Chemical activation of pre-Bötzing complex in vivo
reduces respiratory network complexity

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ABSTRACT

In the in vivo anesthetized adult cat model, multiple patterns of inspiratory motor discharge have been recorded in response to chemical stimulation and focal hypoxia of the pre-Bötzinger complex (pre-BötC), suggesting that this region may participate in the generation of complex respiratory dynamics. The complexity of a signal can be quantified using approximate entropy (ApEn) and multiscale entropy (MSEn) methods, both of which measure the regularity (orderliness) in a time series, with the latter method taking into consideration temporal fluctuations in the underlying dynamics. The current investigation was undertaken to examine the effects of pre-BötC-induced excitation of phasic phrenic nerve discharge, which is characterized by high-amplitude, rapid rate of rise, short-duration bursts, on the complexity of the central inspiratory neural controller in the vagotomized, chloralose-anesthetized adult cat model. To assess inspiratory neural network complexity, we calculated the ApEn and MSEn of phrenic nerve bursts during eupneic (basal) discharge and during pre-BötC-induced excitation of phasic inspiratory bursts. Chemical stimulation of the pre-BötC using DL-homocysteic acid (DLH; 10 mM; 10-20 nl; n=10) significantly reduced the ApEn from 0.982±0.066 (mean±SE) to 0.664±0.067 (p < 0.001) followed by recovery (~1-2 minute after DLH) of the ApEn to 1.014±0.067; a slightly enhanced magnitude reduction in MSEn was observed. Focal pre-BötC hypoxia (induced by sodium cyanide; NaCN; 1 mM; 20 nl; n=2) also elicited a reduction in both ApEn and MSEn, similar to those observed for the DLH-induced response. These observations demonstrate that activation of the pre-BötC reduces inspiratory network complexity, suggesting a role for the pre-BötC in regulation of complex respiratory dynamics.

Key Words: approximate entropy; network dynamics; inspiratory motor discharge; control of breathing
INTRODUCTION

The primary locus for respiratory rhythm generation in mammals is proposed to be located within the ventral respiratory column in a region referred to as the pre-Bötzinger complex (pre-BötC; 43,48,49). Focal activation of this region both in vitro and in vivo has been shown to increase the frequency of inspiratory motor bursts (15,26,33,34,50,52,53,55), and in some cases, modify the pattern of these bursts (50,52,53) while destruction of this region in vivo, including selective lesion of the neurokinin-1 receptor expressing neurons, has been shown to abolish the normal “eupneic” pattern of breathing (25,42).

In the in vivo anesthetized adult cat model, multiple patterns of inspiratory motor discharge have been recorded in response to chemical stimulation and focal hypoxia of the pre-BötC (50,52,53), suggesting that this region may participate in the generation of complex respiratory dynamics. The complexity of a signal can be quantified using approximate entropy (ApEn), a statistical index that measures the regularity (orderliness) in a time series (36,40), thus providing a nonlinear measure for studying the dynamics of complex physiological signals, such as heart rate variability (e.g., 24,27,28,36-38,40,46), endocrine function (14,39,41,54), and respiration (5,11-13,23,57). Higher values of ApEn are associated with irregularity and greater randomness, and thus reflect less system order (i.e., disorder) or higher system complexity. Conversely, lower values of ApEn are associated with a higher degree of regularity and predictability, and thus reflect a more ordered system or lower system complexity. The ApEn method is robust, and it has been used to estimate the complexity of a signal with as few as 100 data points as well as signals that are corrupted by noise (providing that the threshold (r) is set correctly) or signals that contain spurious large or small artifacts and/or outliers (10,40). Furthermore, it can be used to
analyze signals that are deterministic chaos or stochastic as well as signals that exhibit a combination of these two behaviors (40). Thus, ApEn appears to be well suited for application to experimental data that are often short in data length, corrupted with noise, and in many cases, contain underlying dynamics which may exhibit both deterministic and stochastic behaviors.

