SELECTIVE LOSS OF HIGH-FREQUENCY OSCILLATIONS IN PHRENIC AND HYPOGLOSSAL ACTIVITY IN THE DECEREBRATE RAT DURING GASPING

Vitaliy Marchenko and Robert F. Rogers

Department of Electrical & Computer Engineering
University of Delaware
Newark, DE 19716

Running Title: Phrenic & XII Spectral Components During Eupnea and Asphyxia

Contact Information
Robert F. Rogers, PhD
Dept. of Electrical & Computer Engineering
University of Delaware
Newark, DE 19716
email: rrogers@ece.udel.edu
phone: (302) 831-8517
fax: (302) 831-4316

Copyright © 2006 by the American Physiological Society.
ABSTRACT

Respiratory motor outputs contain medium-(MFO) and high-frequency oscillations (HFO) that are much faster than the fundamental breathing rhythm. However, the associated changes in power spectral characteristics of the major respiratory outputs in unanesthetized animals during the transition from normal eupneic breathing to hypoxic gasping have not been well characterized. Experiments were performed on 9 unanesthetized, chemo- and baro-denervated, decerebrate adult rats, in which asphyxia elicited hyperpnea followed by apnea and gasping. A gated Fast Fourier transform (FFT) analysis and a novel time-frequency representation (TFR) analysis were developed and applied to whole phrenic and to medial branch hypoglossal nerve recordings. Our results revealed one MFO and one HFO peak in the phrenic output during eupnea, where HFO was prominent in the first two-thirds of the burst, and MFO in the latter two-thirds of the burst. The hypoglossal activity contained broad-band power distribution with several distinct peaks. During gasping, two high-amplitude MFO-peaks were present in phrenic activity, and this state was characterized by a conspicuous loss in HFO power. Hypoglossal activity showed a significant reduction in power and a shift in its distribution towards lower frequencies during gasping. TFR analysis of phrenic activity revealed the increasing importance of an initial low-frequency “start-up” burst that grew in relative intensity as hypoxic conditions persisted. Significant changes in MFO and HFO rhythm generation during the transition from eupnea to gasping presumably reflect a reconfiguration of the respiratory network and/or alterations in signal processing by the circuitry associated with the two motor pools.

Keywords: respiratory patterns; ZIS analysis; motor neuron synchrony; network plasticity
Introduction

Fast rhythms occur in variety of neural systems at various levels including cortex, thalamus, brain stem, and spinal cord (14). The study of such rhythms is expected to provide insight into neural interactions that produce physiologically important output patterns. Fast oscillatory rhythms are present in all respiratory muscles, nerves, and central neurons in mammals, including humans (1, 11, 13, 40, 45). These and many other studies have shown the presence at least of two general fast respiratory output rhythms present in cat or rabbit phrenic nerve records: medium frequency oscillations (10-50 Hz, MFO) and high frequency oscillations (50-100 Hz, HFO).

Although much of the early work in fast oscillations in respiratory motor nerves was conducted in cats, rodents have become a popular experimental model for respiratory rhythmogenesis. However, a limited number of experiments have examined HFO in rodents (24, 29). These in vivo studies reported that MFO and HFO in rodents are at frequencies twice those in cats and rabbits (1, 11, 44), and they are significantly higher than those in in situ perfused juvenile rats (47, 51) and in vitro neonatal cat preparations (23).

MFO, and especially HFO, are very sensitive to anesthesia, as evidenced by prominent spectral peaks in unanesthetized, decerebrated animals (10). Despite the depressive effects of anesthesia and popularity of the rodent model, to date only one study utilized decerebrate rats (29) during normal eupnea, and no studies have been published regarding fast respiratory motor output rhythms in decerebrate adult rats during anoxia-induced behaviors in vivo. Therefore, in the present study we used precollicularly-decerebrated, unanesthetized adult male rats to characterize the changes in fast respiratory motor outputs rhythms between eupneic and mild to severe hypoxic/hypercapnic states in adult rats.
Respiratory responses to hypoxia in mammals typically progress through four phases: hyperpnea, primary apnea, gasping, and terminal (or secondary in case of successful resuscitation) apnea (16). Gasping, as a distinct motor behavior or functional state, is a very important mechanism of auto-resuscitation in infant and adult mammals, including human (35), rats (15, 18) and mice (22).

Despite its physiological importance, the frequency composition of phrenic nerve activity during gasping is not resolved. Based on results using FFT-derived parametric analysis in decerebrate cats, St.-John (49) suggested that two hallmarks of gasping (compared to eupnea) are the shift of HFO to even higher frequencies and a marked decrease in MFO power. However, other studies (e.g., 2), using autoregressive nonparametric spectral and wavelet analyses, reported a shift in HFO towards lower frequencies, as well as high power in MFO, during gasping in anesthetized adult cats and young piglets. This discrepancy may be explained by preparation (decerebrate vs. anesthetized), analytical methods (parametric vs. non-parametric), or even recording method (mono- vs. biphasic). Since biological signals are often non-stationary in character, other non-parametric methods have been applied for spectra estimation of respiratory output frequencies, including time-variant wavelet analyses (e.g., 3) and time-frequency representation (TFR) smoothed pseudo-Wigner-Ville distribution (SPWVD; see 32). The latter study only analyzed diaphragmatic EMG and hypoglossal nerve recordings in the anesthetized mouse, and time-frequency components of phrenic discharge were not described. Therefore, the time-frequency representations of the phrenic nerve activity in unanesthetized adult rats during eupnea and gasping have not been characterized.

In light of these unknowns, the two major goals of the following study are to: (1) definitively characterize the dynamics of the frequency components of the phrenic and
hypoglossal nerves in the adult rat, without the effects of peripheral feedback and anesthesia; and
(2) resolve the discrepancies regarding the frequency components in the phrenic nerve during severe hypoxia/hypercapnia-induced behaviors, particularly gasping. In order to achieve these goals, we applied classic time-invariant FFT, and a new FFT-based TFR analysis, developed in our laboratory. Using these methods, we tested the hypothesis that gasping is characterized by a shift in HFO towards higher frequencies and a reduction in MFO in the phrenic discharge. In addition, we applied these methods to the hypoglossal nerve, as this is a prominent tool used for respiratory rhythm detection in in vitro (slice or en bloc) studies to monitor network state.
Materials & Methods

**ANIMAL PREPARATION**

**General Surgical Preparation**

Experiments were performed on 9 adult, male Sprague-Dawley rats (340-380 g). Spontaneously breathing animals were anesthetized with isoflurane vaporized in \( \text{O}_2 \) (MDS Matrix; 4-5% induction, 1-2% maintenance). A tracheotomy was performed via a ventral approach, and animals were intubated with an atraumatic glass tube, after which they were artificially ventilated (Columbus Apparatus, 60 cycles/min\(^{-1}\), 2.5 – 3.0 ml tidal volume) with the same gas mixture. The femoral artery and saphenous vein were then cannulated for measurement of arterial pressure and infusion of drugs, respectively. Arterial and tracheal cannula were connected to pressure transducers (CDXII, Argon Medical) for monitoring blood pressure (BP) and inflation pressure using conventional amplifiers (Gould Statham). Signals from three small subcutaneous electrodes were amplified and filtered (Neurolog, Digitimer LTD; Hertfordshire, England), and used to monitor EKG via an audio amplifier (AM10, Grass Instruments). The level of anesthesia was determined by the absence of flexor reflexes and changes in heart rate and blood pressure in response to pinches of the distal hind limbs. End-tidal \( \text{CO}_2 \) was maintained between 4.5 – 5.5 % (Capstar, CWE Inc.) by adjusting minute volume, and rectal temperature was maintained at 37.0 ± 0.4 \(^\circ\)C via a servo-controlled heating blanket coupled to a rectal thermometer (Harvard Apparatus), during all surgical procedures. Left and right phrenic (Ph) and the medial branches of the hypoglossal (XII) nerves were dissected free from surrounding tissues, transected, and desheathed. Both internal carotid arteries were tied off just below the pterygopalatine artery (43) to prevent bleeding following decerebration.

