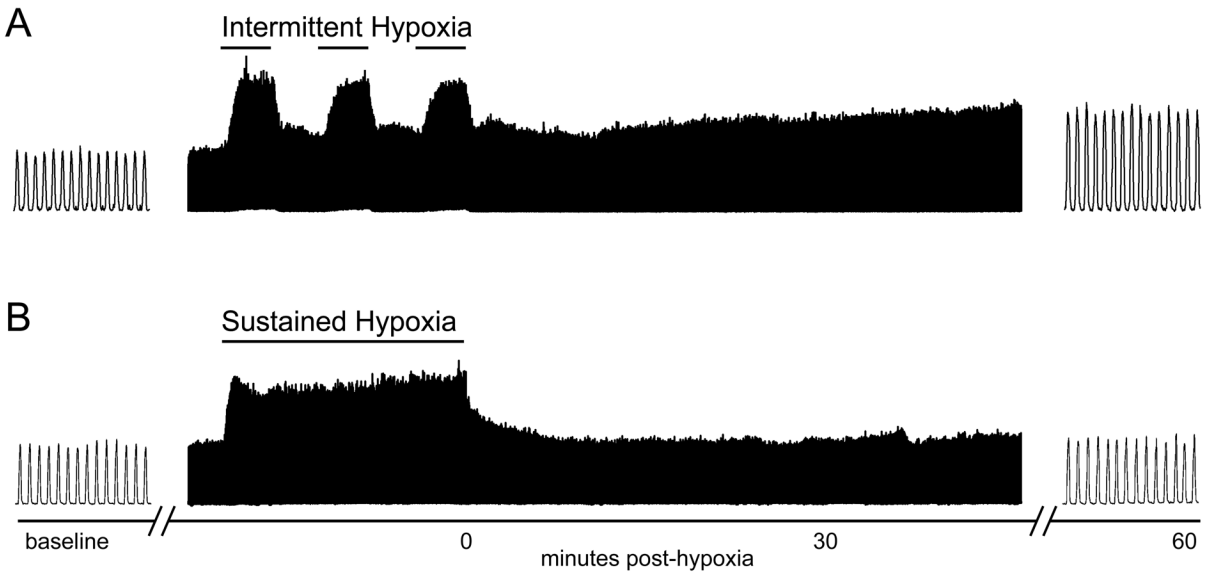

Figure 1.

Representative phrenic neurogram from a rat exposed to (A) acute intermittent hypoxia (AIH) or (B) acute sustained hypoxia (ASH). Rectified, integrated phrenic discharge during baseline and 60 min post-hypoxia are expanded to illustrate amplitude and frequency changes following hypoxia; the time points in between are compressed to illustrate general trends in phrenic amplitude. AIH results in large increases in phrenic amplitude and smaller increases in burst frequency upon return to baseline inspired oxygen conditions.

