Time-Frequency Representation of Inspiratory Motor Output in Anesthetized C57BL/6 Mice In Vivo

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O’Neal, Marvin H., III, Evan T. Spiegel, Ki H. Chon, and Irene C. Solomon. Time-frequency representation of inspiratory motor output in anesthetized C57BL/6 mice in vivo. J Neurophysiol 93: 1762–1775, 2005. First published October 20, 2004; doi:10.1152/jn.00646.2004. Inspiratory motor discharges, in addition to long-time-scale rhythmic oscillatory bursting, exhibit short-time-scale rhythmic oscillations that have been identified, and subsequently characterized, using power spectral analyses [predominantly fast-Fourier transforms (FFT)]. These analyses assume that the signal being analyzed is stationary; however, this is not the case for most biological signals, which exhibit varying degrees of nonstationarity. To overcome this limitation, time-frequency methods, which provide not only the frequency content but also information regarding the timing of these fast rhythmic oscillations (i.e., dynamics of spectral activity), should be used. Thus this study was performed to investigate the dynamic or time-varying features of spectral activity in inspiratory motor output. Both conventional time-invariant and time-frequency (time-varying) spectral analysis methods were performed on recordings of diaphragm EMG, phrenic nerve, and hypoglossal nerve discharges obtained from spontaneously breathing urethane-anesthetized adult C57BL/6 mice. Conventional time-invariant spectral analysis using a FFT algorithm revealed three dominant peaks in the power spectrum, which were located at 1) 20–46, 2) 83–149, and 3) 177–227 Hz. Time-frequency spectral analysis using a generalized time-frequency representation (TFR) with the smoothed pseudo-Wigner-Ville distribution (SPWD) kernel confirmed the general location of these spectral peaks, identified additional spectral peaks within the frequency ranges described above, and revealed a time-dependent expression of spectral activity within the inspiratory burst for each of the frequency ranges. Furthermore, this method revealed that 1) little or no spectral activity occurs during the initial portion of the inspiratory burst in any of the frequency ranges identified, 2) transient oscillations in the magnitude of spectral power exist where spectral activity occurs, and 3) total spectral power exhibits an augmenting pattern over the course of the inspiratory burst. These data, which provide the first description of spectral content in inspiratory motor discharges in adult mice, show that both time-invariant and time-varying spectral analysis methods are capable of identifying short-time-scale rhythmic oscillations in inspiratory motor discharge (as expected); however, the dynamic (i.e., timing) features of this oscillatory activity can only be obtained using the time-frequency method. We suggest that time-frequency methods, such as the SPWD, should be used in future studies examining short-time-scale (fast) rhythmic oscillations in inspiratory motor discharges, because additional insight into the neural control mechanisms that participate in inspiratory-phase neuronal and motoneuronal synchronization may be obtained.

I N T R O D U C T I O N

Fast oscillatory neuronal activity is a prominent feature in many regions of the CNS (e.g., cortex, thalamus, hippocampus, brain stem) (Bragin et al. 1999; Buzsáki et al. 1992; Christakos et al. 1988; Gray 1994; Gray et al. 1989; Llinás 1990; Llinás et al. 1991; Timofeev and Steriade 1997; Traub et al. 1996; Ylinen et al. 1995), including CNS areas associated with motor control systems (Brown 2000; Christakos et al. 1988; Conway et al. 1995; Donoghue et al. 1998; Murthy and Fetz 1992; Salenius 1996, 1997). In the respiratory neural control system, it has long been recognized that fast oscillatory rhythms are present in inspiratory-related muscles, nerves, and neurons (recently reviewed by Funk and Parkis 2002) and that these fast rhythmic oscillations provide an index of inspiratory-phase short-time-scale synchronization (Cohen et al. 1987b; Dittler and Garten 1912; Gasser 1928; Sica et al. 1991). Initial investigations of these fast oscillatory rhythms used visual observation or auto- and cross-correlation techniques to identify fast oscillations in inspiratory-related discharges in the range of 50–100 Hz (e.g., Cohen 1973; Dittler and Garten 1912; Gasser 1928; Mitchell and Herbert 1974); however, more recent studies have used power spectral analyses [using predominantly fast-Fourier transform (FFT) algorithms] that have revealed spectral peaks in two ranges: 20–50 Hz (Cohen et al. 1987b; Richardson and Mitchell 1982), designated as medium frequency oscillations (MFO; Cohen et al. 1987b), and 50–150 Hz, designated as high-frequency oscillations (HFO; Cohen et al. 1987b).

Although prominent oscillations in inspiratory-related discharges have been identified in all mammals studied thus far, only a limited number of investigations have focused on adult rodents (Kocsis and Gyimesi-Pelczer 1997; Marchenko et al. 2002). These investigations have revealed fast oscillations in inspiratory nerve activities in both anesthetized and decerebrate rats in vivo, with the power spectrum most often characterized by a single spectral peak in either the MFO or HFO range (dual spectral peaks were rarely encountered). Furthermore, the peak frequencies associated with HFO activity were reported to be slightly higher in adult rats than those previously observed in other mammals; based on these recent observa-
tions, the upper limit of the original HFO range has been extended to ~160 Hz (Kocsis and Gyimesi-Pelczer 1997; Marchenko et al. 2002). Because the mouse is rapidly becoming a valuable model for studying the contribution of genetic factors in respiratory control (see recent review by Tankersley 2003), and to our knowledge, spectral composition of inspiratory-related activity in adult mouse has not yet been evaluated, we conducted this study, in part, to investigate the presence and location of fast oscillatory rhythms in inspiratory motor discharges in adult mouse in vivo.