The measure of ApEn, however, is based on a single-scale, and therefore, it does not take into account temporal fluctuations in the underlying dynamics, which are inherent in physiological control systems. To overcome this limitation, *multiscale entropy* (MSEn), which is based on the application of ApEn (or sample entropy, 46) for different time scales, has been recently introduced (22); this new method provides measures of both variation and entropy. To compute MSEn, consecutive coarse-grained sequences, determined by the scale factor (τ), are constructed from a time series signal, such that the length of each coarse-grained sequence represents the length of the original time series divided by τ. This is equivalent to smoothing and decimation of the original time series sequence; thus, the MSEn approach is essentially a graded low pass filter with greater smoothing with increasing scales. For a scale factor of one, the time series is simply the original time series, which effectively yields the value of ApEn.

Although both chemical-induced activation and focal hypoxia of the pre-BötC can produce a marked excitation of phasic phrenic nerve discharge, which is characterized by high-amplitude, rapid rate of rise, short-duration bursts, our previous studies have only assessed the timing (*i.e.*, T₁, Tₑ) and patterning (*i.e.*, amplitude, rate of rise) characteristics associated with this type of modulation, and therefore, provide no insight into the underlying dynamics contained within the phrenic motor bursts. It, therefore, remains to be determined whether the underlying dynamics
contained within the inspiratory (phrenic) discharges are altered under these conditions. Thus, the current investigation was undertaken to examine the effects of this pre-BötC-mediated modulation of inspiratory motor discharge on the complexity of the central inspiratory neural controller. We hypothesized that chemical stimulation of the pre-BötC would reduce inspiratory network complexity, and that a similar modulation would be elicited by focal hypoxia in this region. To test this hypothesis, we calculated the ApEn and MSEn of phrenic nerve bursts during eupneic (basal) discharge and during pre-BötC-mediated excitation of phasic inspiratory bursts.
METHODS

General Methods. All data were obtained from chloralose-anesthetized adult cats under protocols approved by the Institutional Animal Care and Use Committee at the State University of New York at Stony Brook in compliance with the Animal Welfare Act and in accordance with The American Physiological Society’s “Guiding Principles for Research Involving Animals and human Beings” (1). Detailed descriptions of the general methods have been published previously (52,53).

In brief, anesthesia was induced in adult cats (3.0 - 4.8 kg) with halothane (5%) in oxygen and maintained with intravenous α-chloralose (initial 35-50 mg/kg; supplemental 3-5 mg/kg). The adequacy of anesthesia was regularly verified by absence of a withdrawal reflex (in the unparalyzed state) or blood pressure response (during muscular paralysis) to a noxious paw pinch. If the cat withdrew its limb during the absence of paralysis or if an increase in blood pressure was evoked, additional anesthesia was given. The right brachial vein and both brachial arteries were cannulated for administration of drugs, measurement of arterial blood pressure (Statham transducer, P23XL), and sampling of arterial blood. The trachea was cannulated, the cat was vagotomized bilaterally, and the lungs were mechanically ventilated with 40% O₂ in a balance of N₂. Bilateral pneumothoraces were established and the expiratory outlet of the ventilator was placed under 2-3 cm H₂O to prevent collapse of the lungs during expiration. The cat was then paralyzed with vecuronium bromide (0.2-0.4 mg/kg iv), supplemented as needed. The dorsal surface of the brainstem was exposed, and the C₅ rootlet of one or both phrenic nerves was isolated for recording. Raw phrenic nerve discharge was amplified (x10k), notch filtered at 60 Hz, and analog filtered to pass frequencies between 1 Hz and 500 Hz. The filtered signal was
rectified, and a moving average was obtained using a third-order Paynter filter with a 100-ms time constant.

*Data Acquisition and Analyses:* We calculated the ApEn and MSEn of phrenic nerve bursts during eupneic (basal) discharge and during pre-BötC-mediated chemical-induced excitation of phasic inspiratory bursts. For these experiments, only pre-BötC sites in which unilateral microinjection of DL-homocysteic acid (DLH; 10 mM; ≤ 20 nl; n=10; Fig. 1) or sodium cyanide (NaCN; 1 mM; 20 nl; n=2) produced a rapid series of high-amplitude, rapid rate of rise, short-duration phrenic bursts under hyperoxic, normocapnic conditions were selected. All sites in the pre-BötC were initially localized using predetermined stereotaxic coordinates relative to the calamus scriptorius, functionally identified using DLH, and histologically confirmed following completion of the experiment as previously described (52,53).