**Decerebration**
Following this initial surgical preparation, the rats were placed prone in a stereotaxic device. Using a variable-speed surgical drill (Foredom Electric Co.; Bethel, CT), the parietal bones were removed, the sagittal sinus venousus was tied off, and the neuraxis was gently transected using a microspatula at the rostral border of the superior colliculus. Brain tissue rostral to the transaction was removed by suction and bleeding was stopped by filling the empty skull space with small pieces of gelfoam (USP, Pharmacia) soaked with cold thrombin solution (Thrombin, Topical, USP, 500 units/ml, dissolved 0.9 % saline). 10 to 15 min after the decerebration, anesthesia was slowly withdrawn and the animals were paralyzed by intravenous bolus injection of 2 mg of vecuronium bromide (Abbott Labs), followed by continuous infusion (3 mg·kg⁻¹·h⁻¹). Nerve recordings were not initiated until at least 1 hour following decerebration, with mean blood pressures >90 mmHg. If necessary, animals were continuously infused with 5.0 % dextrose in 0.9 % saline (1.0 - 1.5 % body weight or 3.5 – 5.0 ml·h⁻¹) to maintain a mean BP of at least 100 mmHg.

**Recording**

In order to prevent peripheral influences on motor nerve outputs, all animals were baro- and chemoreceptor denervated via bilateral transection of the vagus (just below the nodose ganglion) and carotid sinus nerves. This ensured that responses to hypercapnia and hypoxia would be via central chemosensation (4). Bilateral pneumothorax was performed prior to recording to eliminate lung inflation-related movement artifacts, with a positive end expiratory load of 1.5 cm H₂O applied. With the rat in the supine position, the Ph nerves and the medial branches of the XII nerves were placed on bipolar silver electrodes and immersed in a mineral oil pool formed by skin flaps. Monophasic recordings (0.5 – 5,000 Hz; Neurolog, Digitimer) of efferent activity were obtained after crushing the peripheral end of each nerve between the
recording electrodes. The four neurograms, expiratory CO₂ level, arterial blood pressure, EKG and intratracheal pressure were all recorded onto the hard disk of a PC at sampling rate of 10,000 Hz/channel using a 16-bit analog-to digital converter system (ADInstruments; Colorado Springs, CO).

Gasp Induction

Asphyxia is the most common means of inducing progressive hypoxia/hypercapnia in laboratory animals (38) and was utilized in the present study. Asphyxia was produced by shutting off the ventilator, and responses occurred in three phases. The first response to asphyxia (Ph and XII nerve hyperactivity) was observed just after cessation of ventilation. Hyperpneic bursts with interburst intervals longer than control (i.e., eupnea) were omitted from analysis. The next response to asphyxia was complete silence of any nerve activity. Following this silent phase, gasps were observed (see Fig. 2). Asphyxia was induced after 15 minutes of control nerve recording during eupnea.

Data Analysis

Spectral Analysis

Ph and XII inspiratory and expiratory tags, indicating the start and the end of inspiratory activity (I-phases) for each nerve, were obtained from digitally integrated signals generated from the raw neurograms (τ = 50 ms). I- and E- tag demarcations were obtained using level discrimination and verified manually. All nerve signals were digitally filtered by a low pass (300 Hz) and high pass (10 Hz) filters with a stop band at 450 Hz (40 dB attenuation at 345 Hz). Carefully avoiding ground loops and eliminating electrical line noise obviated the need to use a 60 Hz notch filter.
Custom-written software, based on the Fast Fourier Transform (FFT) algorithm, was created in the Spike2 software environment (Cambridge Electronic Design Limited, UK). Script languages were developed in our laboratory for estimating “gated” (only epochs during inspiration) power spectra for all nerves. Each I-phase started at the onset of I-phase (I-tag) and ended at the offset of I-phase (E-tag). This procedure allowed us to analyze inspiratory data only.

To calculate the power spectra from the neurograms of the four different nerves, we used equal-duration epochs (FFT window) and FFT resolution. The FFT-window width was less than the longest mean XII inspiratory duration during eupnea (Table 1), which corresponded to 4096 data points (i.e., 409.6 ms, at a frequency resolution of 2.44 Hz/bin). All data were analyzed with a 50% overlap of the FFT-window. In cases when a 50% overlap extended past the end of the inspiratory burst, we used two windows: one starting at the beginning of the burst and one ending at the end of the burst. This prevented loss of spectral information from the part of nerve activity in excess of the FFT-window. Inspiratory bursts shorter than 409.6 ms were padded with zeros. Results from overlapping regions (FFT window positions) of the burst were averaged. No standard window filtering functions (e.g., Hanning or Hamming) were applied.

**Mechanical Artifact Suppression**

To exclude the influence of cardiovascular mechanical artifacts on the nerve recordings, we used ‘partial autospectral’ analysis (e.g., 27). Partial autospectral analysis assumes the presence of a ‘predictable part’ in the autospectra, AS\textsubscript{S1} and AS\textsubscript{S2}, of each of two signals, S\textsubscript{1} and S\textsubscript{2}, at each frequency \( f \). The ‘predictable part’ (containing the various component frequencies of the blood pressure waveform, including its natural harmonics) is estimated by:

\[
AS_{S1/S2}(f) = AS_{S1}(f)[1 - Coh_{S1-S2}(f)]. \quad (1)
\]
where $\text{Coh}_{S1:S2}(f)$ is the coherence between $S_1$ (nerve signal) and $S_2$ (blood pressure signal). During the gasping phase of asphyxia, BP routinely plummeted to nearly zero with virtually no pulse waves, but we still applied partial autospectral analysis to confirm that there were no cardiovascular-related mechanical influences on the nerve recordings.

**Time-Frequency Analysis: Sliding Zero-Interval Subtraction (ZIS) Method**

In order to address non-stationarities associated with changes in state, we developed time-frequency methods for power spectra estimation. Different software applications generate TFR using either a parametric FFT-based method (e.g., spectrogram.m by MatLab) or non-parametric methods (e.g., SPWVD). Given our application, the various time-frequency analysis programs that are currently available proved sub-optimal. First, the various SPWVD-based algorithms are very slow. Second, some methods (e.g., spectrogram.m) are not valid for computing low-frequency bands. Third, all methods yield results with varying degrees of band widening. Since we needed to analyze a large amount of data (derived from thousands of phrenic and hypoglossal inspiratory bursts), we required a fast, accurate method. To achieve this, we used an FFT-based Matlab function (pwelch.m) for estimating power spectra density, and then created a novel TFR algorithm, which we term the *Zero-Interval Subtraction* (ZIS) method. As depicted in Figure 1C, ZIS calculates the difference between spectrum $S_2$ (calculated from an epoch of data including a zeroed segment) and spectrum $S_1$ (calculated from the same epoch, but without a zeroed segment). The number of segments ($N$) depended on the length of the signal ($S$, data points), sampling rate ($SR$, Hz), on the desired low frequency limit for analysis ($f_{\text{low}}$, set to 10 Hz), and on a constant, $k$, that allows for scaling of the zeroed segment:
\[ N = \text{round}(k \times f_{\text{low}} \times S / (2 \times SR)) \]

The width of the zeroed sliding segment, which was kept approximately constant \((k = 10, \text{ resulting in 190-210 data points for the zeroed segment; varied due to rounding to nearest integer})\), allowed for accurate and consistent low-frequency estimates down to 10 Hz. The length of the segment can be derived by dividing \(S\) by \(N\). The zeroed segment was advanced in steps \(\frac{1}{2}\) of its width (i.e., 50% overlap). This enabled us to analyze adjacent, overlapping data segments. At each temporal position of the zeroed window, spectra \(S_1\) and \(S_2\) were calculated, and the appropriate subtraction performed. The “difference spectra” \((\Delta S)\) values were stored in 2-D matrices containing the values of the amplitudes at each frequency vs. temporal position. Since we slid the zeroed segment with 50% overlap, \(2N-1\) power spectral vectors (time points) per inspiratory burst were created in positions normalized relative to the inspiratory period. The individual vectors at the same time position were averaged over all the analyzed bursts, producing a \(2N-1 \times 164\) array (0-400.16 Hz at 2.44 Hz/bin frequency resolution). Thus, all ZIS data TFR is presented in normalized time axes ranging from burst onset (time = 0) to the end of the burst (time = 1), although we typically extended past this point in order to analyze post-inspiratory activity. These matrices were then graphed as standard isocontour plots, with smooth pseudocolored interpolation. TFR results were grouped and averaged according to state (eupnea, hyperpnea, and gasping) both within individual animals and across all animals.