As noted above, power spectral analyses using FFT algorithms are most commonly used to identify the fast oscillatory rhythms observed in inspiratory-related motor outputs. These conventional spectral analysis methods assume that the signal being analyzed is stationary (i.e., the fast oscillatory components do not change with time), which is not the case for most biological signals, which exhibit varying degrees of nonstationarity. In fact, some studies have used power spectral analyses (using FFT algorithms) to compare spectral content of early versus late segments of the inspiratory burst (e.g., 1st half vs. 2nd half) (Bruce 1986, 1988; Christakos et al. 1989, 1991; Cohen et al. 1987b; Marchenko et al. 2002; Schmid et al. 1990; Webber 1989) or have stepped through the inspiratory burst with a 250-ms window in small steps (e.g., 1/16 s) (Richardson 1988; Richardson and Mitchell 1982) in an attempt to show that spectral content may change during inspiration. Although these studies have revealed changes in power and/or frequency of spectral peaks in the MFO and/or HFO ranges during inspiration, the segmenting procedures used may have been inadequate to produce data segments exhibiting stationarity, which is the underlying assumption for conventional spectral analyses. To overcome this limitation (i.e., stationarity), time-frequency methods, which provide not only the frequency components do not change with time), which is not the case for most biological signals, which exhibit varying degrees of nonstationarity. In fact, some studies have used power spectral analyses (using FFT algorithms) to compare spectral content of early versus late segments of the inspiratory burst (e.g., 1st half vs. 2nd half) (Bruce 1986, 1988; Christakos et al. 1989, 1991; Cohen et al. 1987b; Marchenko et al. 2002; Schmid et al. 1990; Webber 1989) or have stepped through the inspiratory burst with a 250-ms window in small steps (e.g., 1/16 s) (Richardson 1988; Richardson and Mitchell 1982) in an attempt to show that spectral content may change during inspiration. Although these studies have revealed changes in power and/or frequency of spectral peaks in the MFO and/or HFO ranges during inspiration, the segmenting procedures used may have been inadequate to produce data segments exhibiting stationarity, which is the underlying assumption for conventional spectral analyses. To overcome this limitation (i.e., stationarity), time-frequency methods, which provide not only the frequency content but also information regarding the timing of these fast rhythmic oscillations (i.e., time-varying or dynamic features of spectral activity), should be used. To our knowledge, no studies to date have applied time-frequency methods to investigate the dynamic features of spectral activity in inspiratory motor discharges.

Thus the purpose of the current investigation was two-fold: 1) to identify the spectral content of inspiratory motor activity in adult mouse in vivo, because the mouse is rapidly becoming a valuable model for studying the contribution of genetic factors in respiratory control (as stated above), and 2) to investigate the time-varying (i.e., dynamic) features of spectral activity in inspiratory motor discharges, because these fast rhythmic oscillations seem to exhibit nonstationarity (as is true of most biological signals). To address these objectives, both conventional (i.e., time-invariant) and time-frequency (i.e., time-varying) spectral analyses were performed on recordings of diaphragm EMG activity, phrenic nerve discharge, and hypoglossal nerve discharge obtained from spontaneously breathing urethane-anesthetized adult C57BL/6 mice. For time-frequency spectral analysis, a generalized time-frequency representation (TFR) with the smoothed pseudo-Wigner-Ville distribution (SPWD) kernel was employed and is described below (see Spectral analyses). A preliminary account of a portion of this work has been reported in an abstract (O’Neal et al. 2003).

METHODOLOGY

Experiments were conducted in 49 adult C57BL/6 mice of either sex (27 male; 22 female), weighing 15–33 g. All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at the State University of New York at Stony Brook in accordance with Public Health Service Policy on Humane Care and Use of Laboratory Animals. The mice were anesthetized with an intraperitoneal injection of urethane (~2.0 g/kg). The adequacy of anesthesia was regularly verified by absence of a withdrawal reflex to a noxious interdigitary pinch and was supplemented as needed (~0.2 g/kg, ip). The mice were supplied with a gas mixture of 40% O2 (in a balance of N2) from a nose cone, and diaphragm EMG activity was recorded using a thin platinum-iridium wire bipolar electrode inserted into the right side of the diaphragm (just right of the midline) while the mice breathed spontaneously. Alternatively, phrenic or hypoglossal nerve discharge was recorded using a thin platinum-iridium wire bipolar electrode hooked around the C3 phrenic rootlet or hypoglossal nerve. In some experiments, diaphragm EMG activity and phrenic or hypoglossal nerve discharge were recorded simultaneously. Body temperature was measured using a rectal probe and maintained between 35.0 and 38.7°C throughout the experiment using a heating pad and a heat lamp as necessary.

Data acquisition

The diaphragm EMG signal was amplified (1k), notch filtered at 60 Hz, and filtered to pass frequencies between 10 or 100 Hz and 5 kHz; in a subset of experiments (n = 36), the EMG signal was filtered to pass frequencies between 10 Hz and 1 kHz for analysis of spectral composition. The phrenic and hypoglossal nerve signals were amplified (10k), notch filtered at 60 Hz, and filtered to pass frequencies between 10 Hz and 1 kHz for analysis of spectral composition. Filtered signals were rectified, and a moving average was obtained using a third-order Paynter filter with a 50-ms time constant. The raw and moving-averaged inspiratory motor discharges were recorded on digital tape at a sampling rate of ≥2.5 kHz (C-DAT16, Cygnus Technologies, Water Gap, PA) or on VHS tape via pulse-code modulation at a sampling rate of 5.3 kHz (Model 4000A, A.R. Vetter, Rebersburg, PA); the signals were also recorded on a computer at a sampling rate of ≥2 kHz (Chart 4.0, PowerLab, ADInstruments, Mountain View, CA) for off-line analyses.

Temporal analyses

Inspiratory burst frequency, burst duration (T_b), and the duration between bursts (T_p) were determined for the last 10 s of a 1-min recording period (typically 23–34 breaths) or for the last 10 s of each minute of a 9- to 10-min recording period (n = 10). In some experiments, duration to peak amplitude (T_peak), which was normalized to T_b, was also determined for the last 10 s of a 1-min recording period. All temporal data are reported as means ± SE.

Spectral analyses

The experimental data used for spectral analyses were segmented to obtain data lengths corresponding to the entire inspiratory burst (see Fig. 1B for an example), which were set to 360 data points; zero padding was used if necessary. The number of data points was not reduced to 256 because this procedure would have reduced spectral resolution. Furthermore, the data lengths were not increased to 512 data points by additional zero padding, because this procedure, which would not affect spectral content, would only have minimally improved computation time. These data were digitally band-pass filtered between 20 and 250 Hz using a 100th order Hamming window. The lower limit (20 Hz) was selected to minimize potential contamination of the power spectrum by the lower frequency oscillations (~9–12
Hz) accompanying the cardiac (i.e., heart rate) activity recorded in some experiments; the upper limit (250 Hz) was selected because no recent studies have reported frequency domain characteristics in excess of ~200 Hz (although a limited number of earlier studies examining the effects of inspiratory loading on spectral activity have shown higher frequencies; e.g., Bellemare and Grassino 1983; Gross et al. 1979). Each data burst was demeaned and normalized by subtracting out the mean and dividing by the SD of the data record to allow for direct comparisons of spectral composition between experiments. Thus all analyzed data sets had zero mean and unit variance.

Both conventional time-invariant and time-frequency (time- varying) spectral analyses of diaphragm EMG activity, phrenic nerve discharge, and hypoglossal nerve discharge were performed. Time-invariant power spectral analyses were performed using a periodogram method. A Hanning window was applied to the data burst during computation of the periodogram to reduce the effect of side- lobes and decrease the estimation bias. To compute the periodogram, \(P(f)\), the following FFT algorithm was implemented

\[
P(f) = \frac{1}{NT} \sum_{n=0}^{N-1} s[n]d[n] \exp^{-j2\pi nf/T}
\]

where \(w(n) = 0.50 - 0.50\cos(2\pi n/N)\), and the variable \(T\) represents the data sampling interval, which was 0.5 ms for our analyses.