Both raw and averaged phrenic nerve activity were recorded on a computer at a sampling rate of 2 kHz (Chart 4.0, PowerLab, ADInstruments, Colorado Springs, CO) and on VHS tape via pulse-code modulation (Model 4000A, A.R. Vetter, Rebersburg, PA) for off-line analyses (MatLab 6.1, The MathWorks, Inc., Natick, MA). The data used for these analyses were segmented to obtain data lengths corresponding to the inspiratory burst, with little or no post-inspiratory discharge. These data were then digitally band-pass filtered (20-250 Hz) using a 4th order Butterworth filter and re-sampled with a sampling rate of 1 kHz (unless otherwise stated). Thus, the calculated values of ApEn and MSEn (see below) provided an index of the complexity of the central inspiratory neural network, which reflects the underlying dynamics associated with brainstem respiratory rhythm generating and pattern formation circuits as well as phrenic
motoneurons.

The calculation of ApEn requires \textit{a priori} specification of the parameters $m$, the embedding dimension, and $r$, the tolerance (threshold) level (which is in effect a noise filter). The value of $m$ can be estimated using the first minimum value of the nonlinear correlation function called average mutual information and subsequent use of the false nearest neighbor approach (2); however, theoretical and varied clinical applications have shown that either $m=1$ or 2 and $r$ between 0.1 and 0.25 of the standard deviation (SD) of the data provide good statistical validity of ApEn. For our analysis, we used $m=2$ and $r=0.1$ SD, values which were determined from a series of simulation experiments and preliminary analyses of our baseline phrenic nerve discharge data. ApEn was calculated as follows (36,40):

$$ApEn(m, r, N) = \Phi^m(r) - \Phi^{m+1}(r) \quad \text{where} \quad \Phi^m(r) = (N - m + 1)^{-1} \sum_{i=1}^{N-m+1} \ln C_i^m(r)$$

and $C_i^m(r) = N^m(i)/(N - m + 1)$

$$N^m(i) = \text{no. of } d[X(i), X(j)] \leq r$$

where $X(i), X(j)$ are vectors defined by $X(i) = [u(i), u(i+1), ..., u(i+m-1)]$ and $X(j) = [u(j), u(j+1), ..., u(j+m-1)]$ from the original time series $u(1), u(2), ..., u(N)$.

Since ApEn depends on the differences between the current $m$ and the next $m+1$ embedding dimension, no information is provided about feature patterns on scales other than the scale of one. To overcome this limitation and evaluate the temporal variability in the underlying dynamics, we used the measure of MSEn, which is based on a simple modification of the ApEn method. For these analyses, MSEn was calculated from the original time series $u(1), u(2), ...,
u(N) by constructing consecutive course-grained time series, \( w_1^\tau, w_2^\tau, \ldots, w_{N/\tau}^\tau \), determined by the scale factor \( \tau \), according to the following equation:

\[
w_j^\tau = \frac{1}{\tau} \sum_{i=(j-1)\tau+1}^{j\tau} u(i) \text{ where } 1 \leq j \leq N / \tau
\]

Thus, the vector \( W^{(\tau)} = [w_1^\tau, w_2^\tau, \ldots, w_{N/\tau}^\tau] \), for any \( \tau \), can then be quantified by the ApEn method. For our analyses, we used up to 20 scales (\( \tau \)), and the evaluation of all of the selected \( \tau \) was defined as MSEn, with the following relationship:

\[
MSEn(\tau) = ApEn(W^{(\tau)})
\]