**Evaluation of TFR Method**

We tested all available TFR-related software applications, including ZIS, using a 600 ms-long sinusoidal signal, created with 0.1 ms resolution and containing three pure frequencies: 10Hz (start to 200 ms), 10 and 80 Hz (200-600 ms) and 10, 80 and 160 Hz (400-600 ms) all with the equal amplitudes (set to 1.0 as default in arbitrary units, au), as shown in Figure 1A1. The
The results of the parametric FFT-base power spectral estimation are given in Figure 1A2 where the relative amplitude of each spectral peak reflects the running time for each frequency. The results of the ZIS TFR method are provided in Figure 1B1, and match well to the original signal components. In addition, ZIS works ~250 times faster (5.041 vs. 1260.25 s) than SPWVD [Time-Frequency Toolbox for MatLab developed by CNRS (France) and by Rice University, DSP Group, USA, http://gdr-isis.org/Applications/tftb/iutsn.univ-nantes.fr/auger/tftbftp.html] and 5.4 times faster (5.041 vs. 27.2214 s) than WaveMetrics’ (Inc., Lake Oswego, OR) “wigner” function. This slower performance is due to algorithm implementation. The reconstructed (i.e., averaging at each frequency in the time dimension) ZIS power spectral estimate is provided in Figure 1B2 (2.44 Hz/bin resolution). This result compares favorably with the FFT-based method (Fig. 1A2) in that the ZIS-derived peaks are narrower at the three frequencies of interest. Another very important advantage of the ZIS method (compared to SPWVD and wavelet analysis) is its ability to perform gated TFR on whole bursts of different durations. Our analysis of the ZIS algorithm performance shows excellent TFR resolution at zero segment lengths greater than 1/32 (128 points or 3.125 %) of the FFT window width (4098 data points). In order to keep time resolution constant, the number of zeroed segments varied depending on inspiratory burst length. For example, zeroed segments were between ~1/11th (gasps) and ~1/15th (hyperpnea) the length of the FFT window for Ph bursts. Expanding the zeroed segments increases the accuracy of low frequency estimates at the expense of temporal resolution. For this reason, the ZIS-method is more applicable at faster sampling rates (10 kHz in our case). The ZIS-produced results qualitatively similar to those produced by SPVWD methods (not shown).

The ratio of the amplitude spectra S1, S2 and ΔS are shown on Figure 1D. The relative ratio between the result spectra (ΔS) and the control spectra S1 supports the validity of the ZIS
method, since the ratios for all the subtracted frequencies are almost identical (Fig. 1E). This provides strong evidence of the linearity of the ZIS algorithm.

**Statistical Analysis**

The data were obtained from an ensemble of I-phases (n>20 for eupneic breathing and n>10 for hyperpnea and for gasping). In order to demonstrate consistent trend within a given state, the power spectra were averaged over of the number of breaths analyzed within that state for a given animal. In order to identify consistent global trends within the three states, the spectra were grouped into eupneic, hyperpneic, or gasping categories and averaged over all animals. Power spectra were reconstructed from the ZIS results and were compared qualitatively to FFT-calculated spectra. We also calculated the power spectrum during the expiratory phase of nerve activity, and used this as our “background” value. Data are presented as means ± SE, and a p value < 0.05 obtained from any statistical tests was interpreted as significant, as was a 95 % confidence level. To evaluate the statistical significance, the lower confidence value was computed for each frequency bin and was compared to the upper confidence level of background spectra. Depending on how well the data conformed to a normal distribution, either parametric (t-test) or non-parametric (Wilcoxon and Mann-Whitney U) tests were applied to compare two groups of data. For comparing among multiple results (e.g., eupnea vs. hyperpnea vs. gasping, in pairs; see Fig. 7), we used parametric (one-way and repeated-measure ANOVA) and non-parametric (Friedman and Kruskal-Wallis) tests. Only left nerve data were used to display individual spectra (Fig. 3). Bursts from both sides were used to create averaged TFR plots (Figs. 4 and 6).

Finally, the borders of frequency bands and their maximal peaks were identified using a custom-written optimization algorithm, running in Matlab. The kernel of this algorithm uses
simple differential equations for evaluating the maximum and minimum slope of the averaged spectra. The tan(\(\alpha\)) and cot(\(\alpha\)) were used to evaluate slopes, with thresholds set to \(\alpha = 1.2\) for rising slopes, and \(\alpha = 0.9\) for falling slopes.
Results

Bilateral recordings of Ph and XII (medial branch) electrical activity were made in nine peripheral baro- and chemo-denervated, precollicularly-decerebrated, paralyzed rats that were maintained under artificial ventilation without anesthesia. The results reported are based 928 eupneic, 386 hyperpneic, and 186 gasping bursts produced by nine rats. The general characteristics such as respiratory rate, burst amplitude and duration, length of hyperpnea, primary apnea and gasping are described in Table 1. Following cessation of ventilation, three prominent phases of anoxic response were observed in 7 animals: initial hyperpnea (H, Fig. 2), silent phase (S, Fig. 2) and gasps (G, Fig. 2). One animal did not produce any gasps, and the final one produced fewer than 10 gasps. In the remaining seven rats the above sequence of changes in nerve activity were noted, a typical example of which is shown in Figure 2 (expanded traces for details).

Changes in Phrenic Power Spectra from Eupnea to Gasping.

Power spectral analysis was used to characterize the changes in fast rhythms within the Ph activity during different behavioral states. We considered both parametric (FFT) and non-parametric (ZIS) analyses of Ph bursts in the frequency domain (see Methods). All statistics regarding maximal Ph spectral peaks and band ranges during eupnea, anoxic hyperpnea, and gasping are provided in Table 2. Collapsing the ZIS plots in the time dimension provides an average power over the entire breath (Fig. 3A1 – A3; Fig. 4A1-A3, dashed lines), and this result is superimposed on the original FFT-estimated autospectrum (Fig. 4A, thick lines; error bars not shown for clarity) for direct comparison between the two methods.