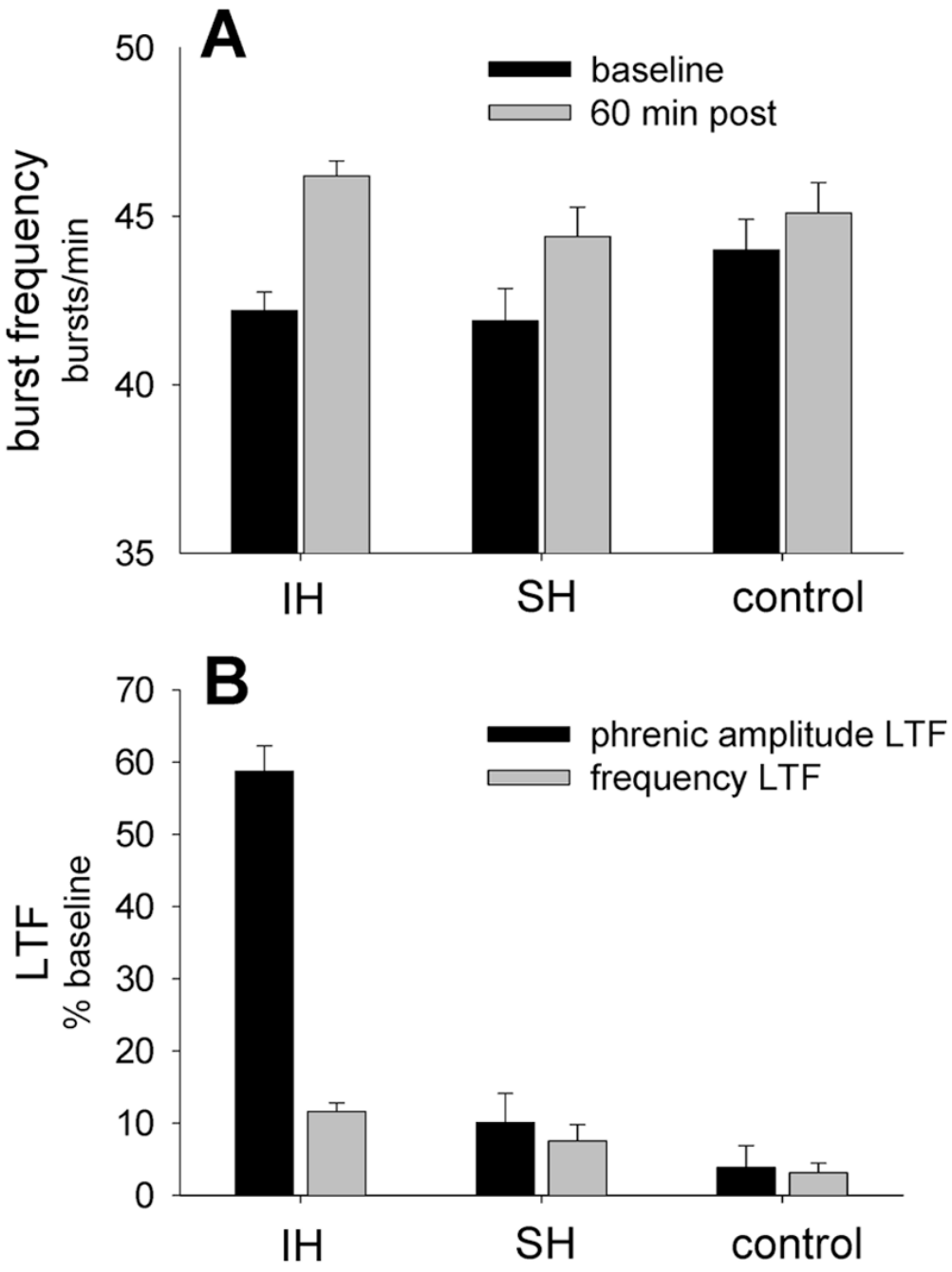

Figure 2.

Changes in burst frequency and phrenic amplitude following acute intermittent (AIH) and sustained (ASH) hypoxia in anesthetized, vagotomized rats. (A) Burst frequency during baseline (black bar) and 60 min post-exposure (white bar) in rats exposed to AIH, ASH, or an equivalent duration of control inspired gas mixtures (no hypoxia). Rats exposed to AIH and ASH, but not controls, had a significant increase in respiratory burst frequency 60 min post-hypoxia. (B) Phrenic amplitude LTF (black bar) and frequency LTF (white bar) in rats exposed to AIH, ASH or controls. AIH elicited significant phrenic amplitude LTF and frequency LTF relative to baseline and the response of control rats. Phrenic amplitude LTF and frequency LTF

following ASH were significant relative to baseline, but not from controls. * significantly different from zero; # significantly different from controls; $p<0.05$