For all experiments, ApEn and MSEn of the total inspiratory burst as well as the 1\(^{st}\) half and 2\(^{nd}\) half were examined in order to characterize the complexity of the central inspiratory network underlying inspiratory motor output, including those associated with generation of early versus late portions of the inspiratory burst. In addition, we evaluated how much of the inspiratory burst is required to capture the dynamics underlying central inspiratory network complexity. For these analyses, we examined the effects of successive 10% reductions in inspiratory duration (\( T_I \)), from the beginning as well as from the end of the burst (\( i.e. \), forward and backward reductions), on ApEn using data obtained during both basal and DLH-induced excitation of phrenic nerve discharge. Finally, since previous studies, predominantly examining heart rate variability, have reported that the ApEn method is fairly insensitive to the number of data points in a time series as long as there are at least 100 data points (40), we also evaluated the effects of down-sampling the original data set on ApEn of the total inspiratory burst during basal and DLH-induced excitation. For these analyses, the original (digitally band-pass filtered) data were down-sampled by a factor of 2, 4, and 8 to yield sampling rates of 1 kHz, 500 Hz, and 250 Hz, respectively.
For all analyses, values for ApEn were determined for individual phrenic bursts, and summary data were calculated as an ensemble average derived from analyses of 10 phrenic bursts under baseline conditions and following chemical stimulation of the pre-BötC as well as during recovery from the effects of chemical stimulation; these summary data are reported as means ± SE. Values for MSEn were determined for individual experiments as an ensemble average derived from analyses of 10 phrenic bursts under baseline conditions and following chemical stimulation of the pre-BötC as well as during recovery from the effects of chemical stimulation; these data are reported as mean ± SD. Summary data for values of MSEn were calculated as an average derived from the analyses of all of the individual DLH experiments, and these data are reported as means ± SE. For ApEn, statistical significance was evaluated using one-way analysis of variance with repeated measures, followed by Bonferroni post hoc analyses for multiple comparisons. In addition, correlation analyses between ApEn and phrenic burst amplitude, rate of rise, and inspiratory duration (T_i) were conducted on a burst-to-burst basis. For MSEn, statistical significance was initially evaluated using one-way analysis of variance with repeated measures, followed by Dunnett’s post hoc analyses for comparisons to scale factor = 1. Once a significant scale factor was identified under baseline, DLH, and recovery conditions, statistical significance was evaluated using one-way analysis of variance with repeated measures, followed by Bonferroni post hoc analyses for comparisons among these conditions. For determination of the inspiratory burst duration required to fully capture the dynamics underlying central inspiratory network complexity, statistical significance was evaluated using one-way analysis of variance with repeated measures, followed by Dunnett’s post hoc analyses for comparisons to the total inspiratory burst. Finally, statistical significance for the effects of down-sampling on ApEn were evaluated using two-way analysis of variance with repeated measures, followed by
Dunnett’s or Bonferroni post hoc analyses for multiple comparisons. The criterion level for determination of statistical significance was set at $P < 0.05$ for all experiments.
RESULTS

*ApEn of pre-BötC-mediated chemical-induced excitation of phasic phrenic bursts.* In the current investigation, we examined the ApEn of inspiratory motor discharge recorded during baseline conditions and in response to pre-BötC-mediated chemical-induced excitation of phasic phrenic nerve discharge in the *in vivo* anesthetized adult cat model. For our analyses, only DLH- and NaCN-induced elicitation of a rapid series of high-amplitude, rapid rate of rise, short-duration phrenic bursts were studied; an example showing a representative response to unilateral microinjection of DLH into the pre-BötC on phasic phrenic nerve discharge is provided in Fig. 1 (similar modulation was evoked by unilateral microinjection of NaCN into this region; not shown). The quantitative details associated with these patterning and timing changes (*e.g.*, burst amplitude, burst frequency, $T_I$, $T_E$, rate of rise) have been characterized in our previous investigations (50,52,53), and will not be summarized again here. Included in Fig. 1 (panel B), however, are the patterning and timing characteristics elicited in response to DLH-induced activation of the pre-BötC on a burst-to-burst basis for the experiment presented in panel A. These data are included in order to demonstrate the degree of variability (coefficient of variation, CV) in the time series data. As can be seen in Fig. 1B, the magnitude of variability associated with these burst-to-burst patterning and timing variables is small during basal phrenic nerve discharge (*i.e.*, CV $\leq 4\%$, except $T_E$), and there is a slight reduction in the magnitude of variability (smaller CV) for the DLH-induced high-amplitude bursts and an enhancement in the magnitude of variability (larger CV) for the DLH-induced rate of rise, $T_I$, and $T_E$. Correlation analysis between these individual patterning and timing variables and ApEn for both the eupneic and the DLH-induced modified burst patterns (on a burst-to-burst basis) demonstrates that these variables are poorly correlated with ApEn (amplitude, $R^2 < 0.12$; rate of rise, $R^2 < 0.14$; $T_I$, $R^2 <$
0.08; $T_e$, not examined), suggesting that even though the patterning and timing characteristics are markedly altered during the DLH-induced modified phrenic bursts, these indicies alone do not directly provide insight into the underlying dynamics contained within the inspiratory burst. Thus, we evaluated the ApEn associated with the total inspiratory burst as well as the 1st half and the 2nd half of the inspiratory burst in order to provide insight into the complexity of the central neural network generating inspiratory motor output, including the network characteristics associated with early versus late portions of inspiratory bursts, exhibiting both eupneic and high-amplitude, rapid rate of rise, short-duration burst patterns.