Eupnea. Figure 3A1 shows the Ph power spectra for individual animals during eupnea. From these overlaid spectra, two major features are consistent across animals: one peak each in
the MFO and HFO bands. Across animals, MFO was larger, but not statistically-significantly so. During eupnea the most prominent ZIS-reconstructed averaged power spectra peaks are located at the 78.1 and 175.8 Hz (see Table 2 for frequency bands). The lower band was categorized as MFO, and the higher band as HFO. Across all animals, the time-averaged ZIS results fit the FFT-estimated spectra well (Fig. 4A1, dashed vs. solid line), with the former displaying more distinct separation between the peaks. The ZIS TFR results, shown in Figure 4B1, indicate that the HFO band begins just after the onset of Ph activity, reaching maximal values by 1/5 of the way through the burst, and maintaining these high levels until ~2/3 of the way through the inspiratory cycle. MFO activity begins later, steadily increasing in intensity from 1/4 through 1/2 of the burst, and reaches sustained maximal values from 1/2-3/4 of the way through the cycle. Although MFO had greater maximum value than HFO, the difference in their amplitudes averaged over the whole cycle was not statistically significant. Taken together, these results demonstrate that the incrementing discharge pattern in the Ph neurogram during eupnea (Fig. 2, bottom panel, ‘E’) results from a dramatic increase in MFO overlaid upon a steady, early-starting and frequency-broadening HFO. In addition, it emphasizes the rather broad frequency power contributions. All frequencies above 40 Hz were significantly greater than expiratory control (Fig. 4A1, bottom plot).

In addition, the onset of inspiration was typically characterized by a very brief (i.e., single cycle) discharge, with duration corresponding to low frequencies (25-70 Hz; low MFO) that is evident in the Ph burst shown in Figure 4C1 (*). This brief feature is barely detected by the TFR analysis over all animals (Fig. 4B1), because it is short-lived and not present in every single eupneic breath. However, this feature is obvious under digital integration (Fig. 4C1, histogram).
**HYPERPNEA.** Figure 3A2 illustrates the variability in ZIS-derived power spectra between animals during hyperpnea, which display relative consistency in that all contain at least one MFO (usually “MFO2”), one HFO, and one upper-HFO peak (“UHFO”). The average power spectra (7 animals, 386 inspiratory bursts) estimated by both FFT- and ZIS-based methods (Figs. 4A2) reveal more complexity in their spectral structures than during eupnea, as does the TFR analysis (Fig. 4B2). This is in part due to inter-animal variability, but also to the presence of more peaks per spectra in individual animals (Figure 3A2) than during eupnea (Fig. 3A1). The lowest major frequency band (53.7 – 151.4 Hz) in the power spectrum was categorized as MFO with two peaks, at 85.5 and 122.1 Hz (see Table 2 for summary). When calculated using FFT-based analysis, only one MFO-related maximal peak was detected (Fig. 4A2, solid line). This FFT-estimated MFO peak was not significantly different in frequency than the single MFO peak during eupnea using either FFT or ZIS analysis. The HFO peak (187 Hz) is slightly (but not statistically significantly) larger than the MFO peak. Another conspicuous difference between the hyperpnea and eupnea states is the presence of a higher, less powerful band (224.6 – 283.2 Hz) detected by both methods, but more clearly shown by the ZIS reconstruction. This was classified as “upper HFO” (UHFO; see 32).

Unlike the case during incrementing eupnea, the ZIS analysis shows that the characteristic “bell-shaped” hyperpnea burst (Fig. 2, bottom panel, ‘H’) consists of a temporally complex combination of power at various frequencies. A lower MFO band (50-90 Hz, Fig. 4B2), otherwise hidden in time-averaged ZIS and FFT representations (Fig. 4A2 and 4C2, shading/*), dominates the earliest portion of the Ph activity. The “start-up” complex now consists of 2-3 cycles of low frequency oscillations, as labeled in the Ph neurogram (Figure 4C2, *). The power in this band is short-lived, mostly dissipating by ~1/5th of the way through the
burst. Beginning just after the start of the burst, a band of HFO (180-220 Hz) increases and sustains the activity through more than 2/3 of the burst length. The two prominent MFO bands make significant contributions starting ~1/3 of the way through the cycle and continuing through to the end, resulting in lengthening of the plateau of the “bell-shaped” activity pattern, and abruptly ending (Fig. 4B2).

**GASPING.** The most conspicuous changes in the power spectra of Ph activity occurred during gasping. Figure 3A3 demonstrates the qualitative consistency in the power spectra across all seven animals that produced >10 gasps. In particular, the majority of the power is contained within two bands (MFO1 and MFO2), although there is variability in the relative distribution between the two. When averaged across all animals, the FFT and ZIS-based analyses (Fig. 4A3) show that almost all the power was delivered via the bimodally-distributed MFO band, with a marked reduction or elimination of higher-MFO and HFO power. All power above 120 Hz, including the HFO-related band (161.7 – 219.3 Hz), was statistically indistinguishable from inspiratory background levels (Fig. 4A3, bottom plot, p-level). Again, the ZIS-reconstructed spectrum shows more pronounced separation of the peaks (41.5 and 87.9 Hz) than does the FFT-derived average power spectrum, indicating the benefits of a non-parametric analysis. The TFR highlights the temporal structure of the power carried by these two distinctive MFO sub-bands, with the higher-frequency, broader sub-band beginning later in the breath, and carrying more sustained power through the first half of the burst (Fig. 4B3). Thus, the rapidly-rising shape of the gasp (Fig. 2, bottom panel, ‘G’) is initiated by the even lower MFO band (compared to eupnea and hyperpnea), and this is evident in the “start-up” component of the neurogram (Fig. 4C3, *), which shows 2-4 low frequency oscillations in its activity. This is supplemented and eventually dominated by the higher-MFO band, and the majority of the power in both is
quenched by 55% of the burst, with a modest resurgence of the low-MFO band from 70-100% of the way through the burst (Fig. 4B3).

Both MFO and HFO activity is readily apparent in the raw data during eupnea and gasping, shown in Figure 5.

**Changes in Hypoglossal Power Spectra from Eupnea to Asphyxia.**

XII Power Spectra were quantified using the same methodological approach applied to Ph data. Figure 6 is organized in exactly the same manner as Figure 4B and 4C, except that we include a marker in the TFR results (black vertical lines, Fig. 6B1 and 6B2) indicating the onset of the phrenic burst. The end of XII burst coincided very closely to that of the Ph in all breaths analyzed.

**Eupnea.** Figure 3B1 shows the XII power spectra for individual animals during eupnea. These overlaid spectra demonstrate the consistency of the major features (multiple peaks at specific locations) across all animals. As shown on Figure 6A1 (solid line), FFT and ZIS-based averaged XII power spectra during eupnea were broad-band and pyramidal-shaped, with constituent frequencies ranging from 50 – 300 Hz. The maximal power spectral value is located at 187 Hz (Table 3). There are other smaller peaks with maxima at 148.5 and 229.5 Hz, respectively (see Tables 3 and 4). The time-averaged ZIS results (dashed line, Fig. 6A1) coincide well with the FFT estimates (solid line).

The results of TFR analysis reveal broad, medium and higher frequency components in the portions of the XII bursts preceding the onset of Ph activity (left of black vertical line in Fig. 6B1). These frequencies increase nearly uniformly as the burst proceeds from pre-Ph to Ph-related activity, with the majority of power contained in 150-250 Hz frequencies. There was one uniquely Ph-related band (120 – 140 Hz) in XII activity (Fig 6B1).
**HYPERPNEA.** Figure 3B2 illustrates the variability in ZIS-derived XII power spectra between animals during hyperpnea, which are relatively consistent in the low and medium frequency features and more variable in the high frequencies. Figure 6A2 illustrates the results of FFT-based (solid line) and ZIS-reconstructed (dashed line) XII analyses, demonstrating that, as with Ph activity, there is a marked increase in overall power (note y-axis scale in Fig. 6A1 vs. 6A2). The XII activity during hyperpnea is distributed in a bell-shaped manner. Under this condition, there are three major XII bands (each partially split between pre-Ph and Ph-related parts, Fig 6B2), with increasing peak magnitudes located at 92.3, 136.7, 185.5 Hz (see Tables 3 and 4).