The power spectrum, as defined above, is of great value in identifying periodic components that do not change with time. However, most biological signals are nonstationary, and thus oscillating components do not occur at all time-points but only at certain time-points within the signal. To overcome the limitations of time-invariant power spectral analyses, time-frequency spectral analyses were performed to assess the spectrum over varying time segments (i.e., TFR). These analyses used a generalized TFR (also known as the Cohen representation; Cohen 1995) with a specialized windowing function called the kernel. All TFR can be obtained from

\[
C(t,\omega) = \frac{1}{4\pi} \int \int s(u-\frac{\tau}{2})s^*(u+\frac{\tau}{2})\phi(\theta,\tau)e^{-j(\omega-t+\omega\tau)}d\tau d\theta
\]

where \(\phi(\theta,\tau)\) is the two-dimensional function or kernel, and \(s(u-\tau/2)\) and \(s^*(u+\tau/2)\) represent the autocorrelation of the real and complex conjugate of the signal, respectively. Given the TFR in Eq. 2, if the kernel, \(\phi(\theta,\tau)\), is equal to 1, the well-known Wigner-Ville distribution is obtained. The Wigner-Ville distribution provides better time and frequency resolution than do other time-frequency methods because the signal is not segmented into small segments; however, when there are many frequencies in a given signal, the Wigner-Ville distribution may show artificial frequencies in addition to the true frequencies present in the signal (Cohen 1995). To overcome this problem, many different kernels have been designed; in this study, the SPWD kernel was used. The SPWD kernel has the form

\[
\phi(\theta,\tau) = \eta\left(\frac{\tau}{2}\right) \eta^*(\frac{-\tau}{2}) G(\theta)
\]

where \(\eta()\) and \(G(\theta)\) are two Hamming windows whose effective lengths independently determine the time and frequency smoothing, respectively. This kernel reduces artificial frequencies yet retains higher time-frequency resolution than other methods used for TFR.

Substituting the kernel in Eq. 3 into the general class (Eq. 2) and integrating over \(\theta\), we obtain

\[
SPWD(t,f) = \int \int g(t-u)H(f-f')W(t,f')du df
\]

where

\[
H(f) = \int \eta\left(\frac{\tau}{2}\right) \eta^*(\frac{-\tau}{2}) e^{j2\pi f\tau} d\tau
\]

and

\[
W(t,f) = \int s(t-\frac{\tau}{2})s^*(t+\frac{\tau}{2}) e^{-j2\pi f\tau} d\tau
\]

Thus in Eq. 4, \(g(t)\) and \(H(f)\) represent time and frequency Hamming windows, respectively, whose window lengths independently determine the time smoothing spread \(\Delta\tau\) and the frequency spread \(\Delta f\); respectively. Thus if \(g(t) = \delta(t)\) (i.e., no time smoothing), Eq. 4 reflects the pseudo Wigner-Ville distribution. In this study, the win-
bow lengths of \( g(t) \) and \( H(f) \) were set to 13 and 129, respectively, to accentuate the time resolution while maintaining frequency resolution at a level similar to that used for the time-invariant spectral analysis approach.

For diaphragm EMG activity, the power spectral density (PSD) and time-frequency (TF) spectrum were calculated as an ensemble average derived from the last 10 s of a 1-min recording period or for a maximum of 29 inspiratory bursts using each of the spectral analysis procedures outlined above. In a subset of experiments, the PSD and TF spectrum were also determined during the last 10 s of each minute for a 9- to 10-min recording period (\( n = 10 \)). Relative power was autoscaled to the largest peak in the power spectrum. For phrenic and hypoglossal nerve discharges, the PSD and TF spectrum were similarly calculated as an ensemble average derived from the last 10 s of a 1-min recording period or for a maximum of 29 inspiratory bursts using each of the spectral analysis procedures outlined above. In the case of simultaneous diaphragm EMG and phrenic or hypoglossal nerve recordings, the frequencies associated with dominant spectral peaks were compared using a Student’s paired \( t \)-test, for which the criterion level for determination of statistical significance was set at \( P < 0.05 \). In addition, the cross-spectrum and squared coherence value were calculated on a burst-by-burst basis for 10 consecutive bursts to evaluate the linear correlation of the two signals (EMG vs. phrenic, \( n = 7 \); EMG vs. hypoglossal, \( n = 4 \)). For these analyses, PSD of the inspiratory bursts was reassessed using Blackman and Tukey’s corroborategram method (Marple 1987), defined as

\[
S_x(\omega) = \sum_{m=-M}^{M} \phi_x(m)w(m)e^{-j\omega m}
\]  

(7)

where \( S_x \) denotes the spectrum and

\[
w(m) = 0.54 + 0.46 \cos (2\pi m/M)
\]  

(8)

is the Hamming window of maximum lag \( M = 128 \). The correlogation function estimate \( \phi_x \) was computed over lags \( m \) from \(-128 \) to \( 128 \), resulting in spectral resolution of 7.8125 Hz. The level of significance for coherence was determined by estimating the coherence on 100 realizations of two uncorrelated white-noise processes with zero mean and unit variance (Jenkins and Watts 1968), which yielded a coherence value of 0.65. Time-frequency data were used to determine the temporal window of spectral activity and total spectrum power, which were normalized to burst duration. All spectral data are reported as means ± SE.

RESULTS

Temporal characteristics of inspiratory motor activity

In each of the experiments conducted, inspiratory motor output exhibited an augmenting discharge pattern, characteristic of eupneic breathing. In some cases, postinspiratory activity could also be detected in the inspiratory motor bursts. When present, it corresponded to 12.4 ± 1.6% of the burst duration based on diaphragm EMG data. The inspiratory motor bursts exhibited some variability in peak amplitude from burst to burst; however, the breathing rate was steady throughout the duration of the recording protocol. An example of an original diaphragm EMG record is included in Fig. 1 (examples of phrenic and hypoglossal bursts may be seen in Fig. 3A). Based on the 46 experiments that included diaphragm EMG recordings, burst frequency was 166.8 ± 5.8 bursts/min, which is similar to the breathing frequency previously reported for conscious freely moving C57BL/6 mice (Han et al. 2001; Tankersley et al. 1997); \( T_i \) averaged 112 ± 4 ms and \( T_E \) was 265 ± 13 ms; thus the inspiratory duty cycle (\( T_i/T_{\text{total}} \)) was ~0.3. \( T_{\text{peak}}/T_i \), which was assessed in 16 of these experiments, corresponded to 87.6 ± 1.6%, which is consistent with an augmenting discharge pattern. In some experiments, a small cardiac artifact could also be detected in the EMG record. Heart rate in these animals corresponded to ~10 Hz (\( \leq 12 \) Hz). Temporal analyses of phrenic and hypoglossal nerve discharges revealed similar patterning and timing characteristics as those described above for diaphragm EMG activity.