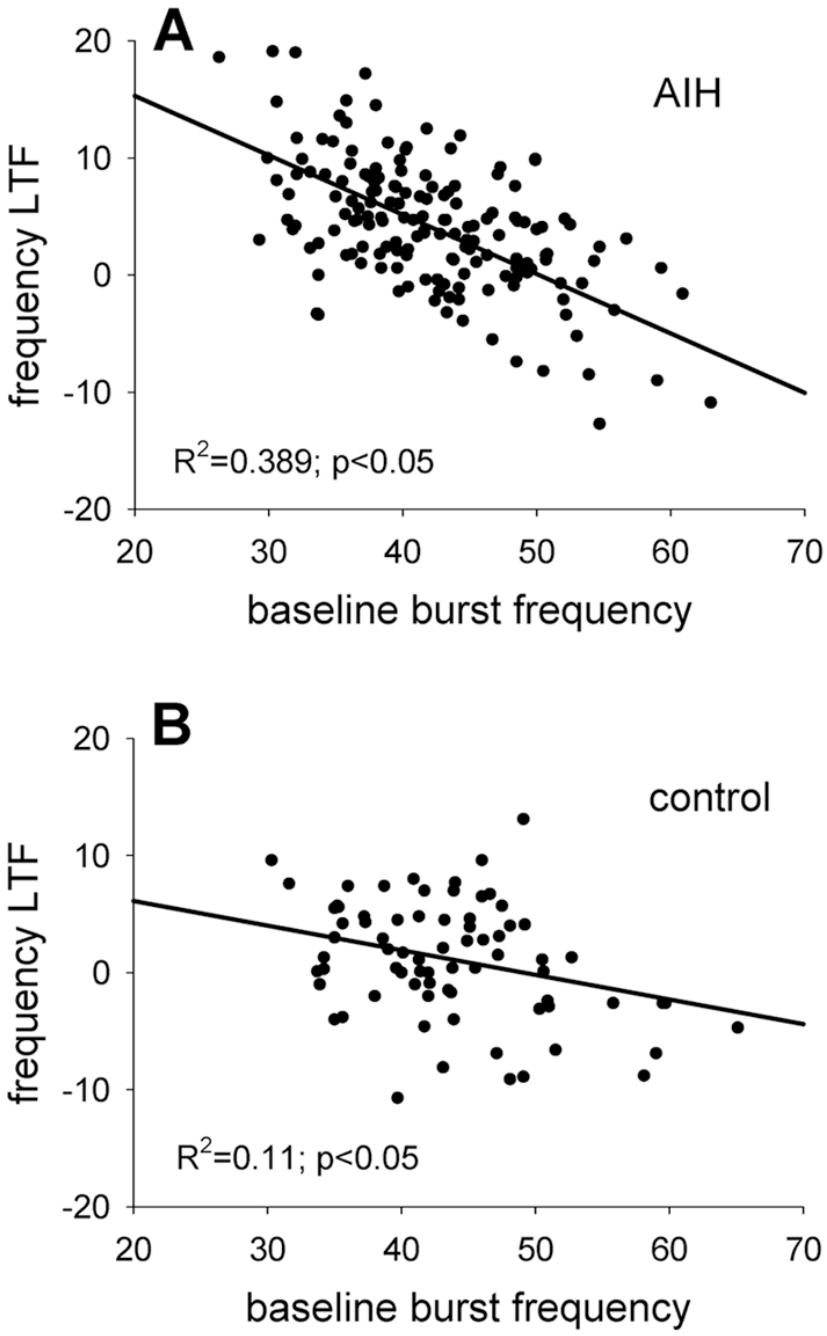

Figure 3.
Relationship between baseline burst frequency and frequency LTF in rats exposed to acute intermittent hypoxia (AIH) or control rats not exposed to hypoxia. (A) Baseline burst frequency is a strong predictor of frequency LTF 60 min post-AIH; rats with lower baseline burst frequencies generally express greater frequency LTF. (B) A significant relationship between baseline burst frequency and frequency LTF was also noted in control rats, but the slope of the regression was significantly lower than for AIH rats ($p<0.05$).