TFR analysis reinforces the dramatic increase in pre-Ph duration of XII activity during hyperpnea (Figs. 2, bottom panel ‘H’, and 6B2). In addition to duration, two qualitative differences are evident when comparing the pre-Ph XII activity in hyperpnea with eupnea: (1) the appearance of an early component at 70-110 Hz; and (2) the progression of frequency recruitment from lower to higher frequencies as the pre-Ph burst advances in time. Together, these create the ramping and plateau-like pre-Ph portion of the XII burst. During Ph-related periods (i.e., to the right of the vertical line in Fig. 6B2), active bands continue to make contributions, and appear to quench in reverse order from recruitment, with progressively lower frequencies ceasing later (70-110 Hz band excepted).

**GASPING.** During gasping, the averaged XII power spectrum is also asymmetrical and broad-band, skewed even more towards lower frequencies (Fig. 6A3). Power in multiple frequency bands were detected, with the largest peak at 109.9 Hz and two smaller peaks at 158.7 Hz and 187.0 Hz. Figure 3B3 displays the XII power spectral patterns across all seven animals that produced gasps. Compared to the two other states, there is much more inter-animal
variability in magnitude of the power spectra contained within the two major bands (25-150 and 150-225 Hz).

During gasping, the XII shows no pre-Ph activity (Fig. 2, bottom panel, ‘G’), and the power appears scattered among various frequency bands (Fig. 6B3). Unlike the case of Ph activity during gasping (Fig. 4B3), at least some power is maintained in medium and higher frequency regions. Interestingly, it appears that activity in most frequency regimes is not stable throughout the entire burst, and there is a marked diminution in frequencies above 240 Hz, as compared to eupnea and hyperpnea.

**Comparative Analysis of States**

Figure 7 provides an analysis of the amplitudes of the frequencies during eupneic, hyperpneic, and gasping states. Pair-wise comparisons in Figure 7A1 show that discrete Ph MFO, HFO and UHFO bands increase significantly when comparing eupnea (solid line) to hyperpnea (dashed line; gray-colored p-values <0.05 in ‘E-H’), whereas there is widespread, significant decrease in power at all frequencies >50 Hz (E-G, H-G) during gasping (dotted line). Thus, during the initial phase of hypoxia/hypercapnia, there is exaggeration of eupneic-related bands (Fig. 7A1).

A similar pattern is illustrated in XII activity (Fig. 7A2). Unlike the band-selective Ph differences, initial responses to hypoxia/hypercapnia show significant increases in all frequency bands above 40 Hz that are present during eupnea (E-H) in XII discharge. Analogous to the Ph data, gasping (E-G) produces a selective loss at frequencies only >60 Hz (ZIS) in XII activity.

Figures 7B1 and B2 provides a comparison between pre-phrenic (pre-Ph; dashed) and phrenic-related (Ph-r; solid) spectral characteristics of XII activity during eupnea and hyperpnea, respectively. During eupnea (Fig. 7B1), the pre-Ph and Ph-r spectra of XII are nearly identical,
and mimic the shape of the entire nerve spectrum (Fig. 7A2). During hyperpnea (Fig. 7B2), the pre-Ph part of the XII spectrum (dashed line) is shifted to lower frequency values, in the form of increased power in the same bands as those present in the Ph-r portion (solid line). The p-value function (Fig. 7B1 and B2, bottom panel) shows many more significant differences between pre-Ph and Ph-r spectra in frequencies below 150 Hz than in those above this level. The results are summarized in Table 4.
DISCUSSION

Our overall findings were that: (1) oscillations are comprised of dynamically changing frequency bands in the unanesthetized “deafferented” adult rat model; (2) the data presented here clarify to a large degree the controversy regarding changes in oscillatory behavior during severe hypoxia/hypercapnia-evoked gasping; (3) an early, low-frequency MFO component emerges in response to progressive hypoxia/hypercapnia; and (4) a good deal of the controversy surrounding these issues is due to the various animal preparations, analytical methods, and definitions of gasping.

Clinical Relevance

Although a lower mammalian species with reduced neuraxis, our experimental model shows all four classic phases of the anoxic response: initial hyperpnea, primary apnea, gasping, and secondary (or terminal) apnea (Figure 2; 15, 19). These classic phases of respiratory response to hypoxia or anoxia have been described in clinical observations: “In response to asphyxia, there is an initial period of arousal and hyperpnea, then primary apnea lasting seconds or minutes, then a gasping stage. The gasps become progressively weaker and finally result in terminal apnea unless external support is provided. Gasping respirations are easily recognized as the presence of a rapid inspiratory rise accompanied by a retarded expiratory phase preceded and followed by a cessation of breathing movements.” (34, p. 167; see also ref. 37).

Principal Findings

Our data suggests that the HFO bands in both nerves (but particularly the Ph), are more sensitive to hypoxic/hypercapnic conditions than are MFO generating mechanisms, as the former are greatly exaggerated during hyperpnea and almost abolished during gasping. Hyperpnea produces significant increases in power at almost all frequencies in XII, and gasping reduces
power almost uniformly in all frequencies (Fig. 7). The mechanisms underlying these shifts remain unclear, and will require careful studies of neuronal behavior in respiratory networks in order to uncover.

**Phrenic Characterization.** Schmid et al. (44) described the changes in Ph MFO and HFO in response to CO₂ stimulation in (anesthetized or decerebrated) vagotomized rabbits, in which they found that HFO power steadily rose with increasing respiratory drive, and our results in the rat agree well with their finding. In contrast to HFO, characterized by a spectral peak of relatively narrow bandwidth, the MFO spectrum usually consisted of a broad complex, in some cases composed of two distinct peaks (i.e. MFO was heterogeneous), and the low- and high-frequency sub-bands of the MFO complex were related predominantly to the first and last third of inspiration, respectively (44). Our data showed similar results during hyperpnea (Figs. 4A2, 4B2). The high sensitivity of Ph HFO power to chemoreceptor stimulation has been noted repeatedly in other species (see 17 for review), and is consistent with our findings. It is possible that Ph MFO observed during gasping results from reconfiguration of central networks, and the initial hyperpnea may provide an early or transitional glimpse of this reconfiguration. Our results demonstrate that this occurs in the absence of peripheral cardiorespiratory sensory inputs.

Another unique finding of this study, revealed by ZIS TFR analysis, included the presence of an early, short-lasting, MFO burst under all states examined (Fig. 4), similar to that shown by Cohen et al. (10) in cats and by Marchenko et al. (29) in rats. The power and duration of this early “start-up” component (Fig. 4C1-C3) grows steadily in comparison to other frequency components as anoxia progresses (Fig. 4B1-B3). It is worth noting that this feature would be masked in a parametric analysis, but is clear in the non-parametric TFR analysis. It is not clear if the same mechanism that produces MFO during this “start-up” period also produces
MFO later in the burst. During eupnea and hyperpnea, it exists at lower frequencies than any others present during the rest of the inspiratory cycle, but during gasping it represents one of the dominant frequencies in the burst. This finding suggests that the start-up components, and the lower MFOs in general, may share a common mechanism (in all states) that is activated with increasing vigor as hypoxia/hypercapnia becomes more severe.