Time-invariant spectral analysis: FFT

Time-invariant spectral analyses of diaphragm EMG bursts revealed three dominant peaks in the power spectrum (Figs. 1D and 2). For the 36 experiments included in these analyses, the location of these peaks was plotted as a function of frequency, and the results obtained are shown in Fig. 2. In brief, two of the spectral peaks corresponded to the frequency ranges typically associated with MFO and HFO activity often observed in inspiratory motor output in adult mammals (Cohen et al. 1987b; Richardson and Mitchell 1982); the remaining spectral peak was observed at higher frequencies, showing an additional HFO-like peak. This peak was designated as upper high-frequency oscillations (UHFO) to distinguish it from the classical HFO peak previously described (Cohen et al. 1987b). In some cases, an additional spectral peak was observed between the HFO and UHFO peaks.

The frequencies corresponding to the dominant spectral peaks identified were 1) 35 ± 1 Hz (range = 20–46 Hz) for the MFO peak, 2) 110 ± 3 Hz (range = 83–149 Hz) for the HFO peak, and 3) 206 ± 2 Hz (range = 177–227 Hz) for the UHFO peak; in cases in which an additional spectral peak was observed between the HFO and UHFO peaks, the frequency corresponding to this peak was 168 ± 5 Hz (range = 155–171 Hz). In all experiments, the HFO peak exhibited higher relative power than that of the MFO peak, and in some cases, multiple peaks were detected in one or both of these frequency ranges (e.g., MFO in Fig. 1D). UHFO activity was typically composed of one or two dominant peaks, and the UHFO peak(s) exhibited higher relative power than the MFO peak, and in most cases,
similar or higher relative power to the HFO peak. The magnitude of power noted for the additional spectral peak observed between the HFO and UHFO peaks varied, but it generally exhibited lower relative power than the HFO and UHFO peaks.

Time-invariant spectral analyses were also performed on phrenic and hypoglossal nerve discharges, and these analyses revealed dominant peaks in the power spectrum within the ranges described above for diaphragm EMG activity (Figs. 2 and 3). The mean frequencies corresponding to the MFO, HFO, and UHFO spectral peaks were 38 ± 2, 110 ± 6, and 203 ± 5 Hz for phrenic nerve discharge (n = 8), respectively; and 34 ± 2, 127 ± 5, and 196 ± 7 Hz for hypoglossal nerve discharge (n = 6), respectively. An additional spectral peak was observed between the HFO and UHFO peaks in three cases (phrenic, n = 2; hypoglossal, n = 1). The locations of spectral peaks for both phrenic and hypoglossal nerve discharges were also plotted as a function of frequency, and the results obtained are included in Fig. 2. Paired comparisons of spectral content between simultaneously recorded diaphragm EMG and phrenic or hypoglossal nerve discharge revealed no statistically significant differences (P > 0.05) for the frequencies associated with the dominant MFO, HFO, or UHFO spectral peaks.

Coherence was evaluated on a burst-by-burst basis for simultaneously recorded diaphragm EMG activity and phrenic or hypoglossal nerve discharges in each of the frequency ranges identified. For these analyses, coherence values for each burst were included only if they corresponded to a peak in the coherence spectrum; bursts with little or no coherence (i.e., squared coherence value of ~0) or bursts with an underlying static level of coherence were not included. In ~50% of these experiments, only a few (i.e., 2–5 of the 10) bursts exhibited a peak in the coherence spectrum in the MFO and 155- to 171-Hz ranges between diaphragm EMG activity and phrenic or hypoglossal nerve discharge; thus the squared coherence values reported for these ranges represent only bursts clearly exhibiting coherence peaks. The squared coherence values obtained from these analyses are provided in Table 1.

Significant coherence (i.e., squared coherence value ≥ 0.65) was observed between diaphragm EMG activity and phrenic nerve discharge in each of the frequency ranges examined and between diaphragm EMG activity and hypoglossal nerve discharge in the HFO and UHFO ranges.

**Comparison of time-invariant and time-varying spectral analyses**

Because it has been suggested that spectral content in inspiratory motor discharges may change over the course of the inspiratory burst (Bruce 1986, 1988; Christakos et al. 1989, 1991; Cohen et al. 1987b; Marchenko et al. 2002; Richardson 1988; Richardson and Mitchell 1982; Schmid et al. 1990; Webber 1989), time-frequency analyses using a generalized TFR with the SPWD kernel were performed on recordings of diaphragm EMG activity, phrenic nerve discharge, and hypoglossal nerve discharge to evaluate the time-varying (i.e., dynamic) features of spectral activity (see *Time-frequency spectral analysis: SPWD*). There existed, however, two possible outcomes regarding the TFR of the inspiratory motor discharges: 1) the inspiratory motor discharges would exhibit fast oscillatory components that are continuous during the inspiratory burst (i.e., time-invariant) or 2) the inspiratory motor discharges would exhibit fast oscillatory components that exist at particular times during the inspiratory burst (i.e., time-varying). It should be noted that a combination of time-invariant and time-varying fast oscillatory components could

**TABLE 1. Squared coherence values for MFO, HFO, UHFO, and 155 to 171-Hz ranges from simultaneously recorded diaphragm EMG activity and phrenic nerve discharge and diaphragm EMG activity hypoglossal nerve discharge**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>MFO</th>
<th>HFO</th>
<th>UHFO</th>
<th>155–171 Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phrenic</td>
<td>7</td>
<td>0.70 ± 0.07</td>
<td>0.69 ± 0.04</td>
<td>0.75 ± 0.03</td>
<td>0.75 ± 0.04</td>
</tr>
<tr>
<td>Hypoglossal</td>
<td>4</td>
<td>0.51 ± 0.06</td>
<td>0.72 ± 0.04</td>
<td>0.69 ± 0.10</td>
<td>0.52 ± 0.12</td>
</tr>
</tbody>
</table>

Values are means ± SE. MFO, medium frequency oscillation; HFO, high frequency oscillations; UHFO, upper HFO.
also be observed. These possible outcomes were simulated by generating a sinusoidal time series with frequencies of 36 and 110 Hz, approximating MFO and HFO activities, respectively (Fig. 4A). In the first simulation, the activities of the 36- and 110-Hz signals were continuous over the entire simulation duration (Fig. 4A1), whereas in the other simulation, the activities of the 36- and 110-Hz signals were present over finite discrete portions of the simulation duration (Fig. 4A2). Application of the FFT algorithm to both simulated data signals revealed peaks in the power spectrum at 36 and 110 Hz with a similar power distribution (as expected; Fig. 4B); however, the time-frequency information (depicted in Fig. 4A) was not identified by this approach. This shows that time-invariant spectral analysis provides only frequency information, and therefore it is not suited for analyzing nonstationary signals. In contrast, application of a time-frequency method, such as the SPWD, to both simulated data signals not only revealed peaks in the power spectrum at 36 and 110 Hz (as expected; Fig. 4C), but also correctly provided the TFR of the signals (depicted in Fig. 4A). This shows that time-frequency methods provide a powerful tool for analyzing time-varying (i.e., dynamic) signals for which separate time-domain and frequency-domain (power spectrum) representations are not adequate.