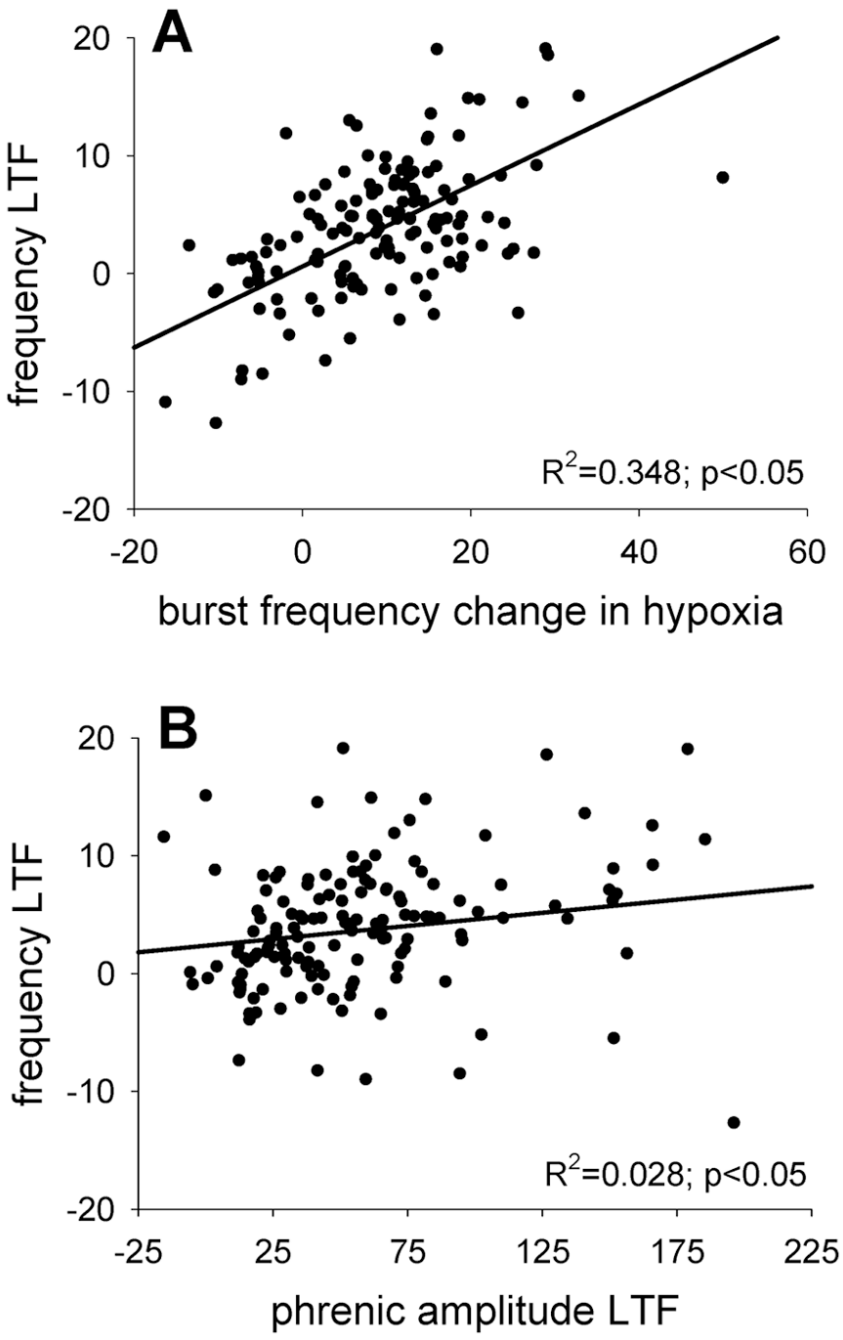

Figure 4.
Regression analysis of frequency LTF following acute intermittent hypoxia (AIH) with the change in burst frequency in hypoxia and phrenic LTF. (A) Hypoxic burst frequency is a strong predictor of frequency LTF 60 min following AIH, such that rats with high hypoxic burst frequencies also tend to have greater frequency LTF. (B) A weaker relationship was noted between the magnitude of phrenic LTF and frequency LTF.