Our general finding is that Ph power is dynamically distributed over the course of inspiration, but the details of these dynamics and their constituent frequencies differ from other published accounts. For example, our ZIS TFR results are in stark discord with SPWVD-based TFR performed on diaphragmatic EMG records reported by O’Neal et al. (32) in anesthetized mice. These investigators reported the absence of any significant power (at any frequency) during the entire first half of eupneic inspiration. Unfortunately, O’Neal et al. (32) did not report a definitive data sampling rate, so their SPWVD window of 360 data points represents an unspecified time window. This lack of specificity translates into an unknown fraction of the inspiratory period analyzed. Thus, the TFR data shown in their figures, normalized between 0 and 1, end at some unknown point into the respiratory cycle, perhaps cutting off the latter portions of inspiration, and this may account for some discrepancies between our results and theirs. By contrast, we analyzed (and represented in our ZIS TFR plots) entire bursts, regardless of their length. Moreover, O’Neal et al. (32) used biphasic recordings in anesthetized mice, and both factors may be sources of differences between our results and theirs. Biphasic recording methods are deemed inappropriate for spectral analysis (9, 39), which is why we utilized monophasic recording techniques for both Ph and XII. On the other hand, our results support the general findings of early HFO and late MFO predominance in decerebrate animals, described in cats by Christakos et al. (7, 8), and in (a subset of) the rats described in Marchenko et al. (29).
HYPOGLOSSAL CHARACTERIZATION. To our knowledge this is the first study to examine the frequency components of the activity of the medial branch of XII in decerebrate, unanesthetized, peripherally-denervated rats *in vivo*. In addition, this is the only study to analyze time-frequency dynamics of XII activity in any rat preparation, with particular reference to the onset of Ph activity. Our results demonstrate that during eupneic breathing the pre-Ph and Ph-related activity are composed of different combinations of frequencies, with a dramatic power increase in the 130-160 Hz band during the beginning of the Ph-related epochs (Figure 6B1) compared to any time during the pre-Ph epochs. It is important to note that this band-specific feature is lost when one averages over the entire pre-Ph and Ph-related epochs because the power in this band is relatively modest, but constant, during pre-Ph, and has both dramatic increases and decreases during the Ph-r activity (Fig. 6B1). Furthermore, under hyperpneic conditions, there is a shift in temporal pattern of the 180-210 Hz band, particularly with regard to the timing of the peak (Figs. 6B1 vs. 6B2). In addition, during hyperpnea, the 130-160 Hz band shows increased relative discharge during pre-Ph activation, as compared to eupnea. This may indicate recruitment of additional pools of pre-motor neurons that are activated during hyperpnea, or may be a reflection of the prominent depolarizing effects of hypoxia on XII motor neurons (20, 36). Interestingly, almost all major XII frequency bands during hyperpnea (especially ~ 120-160 Hz) are temporally split between pre-Ph and Ph-related parts of the burst, a finding that supports the possibility of two independent mechanisms producing oscillations during different periods of the burst (42, 52).

The only other study to perform TFR analysis on XII activity was done by O’Neal et al. (32) in the anesthetized mouse (no particular XII branch was identified). These investigators reported very little power during early inspiration in XII activity. This result differs dramatically
from our findings, but does not belie some of the raw nerve records presented (e.g., figure 7 in that study), which appear qualitatively different from those we recorded. Although this may be a species difference, it is possibly due to other factors, including the course frequency resolution used (7.8 Hz per bin), or other factors in animal conditions (e.g., anesthesia).

**Anoxic Responses in Other Models**

There are considerable difficulties in comparing our data with others that address gasping mechanisms. Unfortunately, many papers do not show continuous Ph recordings (Fig. 2) during control periods through gasping during hypoxic, anoxic, ischemic or asphyxic tests in decerebrate, perfused juvenile rats (e.g., 33, 53), anesthetized cats (2, 38, 46), or decerebrate cats (54). Since only selected epochs of records were shown, it is difficult to compare their results with ours because it is not clear what specific gasping characteristics were present. Under normal anesthesia, initial hyperpnea is followed by depression if the hypoxia becomes severe, but this response can rarely be demonstrated in deeply anesthetized animals at any level of hypoxia (31). Subsequent recovery of eupnic Ph pattern is much more successful during reoxygenation before the gasping phase (31). Therefore, it is possible that late hyperpneic bursts have been reported as gasping (Figure 3 of ref. 33; Figure 1 of ref. 53), even though primary apnea never occurred. Without (a description of) the entire experimental record, it is very difficult to evaluate these behaviors definitively.

Using α-chloralose anesthetized cats, Solomon (46) did describe the full time course of the response to hypoxia (94% N₂, 6% O₂), and it consisted of primary apnea, gasps, and secondary apnea, without any initial hyperpnea. Richter et al. (41) ventilated nembutal-anesthetized cats with 93% N₂-7%O₂ for 5 minutes to produce hypoxic responses. In contrast to Solomon (46), they reported only an initial hyperpnea and primary apnea with no gasps.
Therefore, the anesthetic agent may profoundly influence the responses to hypoxia in the same species. This is likely due to the varying effects on neurotransmitter systems that are affected by commonly-used anesthetics (12, 41, 48).

A controversy exists regarding the Ph spectral characteristics during gasping. In a recent review, St. John (49) postulated that one distinction between eupnea and gasping is a shift in HFO to higher frequencies (39, 54). By contrast, Akay et al. (2) reported that Ph HFO power was diminished relative to MFO, with a shift in both towards lower frequencies during gasping in response to progressive hypoxia. Our data are in general accord with the latter view, and we observed that Ph HFO is all but eliminated during gasping (Figs. 4A3 & B3, and 7A). We note that in the case of the study by Tomori et al. (54), these differences may arise from species differences (cats vs. rats), or from differences in the preparation (sino-aortic intact vs. denervated). The integrated Ph activity presented as prototypical (Figure 1 of ref 54) appears more akin to our hyperpnea response in that it is bell-shaped rather than decrementing (Fig. 2). On the other hand, the burst is shorter in duration than during eupnea, and there is no pre-Phrenic portion of XII activity, both of which characterize our gasping behavior (Fig. 2). Our data suggests that the most prominent feature of gasping is the systemic shift in Ph power to lower frequencies, which directly contradicts the characterization made by St. John (49). This difference is probably due to differences in gasping models.

Concerning these differences, the most interesting and important data regarding the evolution of gasping during anoxia were obtained from freely behaving rats at postnatal day 2, 5, 10 and 15 (e.g., 15, 18). Respiratory responses to anoxia through all of these ages have prototypical patterns, which begin with initial hyperpnea followed by primary apnea. After primary apnea, three stages of gasps (stage I, II and III) were observed, separated by apneas.
The first two stages are characterized by the existence of an extended decrementing ramp (“tail”) that follows the initial rapid rise in Ph activity. Stage III has only a rapidly rising peak without any ramp-like tail. All these stages are shown in Fig. 5 of Fewell et al. (15). Our results resemble stage III gasping, where only the initial “start-up” portion of the Ph discharge was observed with a very brief (if any) decrementing tail.

Taking into account the results obtained from developing rats, from adult anesthetized and decerebrate cats, and from the present data, we summarize the Ph response to anoxia or severe hypoxia in Figure 8. All gasping stages have a very fast and high amplitude increase in Ph activity (Fig. 8, shadowed). Our data demonstrates the “G” pattern without a significant decrementing ramp tail (“dr” in Fig. 8). We suggest that the gasping described herein reflects the core or kernel of oscillatory behavior of the respiratory rhythm generator in this state, and is essentially one of pure MFO.

In contrast to our results, all studies (see above) performed on in vivo anesthetized or decerebrate cats, or in situ artificially perfused decerebrate juvenile rats, reported Ph gasping discharges containing a considerable decrementing tail-like ramp after the initial increase in amplitude (‘G+dr’ in figure 8). This may account for the differences in results of spectral analyses, where the ramp is produced by the brain-stem HFO-generating system while the initial discharge (“start-up”) is produced by an MFO-generating circuit. The gasps in the present study show virtually no Ph HFO during gasping, and have no decrementing tail in the Ph activity, while other studies that produced a decrementing tail do contain HFO. For example, the preferentially-increasing MFO band observed in anesthetized cats during gasping elicited by severe hypoxia (2) could be due to the relative absence of a significant decrementing tail. Considering the studies conducted in freely behaving rats (15, 18), our results suggest that the
principal difference between eupnea and gasping is that during eupnea the incrementing ramp is responsible for smooth lung expansion, whereas during gasping the “start-up” discharge is responsible for rapid lung expansion and the tail-like decrementing ramp (if present) is responsible for maintaining lung inflation, thereby prolonging the time for gas exchange.