**Time-frequency spectral analysis: SPWD**

In this study, time-frequency signal analyses confirmed the general location of the dominant spectral peaks (obtained using the FFT algorithm), identified additional spectral peaks within the frequency ranges described above, and revealed a time-dependent expression of spectral activity within the inspiratory burst for each of the frequency ranges. An example of the TF spectrum for diaphragm EMG activity is provided in Fig. 5. In this example, the TF spectrum is shown as a contour plot scaled from zero to maximum for all spectral activity (Fig. 5B1) and as mesh plots rotated to two different angles about the power axis (90° rotation; Fig. 5C).

The time-frequency method revealed little or no spectral activity within the first ~20% of the inspiratory burst. In contrast, spectral activity contained within the latter ~50% of the burst exhibited high relative power, with the highest relative power concentrated between 60 and 90% of the burst duration ($T_i$). Time-frequency analyses also revealed transient oscillations in the magnitude of spectral power in each of the frequency ranges identified. These transient oscillations allowed for detection of multiple peaks of spectral activity over the course of the inspiratory burst within each of the frequency ranges (Fig. 5; also see Fig. 8, B and C). As shown in Fig. 5, multiple peaks were resolved at a given frequency over numerous time-points (e.g., 4 HFO peaks were observed at ~106 Hz at 53, 66, 84, and 90% $T_i$; the dominant HFO peak was observed at ~123 Hz at 76% $T_i$), as well as at various frequencies for a given time-point (e.g., 3 spectral peaks corresponding to ~106, ~158, and ~188 Hz were observed at 84% $T_i$).

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**FIG. 4.** Application of time-invariant and time-frequency spectral analyses methods to simulated data signals. **A**: simulated sinusoidal time series data signal with underlying activities of 36 and 110 Hz that are (A1) continuous over the entire simulation duration and (A2) present over finite discrete portions of the simulation duration. **B**: application of the time-invariant FFT algorithm to both simulated data signals revealed peaks in the power spectrum at 36 and 110 Hz (as expected); however, the difference in time-frequency information (depicted in A) was not identified by this approach. **C**: application of a time-frequency method to both simulated data signals revealed peaks in the power spectrum at 36 and 110 Hz (as expected) and correctly identified the time-frequency representation of the signals (depicted in A).
In all experiments, spectral activity in the MFO range exhibited low relative power (compared with HFO and UHFO activities), and therefore spectral peaks within this range were often difficult to resolve. To better characterize the TFR of spectral activity within the MFO range, it was necessary to adjust the threshold of the power (color) scale from zero to maximum within this range instead of from zero to maximum for the entire TF spectrum. An example of a time-frequency contour plot scaled from zero to maximum for each frequency range is provided in Fig. 5.

Under these conditions, time-frequency analyses confirmed that spectral activity within the MFO range occurred predominantly as multiple peaks and revealed that the highest relative power occurred at ~60–90% of $T_I$, followed by a slight decrease. Within the HFO range, time-frequency analyses showed that spectral power increased until ~70–80% of $T_I$ and then remained constant or decreased slightly. Finally, within the UHFO range, spectral power increased toward the end of the inspiratory burst, with peak power being observed at ~70% of $T_I$. Thus there was an augmenting pattern of total spectral power over the course of the inspiratory burst (data not shown). Although spectral power exhibited changes in magnitude during the inspiratory burst, the frequencies of the spectral peaks were stable over the course of the inspiratory burst, although, as described above, multiple peaks were resolved at various frequencies within each frequency range.

Time-frequency spectral analyses were also performed on phrenic and hypoglossal nerve discharges using the SPWD algorithm. These analyses revealed time-varying characteristics similar to those described above for diaphragm EMG activity (e.g., little or no spectral activity within the first ~20% of the inspiratory burst; highest relative power concentrated between 60 and 90% $T_I$, etc.); however, in some cases, the duration of spectral activity in the hypoglossal burst was shorter than that observed for diaphragm EMG activity. An example of the TF spectrum for hypoglossal nerve discharge is shown in Fig. 6. For simplicity, the presentation of these data is similar to that provided in Fig. 5 for diaphragm EMG activity. As shown in Fig. 6, time-frequency analyses revealed a time-dependent expression of spectral activity, with the highest relative power concentrated between ~70 and 75% $T_I$ for the HFO and UHFO ranges and at ~80% for the MFO range. MFO activity was more easily resolved by adjusting the threshold of the power (color) scale from 0 to maximum within this range (data not shown). Although multiple spectral peaks could be resolved within each of the frequency ranges identified, there was a tendency for expression of fewer spectral peaks in hypoglossal nerve discharge compared with diaphragm EMG discharge (based on simultaneously recorded activity)
Temporal and spectral activity during a 9- to 10-min recording period

The application of the time-frequency analysis method clearly showed that spectral activity is highly dynamic over the course of the inspiratory burst. To evaluate these dynamic features over a longer time period, the time-frequency analysis method was applied to the last 10 s of each minute during a 9- to 10-min recording period (n = 5), and spectral content was examined. Prior to beginning spectral analyses, temporal analyses were performed on diaphragm EMG activity to confirm that the temporal characteristics were stable. These analyses showed minimal variability in burst frequency (Fig. 7; n = 10), T1, and T2.

Time-invariant spectral analyses of diaphragm EMG activity revealed some variability in the relative power associated with the dominant spectral peaks while the frequencies associated with the spectral peaks remained fairly stable (Fig. 8A). In general, the relative power associated with the spectral peak(s) in the UHFO range were the most variable, and differences were noted in the number of the dominant UHFO peaks detected in a single experiment over the course of the 9- to 10-min recording period (Fig. 8A). Time-frequency analyses confirmed the variability in relative power and revealed a high degree of variability in the number of the dominant spectral peaks observed in each of the frequency ranges examined over the 9- to 10-min recording period (Fig. 8, B and C). In contrast, the frequencies associated with the dominant spectral peaks remained fairly stable, and there was little variability associated with the duration of spectral activity over the course of the inspiratory burst. Regardless of the minute-to-minute variability in relative power and number of dominant spectral peaks, the TF spectrum remained highly dynamic, with transient oscillations in spectral activity being observed over the course of the inspiratory burst (see Time-frequency spectral analysis: SPWD), at each minute in the 9- to 10-min recording period (Fig. 8, B and C).