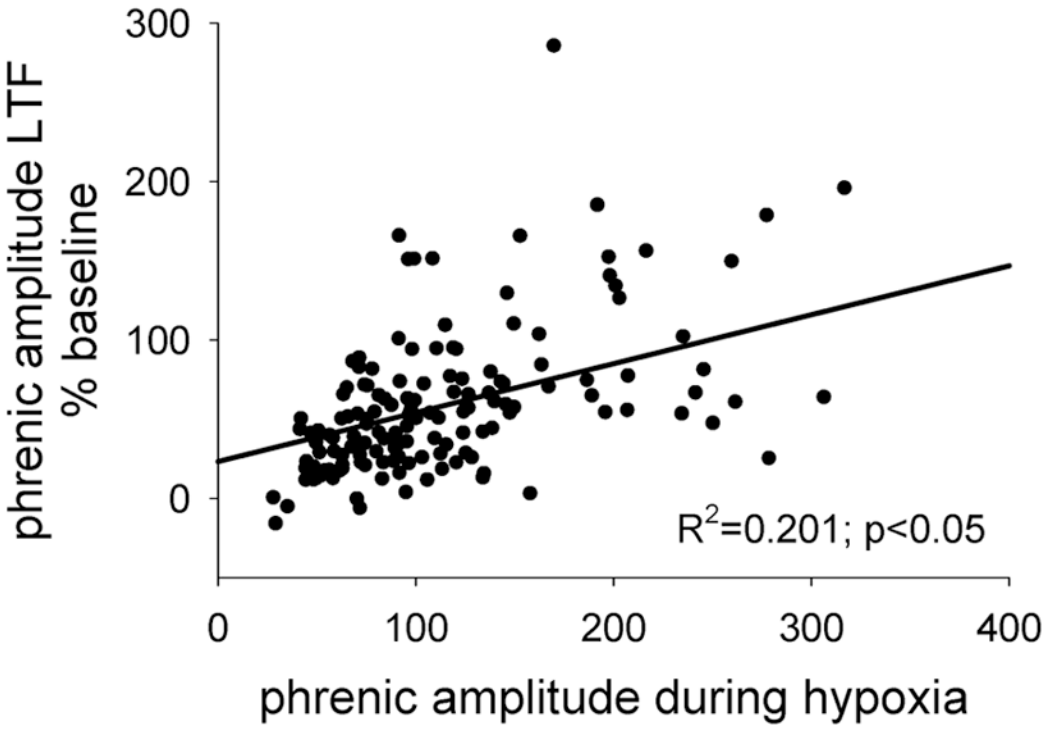

Figure 5.
Relationship between phrenic amplitude response during hypoxia and phrenic amplitude LTF 60 min post-acute intermittent hypoxia (AIH). Phrenic hypoxic response significantly predicts phrenic amplitude LTF 60 min post-AIH, such that rats with higher hypoxic responses tend to have greater phrenic LTF.

Table 1

Summary of literature reports of frequency LTF (freq LTF) and burst amplitude or tidal volume LTF (Amp LTF) in anesthetized and unanesthetized rats exposed to acute intermittent hypoxia. Yes/No indicates that LTF was present in some control groups, but not others within the same study.

Paper	State	Freq LTF	Amp LTF	Duration
Mahamed and Mitchell, 2008	Anesth	Yes	Yes	>60 min
Julien et al., 2008	Unanesth	No	Yes	>60 min
Hsieh et al., 2008	Anesth	Not reported	Yes	>60 min
Macfarlane and Mitchell, 2007b	Anesth	Yes	Yes	>60 min
Tadjalli et al., 2007	Unanesth	Yes	No	>60 min
Dick et al., 2007	Anesth	No	Yes	>60 min
Reeves and Gozal 2007	Anesth	Not reported	Yes	>60 min
Neverova et al., 2007	Anesth	Not reported	Yes	>60 min
McGuire et al., 2007	Anesth	Not reported	Yes	>60 min
Zabka et al., 2006	Anesth	Yes	Yes	>60 min
Doperalski and Fuller, 2006	Anesth	Yes	Yes	>60 min
Reeves et al., 2006	Anesth	Not reported	Yes	>60 min
McGuire et al., 2005	Anesth	Yes/No	Yes	>60 min
Golder and Mitchell, 2005	Anesth	Yes	Yes	>60 min
Fuller, 2005	Anesth	No	Yes	>60 min
Zabka et al., 2005	Anesth	Yes	Yes	>60 min
McGuire and Ling, 2005	Unanesth	Yes	Yes	15–45 min
McKay et al., 2004	Anesth	No	Yes	>60 min
Baker-Herman et al., 2004	Anesth	Not reported	Yes	>60 min
McGuire et al., 2004	Unanesth	Yes	No	~45 min
Bavis and Mitchell, 2003	Anesth	No	Yes	>60 min
McGuire et al., 2003	Unanesth	Yes	No	~45 min
Behan et al., 2003	Anesth	Not reported	Yes	>60 min
Zabka et al., 2003	Anesth	Yes	Yes	>60 min
Peng and Prabhakar, 2003	Anesth	Yes	No	>60 min
McGuire et al., 2002	Unanesth	Yes	No	15–75 min
Baker-Herman and Mitchell, 2002	Anesth	Yes	Yes	>60 min
Zabka et al., 2001b	Anesth	Yes/No	Yes/No	>60 min
Olson et al., 2001	Unanesth	Yes	Yes/No	>60 min
Ling et al., 2001	Anesth	Not reported	Yes	>60 min
Fuller et al., 2001	Anesth	No	Yes	>60 min
Zabka et al., 2001a	Anesth	Yes	Yes	>60 min
Fuller et al., 2001	Anesth	No	Yes	>60 min
Baker and Mitchell, 2000	Anesth	Yes	Yes	>60 min
Bach and Mitchell, 2000	Anesth	Yes	Yes	>60 min
Janssen and Fregosi, 2000	Anesth	No	No	
Kinkead and Mitchell, 1999	Anesth	No	Yes	>60 min
Kinkead et al., 1998	Anesth	No	Yes	>60 min
Bach and Mitchell, 1996	Anesth	Yes	Yes	>60 min
Hayashi et al., 1993	Anesth	No	Yes	>60 min