**Consideration of Analytical Methods**

In addition to different experimental models, differences in data analysis methods can profoundly affect results. The study most relevant to the present one is that of Marchenko et al. (29), performed in decerebrate rats. These investigators estimated HFO power (via parametric FFT analysis) at a lower frequency (106-160 Hz, mean 132 Hz) during eupnea than our present results. One reason for this discrepancy can be attributed to the use of “bin combining/summing” (8), which results in an artificial reduction in frequency. In addition, they reported a higher variability with regard to which half of inspiration (first vs. second) contained which bands (MFO vs. HFO). In some animals, their results were very similar to ours, while in others they were not. This may have reflected variability in the condition of the animals, particularly with regard to the method of decerebration.

Regardless of method, another source of variability resides in our assumption that the system is stationary (within a given state), particularly during anoxia. We grouped bursts that fit our general shape criteria together, but it is likely that the system changes between, for example, the first and last hyperpnoic bursts. These concerns can only be addressed by more detailed analyses than we offer here.

**Neural System Implications**

The available data from anesthetized rats suggests that HFO is not a feature of individual rat phrenic motor neurons. Kong & Berger (25) recorded from individual Ph motor neuron fibers,
but rarely observed interspike intervals <10 ms (i.e., 100 Hz), even during hypercapnic conditions. Hayashi & Fukuda (21) reported that the highest Ph motor neuron firing rates in anesthetized rats were 55 ± 14 Hz, providing little basis to claim that some neurons may fire at 180 Hz. One study, performed in anesthetized cats (8) did seem to show that HFO (65 Hz) was present in the autospectrum of individual cat Ph motor neuron firing (figure 3 of that paper). However, interspike interval analysis of the same unit revealed a peak only at ~33 ms (figure 4 of that paper), corresponding to ~30 Hz (i.e., MFO), and almost no intervals at or below the 15.4 ms required for 65 Hz HFO oscillations. Other cat studies (e.g., 30, 50) also failed to demonstrate HFO in Ph motor neuron discharges. These studies suggest that HFO is a population-based phenomenon, rather than a property of individual Ph motor neurons. Nonetheless, the lack of data derived from unanesthetized decerebrate rats demands that we reserve judgment regarding the ability of individual motor neurons/units to produce HFO. It is likely that MFO produced later in the burst results from increased recruitment and firing rate of individual motor neurons, whereas early HFO is due to activity of multiple neurons with lower individual rates but constant phase shifts.

**Neuromuscular Implications**

Fast respiratory rhythmic output in the early phase of Ph discharge may promote efficiency in muscle contraction (17). Thus, HFO, at least in activation of diaphragm motor units, may create a “catchlike effect” described by Lunteren and Sunkey (28). These authors used rat diaphragm muscle strips, stimulating them with 2-4 shocks at 100-200 Hz “bursts” at the onset of 10 to 50 Hz subtetanic trains. Their results revealed that a high-frequency burst of pulses at the onset of a subtetanic train of stimulation promotes the diaphragm to hold its contractile force at a higher level than expected from the subtetanic trains alone, because of the catch-like property of
muscle. This property has been well documented in other skeletal muscles (6), and plays an important role in prevention of muscle fatigue in humans (5). Our general finding of the domination of HFO during the early portion, and MFO during the latter portion, of Ph discharge (Fig. 4B1) supports the findings of studies in rats (29) and cats (7, 8) and may represent the underlying neural mechanism of this phenomenon in vivo.

**Conclusions**

In this study, we characterized the dynamic spectral components contained within the bursts of Ph and XII activity under three different conditions. Our primary conclusion is that, compared to eupnea, there exists a clear shift in power distribution towards lower frequencies in during gasping. This may reflect the activation of a “core” gasping system that may or may not be a subset of the central pattern and rhythm generator within the CNS. When pushed into this state, it appears that there is some conservation of effort in attempts to recover from (what would be in the natural state) ventilatory insufficiency or severe hypoxia. Alternatively, we may view this behavioral output as the remnant of the original system that is less susceptible to anoxic shock or acute metabolic stress.

Having defined the changes in power spectra in the XII and Ph activity under three conditions, it remains unclear as to how these are synchronized or coordinated among the four motor nerves considered. Examining the coordination and potential shared oscillatory drive can be achieved by analyzing dynamic coherence levels between the nerves during the bursts. In another study, we will present novel TFR analysis of Ph-Ph, XII-XII, and Ph-XII coherence, thereby providing a dynamic representation of coherence during inspiration under the same three behavioral states examined herein.

**Grants**
This work was supported by a grant (RFR) from the National Institutes of Health, R01 HL68143.
References


40. Richardson CA and Mitchell RA. Power spectral analysis of inspiratory nerve activity in the

41. Richter DW, Schmidt-Garcon P, Pierrefiche O, Bischoff AM and Lalley PM.
Neurotransmitters and neuromodulators controlling the hypoxic respiratory response in

42. Saito Y, Ezure K and Tanaka I. Difference between hypoglossal and phrenic activities

43. Sapru HN and Kreiger AJ. Procedure for the decerebration of the rat. *Brain Res Bull* 3: 675-

44. Schmid K, Böhmer G and Weichel T. Concurrent fast and slow synchronized efferent

45. Smith A and Denny M. High-frequency oscillations as indicators of neural control
mechanisms in human respiration, mastication, and speech. *J Neurophysiol* 63: 745-758,
1990.

46. Solomon IC. Modulation of gasp frequency by activation of pre-Botzinger complex in vivo. *J

47. Solomon IC, Chon KH and Rodriguez MN. Blockade of brain stem gap junctions increases
phrenic burst frequency and reduces phrenic burst synchronization in adult rat. *J