To provide further insight into the dynamic features of spectral content over the longer duration recording protocol (i.e., temporal variability of spectral activity), an ensemble average of spectral activity was compiled for the entire 9- to 10-min recording period; this ensemble average was evaluated using both time-invariant and time-varying spectral analyses methods. The ensemble-averaged data associated with the 10-min recording period depicted in Fig. 8 are provided in Fig. 9 (i.e., average of 290 diaphragm EMG bursts). As seen in this example, both time-invariant (FFT; Fig. 9A) and time-frequency (i.e., SPWD; Fig. 9, B and C) spectral analysis methods reveal dominant peaks in the power spectrum in the MFO, HFO, and UHFO ranges; however, there is a loss of spectral resolution, resulting in broad band spectral activity within each of the frequency ranges (which is more apparent with the time-varying approach). The time-frequency method further reveals that, under these conditions, there is a marked reduction in the number of bursts of spectral activity (i.e., transient oscillations) in the TF spectrum, which presumably results from averaging signals with a high degree of variability (e.g., transient spectral bursts that are out-of-phase). Although the TFR has been altered (i.e., loss of spectral resolution; reduction in the dynamic features within the TF spectrum), there seems to be a high degree of temporal consistency, such that the duration of spectral activity within the inspiratory burst is negligibly affected. Furthermore, the TF spectrum still exhibits little or no activity within the first ~20% of the inspiratory burst, the highest relative power is seen within the latter 60–90% T1, and the total spectral power still exhibits an augmenting pattern over the course of the inspiratory burst (Fig. 9D).
DISCUSSION

This study set out to 1) identify the spectral composition of inspiratory motor discharge in anesthetized adult mouse in vivo and 2) investigate the time-varying nature of spectral activity within the inspiratory motor burst using a robust time-frequency spectral analysis method. With respect to the first objective, spectral analyses of inspiratory motor discharges revealed fast oscillations in three frequency ranges, two of which correspond to previously identified MFO and HFO activities in some other adult mammals (e.g., cat, rabbit) in vivo; the remaining fast oscillatory rhythm occurred within a slightly higher frequency range and was designated as UHFO. With respect to the second objective, time-frequency analyses of inspiratory motor discharges revealed that spectral activity was highly dynamic or transient within each of the frequency ranges identified, thus confirming previous observations that spectral activity is not constant over the course of inspiration and extending these observations by characterizing the time-varying (i.e., nonstationarity) nature of these fast oscillations within the inspiratory motor burst. Thus the time-frequency analysis method not only confirmed the general location of dominant spectral peaks, but this approach also yielded new information, allowing for better characterization of the dynamics of spectral activity occurring within the inspiratory motor discharge.

Time-invariant spectral analyses: comparison to other studies

This study is the first to characterize spectral composition of inspiratory motor discharge in the adult mouse in vivo; however, spectral composition has been evaluated for hypoglossal and phrenic nerve discharges in medullary slice and en bloc brain stem-spinal cord preparations obtained from neonatal mice (Bou-Flores and Berger 2001). In these neonatal preparations, dominant spectral peaks have been reported in the 10- to 20- and/or 30- to 40-Hz ranges (Bou-Flores and Berger 2001). In this study, a dominant spectral peak was observed in the range of 20–46 Hz (MFO) in diaphragm EMG, hypoglossal nerve, and phrenic nerve discharges; however, dominant spectral peaks were also observed at higher frequencies (e.g., 83–149 and 177–227 Hz) in all experiments. Since previous studies conducted in other mammalian models (e.g., piglet, Cohen et al. 1987a; Gootman et al. 1985; cat, Bruce 1986; Kato et al. 1996; Sica and Gandhi 1990) have shown that peak frequency increases with development and that this lower frequency spectral activity exhibits significant coherence (Kato et al. 1996; Sica et al. 1988, 1991; Tarasiuk and Sica 1997) (which is considered a characteristic of HFO, but not MFO activity, Christakos et al. 1991, 1994; Cohen et al. 1987b), it is unclear whether the MFO peak identified in this study corresponds to the spectral peaks observed in these neonatal mouse preparations or whether the spectral peaks observed in the neonatal mouse preparations correspond to the HFO peak seen in our experiments, albeit at a reduced frequency. Furthermore, it is unclear what influence, if any, the reduced temperature (e.g., 27–28°C) used in the in vitro neonatal mouse preparations contributed to the lower spectral frequencies observed compared with the frequencies seen in the in vivo adult mouse preparation since lower temperatures are associated with lower spectral frequencies and the converse is true for higher temperatures (Kato et al. 1996; Richardson and Mitchell 1982).

Although marked differences in spectral activity were observed in our study in the adult in vivo mouse compared with the neonatal in vitro mouse (Bou-Flores and Berger 2001), the dominant spectral peaks observed in this study do share some similarities with those identified in other adult mammals. For example, in anesthetized and decerebrate adult cats (which have been used most often for these types of analyses), dominant spectral peaks have been reported in the 15- to 52- (MFO) and 50- to 122-Hz (HFO) ranges (Bruce 1986, 1988; Christakos et al. 1989, 1991, 1994; Cohen et al. 1987b; Richardson 1988; Richardson and Mitchell 1982; Webber 1989), whereas in anesthetized adult rabbits, dominant spectral peaks have been seen between 39–56 (MFO) and 60–144 Hz (HFO) (Bruce 1988; Romaniuk and Bruce 1991; Schmid and Böhmer 1989; Schmid et al. 1990). Furthermore, in most of these
studies, relative power was noted to be higher for the HFO peak than for the MFO peak. In contrast to the dual peaks usually noted in adult cat and rabbit in vivo, in anesthetized and decerebrate adult rats, usually only a single peak located between 45 and 160 Hz is observed (Kocsis and Gyimesi-Pelczer 1997; Marchenko et al. 2002), and the designation of MFO or HFO is determined by coherence analysis. In a few cases, however, in the barbiturate- but not urethane-anesthetized adult rat (n = 4/17) and in the decerebrate adult rat (n = 4/16), dual peaks with the lower frequency peak seen between 35 and 78 Hz (MFO) and the higher frequency peak seen between 97 and 160 Hz (HFO) have been detected (Kocsis and Gyimesi-Pelczer 1997; Marchenko et al. 2002); in these cases, relative power of the MFO and HFO peaks are usually similar. These observations have led to the suggestion that, in the adult in vivo rat, the frequency ranges associated with MFO and HFO activities may be shifted to higher frequencies (Kocsis and Gyimesi-Pelczer 1997; Marchenko et al. 2002), which for the HFO may be a consequence of the high discharge frequencies (>200/s) observed in dorsal and ventral respiratory group neurons (Tian and Duffin 1998). It should be noted, however, that in the in vitro arterially perfused (decerebrate) adult rat

FIG. 8. Spectral composition of diaphragm EMG activity for a 10-min recording period based on time-invariant and time-frequency spectral analyses. A: power spectrum obtained using time-invariant spectral (FFT) analysis. B and C: time-frequency spectrum obtained using the SPWD algorithm represented as (B) contour and (C) mesh plots. Spectral composition of diaphragm EMG activity is shown for minutes 1, 3, 5, 7, and 9 (designated as T1–T9) of the 10-min recording period. Power is normalized to maximal power obtained during the entire 10-min recording period; maximal power was observed in the UHFO peak at T3. Color scales correspond to color bar provided in Fig. 4, which shows spectral power in arbitrary units (au).
preparation maintained at $\sim$31°C, dual peaks located within the classically defined MFO (i.e., 40–50 Hz) and HFO (i.e., 90–110 Hz) ranges have been observed (Solomon et al. 2003). Based on the observations described above, our current findings in urethane-anesthetized adult mice seem to be more consistent with those obtained from in vivo adult cat and rabbit than those obtained from in vivo adult rat. The power spectrum in adult in vivo mice, however, does exhibit some differences. Most notable were the presence of an additional peak located at a slightly higher frequency than the HFO, which we designated as the UHFO, and in some experiments, another additional peak located between the HFO and UHFO. Although it is tempting to suggest that the HFO and UHFO spectral peaks observed in our experiments represent a shift of the MFO and HFO spectral peaks to higher frequencies (as suggested for rats), this is unlikely since in each of the experiments conducted, we observed a dominant spectral peak in the classically defined MFO and HFO ranges as well as in the UHFO range. Thus we suggest that inspiratory motor discharges in the adult in vivo mouse exhibit two distinct HFO-like spectral activities; a conclusion that is also supported by our coherence analyses (see following paragraph).

Previous studies have used coherence analysis to define and/or distinguish between MFO and HFO activities, which are believed to have different origins (Christakos et al. 1991, 1994; Cohen et al. 1987b; Kocsis and Gyimesi-Pelczer 1997; Marchenko et al. 2002; reviewed by Funk and Parkis 2002). These analyses have shown that MFO peaks typically occur at different frequencies and exhibit little or no correlation, whereas HFO peaks occur at a common frequency and are highly correlated (Christakos et al. 1991, 1994; Cohen et al. 1987b). In this study, significant coherence between diaphragm EMG and phrenic nerve discharge was observed in each of the frequency ranges examined, whereas significant coherence between diaphragm EMG and hypoglossal nerve discharge was only noted in the HFO and UHFO ranges. Although we are confident that the coherence values for HFO and UHFO ranges accurately reflect the underlying degree of linear correlation between the inspiratory motor outputs, we are cautious in the interpretation of our observations in the MFO and 155- to 171-Hz ranges because the coherence values reported in these ranges reflect only a small portion of the data obtained (i.e., bursts exhibiting peaks in the coherence spectrum). Furthermore, although significant coherence in the HFO range is believed to reflect medullary inspiratory neuron synchronization, it is unclear what specific neuronal population(s) UHFO activity represents. We suggest, however, that UHFO activity also reflects medullary inspiratory neuron synchronization since this fast oscillatory rhythm was observed in all of the inspiratory motor outputs evaluated and it exhibited significant coherence, suggesting a common input. Additional experiments, however, will be necessary to determine whether medullary inspiratory neurons in mice exhibit UHFO-like activity.

Time-frequency spectral analyses: changes in spectral activity over the course of the inspiratory burst

As with previous studies, we relied on a time-invariant spectral analysis method to initially identify dominant peaks in the power spectrum. Although time-invariant spectral analysis methods provide no information about the dynamics of spectral activity, numerous laboratories have used FFT algorithms in an attempt to show that spectral activity may change over the course of inspiration. In the first study to examine this possibility, Richardson and Mitchell (1982) stepped a 250-ms window through the phrenic nerve burst (recorded from decerebrate cat) in 62.5-ms intervals. Based on this approach, they showed that the amplitudes of the spectral peaks in both the
HFO and MFO ranges increased as inspiration progressed. Subsequent studies, predominantly examining spectral content of early versus late segments of the inspiratory burst (e.g., 1st half vs. 2nd half), have produced somewhat more variable results. For example, in the chloralose-anesthetized cat, Bruce (1986, 1988) found that the amplitude of the spectral peak in the range of 60–120 Hz was greatest during early to mid-inspiration and markedly diminished (and in some cases disappeared) near the end of the inspiratory burst, whereas the spectral peak in the range of 36–52 Hz was only minimally altered. In contrast, Webber (1989) reported that, in the chloralose-anesthetized cat, the amplitude of the spectral peak in the >50 Hz range was larger in the second half of the phrenic burst than that observed during the first half and that the spectral peak in the <50-Hz range was absent during the early phase and only seen during the later portion (or entire inspiratory phase) of the inspiratory burst. Observations by Cohen and colleagues also show that PSD differs between the two halves of the inspiratory motor burst in both the pentobarbital-anesthetized and decerebrate cat (Christakos et al. 1989, 1991; Cohen et al. 1987b) and the decerebrate rat (Marchenko et al. 2002); however, they show that peak spectral power in the HFO range is less in the second half than in the first half, although total HFO power in the second half is greater, whereas peak spectral power in the MFO range is greater during late inspiration; a similar alteration in spectral activity was observed in urethane-anesthetized rabbit (Schmid et al. 1990). Interestingly, segmenting the inspiratory burst into smaller fractions revealed that the amplitude of the HFO spectral peak increases for up to about two-thirds of the inspiratory phase, and then it declines (Christakos et al. 1989). In some of the above studies, changes in magnitude of relative spectral power were accompanied by an increase in the frequency of the MFO spectral peak (Christakos et al. 1989, 1991; Marchenko et al. 2002; Schmid et al. 1990) and/or a decrease in the frequency of the HFO spectral peak (Marchenko et al. 2002), whereas in other studies, spectral frequencies remained fairly stable (Richardson and Mitchell 1982; Webber 1989). Although the above observations clearly support the idea that spectral activity is not constant over the course of the inspiratory burst, these studies only provide insight into differences in spectral activity between early and late portions of the inspiratory burst (perhaps with the exception of the study by Richardson and Mitchell 1982), and the time-invariant methodology applied was inadequate for identifying and/or providing an accurate representation of the underlying dynamics of spectral activity over the course of inspiration. Furthermore, it should be noted that all of these studies used FFT algorithms, which assume stationarity of the signal; however, no details were provided, showing that the segmenting procedures applied produced data segments exhibiting stationarity, and based on the observations by Christakos et al. (1989), in which segmenting the inspiratory burst into smaller fractions yielded somewhat different observations, it is unlikely that the larger data segments met the stationarity assumption.