48. Soto-Arape I, Burton MD and Kazemi H. Central amino acid neurotransmitters and the

49. St.-John WM. Medullary regions for neurogenesis of gasping: noeud vital or noeuds vitaux? *J


Figures

**Figure 1.** Zero-interval subtraction (ZIS) algorithm. **A1:** generated sine-wave signal (0.1 ms resolution) with 3 different frequencies (10 + 80 + 160 Hz). **A2:** power spectrum of test signal (A1) calculated by standard MATLAB pwelch.m function. **B1:** time-frequency representation calculated by ZIS method for test signal in A. **B2:** time-averaged ZIS estimate of power spectral reconstruction, calculated from TFR result in B1. **C:** Graphical depiction of ZIS algorithm (not to scale), with zeroed sliding segment, which is moved in 50% overlapping steps along the analyzed epoch (‘FFT window’). See Methods for details. **D:** superimposed amplitudes of reconstructed spectra S1, S2 (shaded), and ΔS. **E:** relative ratio between result spectra ΔS and control spectra S1.
Figure 2. Archetypical respiratory motor responses to asphyxia. **Upper 7 traces, from top:** BP, blood pressure (mmHg); ITP, intra-tracheal pressure (mmHg); RR, respiratory rate; Ph, phrenic nerve (mV); XII, hypoglossal nerve (mV); $\int$, integrated nerve activity (time constant = 50 ms). **Expanded traces:** nerve activity during eupnea (E), hyperpnea (H), and gasping (G) bursts. **Bottom:** across-animal averaged and normalized integrated Ph (black) and XII (gray) activity in the three states examined.
Figure 3. Superimposed averaged ZIS-reconstructed Phrenic and XII power spectra during 3 different states. **Column A**: Ph, **Column B**: XII. Power spectra are provided for eupnea (row 1, 9 animals, 928 breaths), anoxic hyperpnea (row 2, 7 animals, 386 breaths) and gasping (row 3, 7 animals, 186 breaths), respectively. Only spectra derived from left nerves are shown for clarity. TFR estimates (Figs. 4 and 5) use all nerve data (left and right).
Figure 4. Averaged Ph power spectra during 3 different states, estimated by FTT and ZIS methods. A1-A3: averaged power spectra for eupnea (A1), anoxic hyperpnea (A2) and gasping (A3) estimated by FFT (solid line, left y-axis, V²) and ZIS time-averaged power spectrum reconstruction (dashed line, right y-axis). Dashed gray line at the bottom of each graph shows the 95% confidence level during background (expiratory) activity. Lower plot shows bin-by-bin p-values (p = 0.05, dashed line) between spectra and expiratory activity. B1 – B3: across-animal averaged and smoothed isocontour plot of TFR, estimated by ZIS, for eupnea (B1), anoxic hyperpnea (B2) and gasping (B3). Time duration is normalized to breath lengths within each category. Color bar in B1-B3 and right y-axis in A1-A3 in arbitrary units (au). C1-C3: Examples of single Ph bursts during eupnea, hyperpnea and gasping, respectively, highlighting early MFO “start-up” component (*) and complexes (marked above trace). Traces include discrete digital integration (histogram), leaky integrator (τ = 50 ms; smooth line), and raw neurogram (bottom). Shading and vertical lines in column A: frequency bands and peaks estimated from time-averaged ZIS.
Figure 5. Oscillatory behavior in the raw phrenic burst. Near-coincident spikes produce phrenic MFO and HFO. **A:** one eupneic burst. Boxed regions are shown in greater detail in B and C. **B:** expanded view of region near the beginning of the burst, showing dominant HFO rhythms, likely produced by modest synchronization of multiple units. Arrows indicate major wave troughs in smoothed trace (thick line, Savitsky-Golay smoothing, 2.5 ms, 5th order polynomial) with HFO-related periods. **C:** expanded view of epoch in latter portion of burst. Arrows denote troughs in longer complex waves related to MFO. Thin lines in B and C are raw data. **D:** example of a typical gasping Ph burst, with details of boxed region shown in E. **E:** original data (thin line) and smoothed trace (thick line, smoothing as in B and C) clearly indicate ~75 Hz (~13 ms) MFO rhythmicity. Y-axis units in all panels is µV; x-axis units in all panels are ms.
Figure 6. Averaged XII power spectra during 3 different states, estimated by FTT and ZIS methods. A1-A3 and B1-B3 as in Fig. 4. Vertical line in B1 and B2 indicates approximate onset of Ph activity.
Figure 7. Paired-state comparisons of Ph and XII power spectra. **A1 & A2:** Ph and total XII data ZIS-reconstructed power, respectively. **Solid, dashed, and dotted lines:** eupneic, hyperpneic, and gasping states, respectively. **Lower plots:** bin-by-bin pairwise comparison p-values (one-way ANOVA) for eupnea vs. hyperpnea (E-H), eupnea vs. gasping (E-G) and hyperpnea vs. gasping (H-G). **B1 & B2:** pre-phrenic (dashed) and phrenic-related (solid) XII power, during eupnea (B1) and hyperpnea (B2), respectively. **Lower plots:** bin-by-bin pairwise comparison p-values (one-way ANOVA) for pre-Ph vs. Ph-related XII power. **Gray vs. unshaded p-values <0.05:** statistically significant increases or decreases in power, respectively, when comparing the second in the pair to the first. Y-labels apply to all traces.
Figure 8. Final scheme of integrated phrenic nerve activity changes from eupnea (E, with an incrementing ramp, ir) to terminal apnea (2t). Hypoxia/hypercapnia produces hyperpnoic breathing (H), characterized by a bell-shaped ramp (br) in phrenic discharge. This is followed by primary apnea (1t). Gasping follows primary apnea and may take on various forms, depending upon the preparation. In anesthetized adults or in unanesthetized neonates, gasping (rapid onset, G) will include a significant decrementing ramp (+dr). In adult decerebrate and unanesthetized preparations, gasping proceeds directly to a form in which there little or no decrementing ramp (-dr). See text for details.
Tables

### Table 1
Differences in respiratory parameters of phrenic and hypoglossal (XII) nerves during eupnea and hypoxia/hypercapnia.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Eupnea</th>
<th>Hyperpnea</th>
<th>Silent Phase</th>
<th>Gasping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phrenic, msec</td>
<td>263 ± 22</td>
<td>288 ± 31</td>
<td>N/A</td>
<td>224 ± 28</td>
</tr>
<tr>
<td>XII, sec</td>
<td>581 ± 57</td>
<td>874 ± 94</td>
<td>N/A</td>
<td>233 ± 32</td>
</tr>
<tr>
<td>XII-pre-Phrenic, msec</td>
<td>279 ± 26</td>
<td>581 ± 43</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>RR, cycles*min⁻¹</td>
<td>59.15 ± 0.03</td>
<td>71.16 ± 5.11</td>
<td>N/A</td>
<td>48.69 ± 7.26</td>
</tr>
<tr>
<td>Anoxic Phases, sec</td>
<td>N/A</td>
<td>81.83 ± 11.62</td>
<td>55.25 ± 9.74</td>
<td>43.68 ± 18.13</td>
</tr>
<tr>
<td>( \dot{\text{X}}} \text{Phrenic, %}</td>
<td>Control</td>
<td>+ 20.34 ± 2.54</td>
<td>N/A</td>
<td>- 16.86 ± 1.81</td>
</tr>
<tr>
<td>( \dot{\text{X}}} \text{II, %}</td>
<td>Control</td>
<td>+ 19.52 ± 2.81</td>
<td>N/A</td>
<td>- 34.35 ± 2.55</td>
</tr>
</tbody>
</table>

**Phrenic, XII-pre-Phrenic and XII:** averaged duration of inspiratory discharge for phrenic, pre-Phrenic hypoglossal burst, and entire hypoglossal burst.

**RR:** respiratory rate. **Anoxic Phases:** duration of different anoxic phases (Hyperpnea, Silent Phase and Gasping). \( \dot{\text{X}} \) - changes in amplitude of averaged integrated nerve activity (percent increase, ‘+’ and decrease, ‘-’) compared to control.

### Table 2
Average ZIS-reconstructed phrenic power spectral peaks under three conditions.

<table>
<thead>
<tr>
<th>Bands</th>
<th>MFO</th>
<th>HFO</th>
<th>UHFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal Peak, Hz</td>
<td>E</td>
<td>H</td>
<td>G</td>
</tr>
<tr>
<td>78.1</td>
<td>85.5</td>
<td>122.1</td>
<td>41.5</td>
</tr>
<tr>
<td>Amplitude, au x 10⁻¹</td>
<td>7.97 ± 0.81</td>
<td>10.56 ± 1.15</td>
<td>9.34 ± 1.23</td>
</tr>
<tr>
<td>Band Range, Hz</td>
<td>36.6 - 109.7</td>
<td>53.7 - 102.5</td>
<td>109.9 - 151.4</td>
</tr>
</tbody>
</table>

**Abbreviations:** E – eupnea, H – anoxic hyperpnea, G – gasping; au – arbitrary units (Mean ± SE).
Table 3. Distribution of averaged ZIS-reconstructed XII power spectra peaks under three different conditions. Abbreviations as in Table 2.

![Table 3](image)

Table 4. Distribution of time-averaged ZIS-reconstructed XII power spectra peaks during different phases of inspiration. Abbreviations as in Table 2. **Additional Abbreviations:** pre-Ph_P, Ph-r_P – pre-phrenic and phrenic-related maximal peaks mean (Hz); pre-Ph_A, Ph-r_A – pre-phrenic and phrenic-related amplitude of maximal peaks in arbitrary units (Mean ± SE x 10^-1); pre-Ph_B, Ph-r_B – pre-phrenic and phrenic-related band width range (Hz).