To overcome the limitations described above, in this study, we used a robust time-frequency spectral analysis method to evaluate spectral activity over the course of inspiration. This method not only investigated whether spectral activity was different between various portions of the inspiratory burst but also identified the underlying dynamic features of spectral activity. As expected, time-frequency analysis using the SPWD algorithm confirmed the general location of the dominant spectral peaks (obtained using the FFT algorithm); however, this method also revealed a time-dependent expression of spectral activity within the inspiratory burst. These analyses further showed that the onset of HFO and UHFO activities generally preceded that of MFO activity and that maximal relative power for the MFO, HFO, and UHFO peaks did not occur at the same temporal location (i.e., %T1) for each of the frequency ranges, with HFO and UHFO peaks often occurring later in the inspiratory burst than the MFO peak. This temporal patterning of spectral activity may be interpreted to suggest that the underlying dynamics associated with HFO and/or UHFO activities provide the necessary correlated input for generation of the increasing (i.e., ramp-like) pattern of inspiratory discharge. Furthermore, the time-frequency analysis method was able to resolve multiple peaks of spectral activity (i.e., transient oscillations in the magnitude of spectral power) over the course of the inspiratory burst within each frequency range, showing that the underlying signal contained within the inspiratory discharge is highly dynamic. We believe that these transient oscillations represent bursts of synchronized neuronal activity within the underlying signal, which vary in time over the course of inspiration or over different frequencies at a particular time during inspiration. Our analyses, however, did not identify any consistent changes in the frequencies associated with the dominant spectral peaks as the inspiratory burst progressed, suggesting that the underlying frequencies were fairly stable, even though, as noted above, multiple peaks were resolved at various frequencies within each of the frequency ranges.

The general characteristics of the TF spectrum, as described above, were observed for each of the inspiratory motor discharges (i.e., diaphragm EMG, phrenic nerve, and hypoglossal nerve) examined in this study; however, some differences in the time-frequency spectra were noted between diaphragm EMG activity and hypoglossal nerve discharge recorded simultaneously. Specifically, spectral activity, which occurred over shorter duration of the hypoglossal burst, exhibited fewer transient oscillations in the magnitude of spectral power, suggesting that the spectral activity underlying hypoglossal motor output, although dynamic in nature, may be less dynamic than that underlying diaphragm EMG and phrenic nerve activities. Thus the neuronal population(s) underlying fast oscillations associated with hypoglossal motor output may exhibit a higher degree of synchronized activity than those underlying diaphragm activity. Whether these differences in spectral activity are a result of the functions of hypoglossal motor output in regulating upper airway patency versus those of phrenic/diaphragm motor output in generating the forces required to generate airflow is unclear.

**SPWD as an alternative to FFT for analysis of fast oscillations**

The study of fast (i.e., high-frequency) oscillations in inspiratory motor discharges dates back to the early 1900s (Dittler and Garten 1912; Gasser 1928); however, it was not until Richardson and Mitchell (1982) applied power spectral analysis to recordings of phrenic and recurrent laryngeal nerve discharges from decerebrate cat that a renewed interest in the
investigation of high-frequency oscillations in inspiratory motor discharges began. Subsequently, numerous studies (including work from our laboratory) began to use power spectral analysis to identify and further characterize high-frequency oscillations in inspiratory-related discharges (see review by Funk and Parkis 2002), and FFT algorithms became the “gold-standard” for such studies. The primary assumption underlying these analyses is that the fast oscillatory components in the signal being analyzed do not change with time, which is usually not the case for most biological signals. Thus it is common to segment the signal into short intervals and to assume that these short-interval signals exhibit stationarity (Marple 1987). For inspiratory-related discharges, Christakos et al. (1991) have suggested that spectral analysis be performed on short segments of the data (i.e., different portions of the inspiratory phase) in addition to the analysis on the entire inspiratory burst to overcome the stationarity limitation, and allow for correct interpretation of fast oscillatory components as well as identify spectral changes that may occur over the course of inspiration (see APPENDIX of their manuscript for additional information). In this study, however, this approach would be difficult since the inspiratory burst in the adult in vivo mouse has such a short duration (i.e., $T_i \sim 110$ ms). Furthermore, this approach still requires stationarity of the signal (which may not be the case for all data segments), and as noted above, it allows only for identification of the frequency components contained within the data segment being analyzed, not the temporal location in which these frequencies are present within the signal. Thus for the last 20 yr, time-invariant (FFT-based) spectral analysis methods, which in some cases may not have been appropriate, have been widely used for assessment of spectral activity in inspiratory-related discharges.

Based on the above comments, it is clear that there is a need for better approaches that are designed to not only identify frequency components contained within inspiratory-related discharges but also provide information on how these frequency components change in time over the course of inspiration. To this end, application of time-frequency spectral analysis methods should be considered. These methods have been developed to allow for simultaneous evaluation of time and frequency information, and they are rapidly becoming a powerful alternative to FFT-based spectral analysis methods for identifying and characterizing dynamic nonstationary signals. In this study, we used a generalized TFR with the SPWD kernel to compute a high-resolution TF spectrum on short-duration (i.e., $T_i \sim 110$ ms) inspiratory motor discharges recorded from urethane-anesthetized adult mice. Application of the SPWD algorithm showed the primary advantage of using time-frequency spectral methods, that is, it identified the frequency content and provided information regarding the timing of these fast rhythmic oscillations. Thus the SPWD algorithm yielded a dynamic representation of the signal in both the time and frequency domains, thus providing new insight into the dynamic (i.e., time-varying) features underlying spectral activity in inspiratory motor discharges. Furthermore, since this method produces a dynamic representation of the signal, the assumption of data stationarity is unnecessary, thus time-frequency spectral analysis methods seem more suitable for studying fast oscillations in inspiratory-related discharges.

**Summary**

In this study, both time-invariant and time-varying (i.e., time-frequency) spectral analysis methods were used to identify and characterize spectral content in inspiratory motor discharges in adult mouse. Although both approaches revealed spectral peaks in three frequency ranges, which corresponded to the MFO, HFO, and UHFO, only the time-frequency spectral analysis method identified the dynamic (i.e., timing) features of this oscillatory activity; thus simply assigning spectral peaks into three discrete categories (i.e., MFO, HFO, and UHFO) may not be sufficient to accurately characterize spectral activity underlying inspiratory motor discharges. Based on these observations, we suggest that in addition to, or instead of time-invariant (FFT-based) spectral analysis methods, time-frequency spectral analysis methods, such as the SPWD, should be used in future studies examining fast (high-frequency) oscillations in inspiratory motor discharges, as additional insight into the neural control mechanisms that participate in inspiratory-phase neuronal and motoneuronal synchronization may be obtained.

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