In conjunction with the shift from an augmenting pattern of phrenic nerve discharge, which was observed under baseline conditions, to the high-amplitude, rapid rate of rise, short-duration phrenic bursts elicited by unilateral microinjection of DLH or NaCN into the pre-BötC, there was a marked reduction in ApEn. This decrease in ApEn was maintained throughout the duration of modified phrenic nerve discharge, and returned to (and in some cases, transiently exceeded) baseline values with recovery of phrenic nerve discharge back to the eupneic discharge pattern. Examples showing these burst-to-burst pre-BötC-mediated DLH- and NaCN-induced changes in ApEn are provided in Figs. 2 and 3, respectively, for the total inspiratory burst (panel A) and the 1st half and 2nd half of the inspiratory burst (panel B). It should be noted that the ApEn data provided in Fig. 2 correspond to the experimental observations shown in Fig. 1. In these examples, the ApEn values for the total inspiratory burst were reduced in response to microinjection of DLH and NaCN into the pre-BötC by ~53% and ~45%, respectively. A similar reduction in the magnitude of ApEn was observed for the 1st half versus the 2nd half of the inspiratory burst, and little burst-to-burst variation was noted between the two halves of the
inspiratory burst. The overall effect of DLH-induced activation of the pre-BötC on ApEn is summarized in Fig. 4 (panel A, total inspiratory burst; panel B, 1st half and 2nd half of inspiratory burst) for all 10 experiments using DLH. In each of the experiments conducted, DLH-induced stimulation of this region significantly reduced ApEn, resulting in a decrease of ~33% for the total inspiratory burst (P < 0.001) and ~39% for both the 1st half and 2nd half of the inspiratory burst (P < 0.001). The reduction in ApEn returned to baseline levels within ~1-2 minute after DLH microinjection, and this corresponded to the return of the eupneic discharge pattern.

MSEn of pre-BötC-mediated chemical-induced excitation of phasic phrenic bursts. Although the above results demonstrate a reduction in ApEn in response to microinjection of DLH or NaCN into the pre-BötC, the measure of ApEn is based on a single-scale, and therefore, it does not take into account temporal fluctuations in the underlying dynamics. To overcome this limitation, we used the measure of MSEn to further characterize the complexity of the central inspiratory network and its corresponding temporal variability in the underlying dynamics. As with our analysis of ApEn, we examined the MSEn of inspiratory motor discharge recorded during baseline conditions and in response to pre-BötC-mediated chemical-induced excitation of phasic phrenic nerve discharge. The results of these MSEn analyses are provided in Fig. 5 (same data sets shown in Figs. 2 and 3) and Fig. 6. Similar to the observations using the ApEn method, microinjection of DLH (panel A) or NaCN (panel B) into the pre-BötC resulted in a marked reduction in MSEn; however, increasing the scale factor above “scale 1” resulted in an increased MSEn value for both baseline and recovery data, with less of an effect on the DLH and NaCN data. The effect of increased scale factor was observed for the total inspiratory burst as well as for the 1st half and the 2nd half of the inspiratory burst, and this effect appeared to be slightly
smaller (or delayed in onset) for the 2nd half of the inspiratory burst as compared to the 1st half. The overall effect of DLH-induced activation of the pre-BötC on MSEn is summarized in Fig. 6 (panels A1-B1, total inspiratory burst; panels A2-B2, 1st half of the inspiratory burst; panels A3-B3, 2nd half of the inspiratory burst) for all 10 experiments using DLH. For these data, increasing the scale factor to ≥2 significantly increased the value of MSEn, with the greatest effect being observed on baseline and recovery values. Thus, although MSEn was reduced during DLH-induced high-amplitude, rapid rate of rise, short-duration phasic phrenic bursts at all scale factors, including a scale factor of 1, at the first significant scale factor, the magnitude of this reduction was enhanced (Fig 6B).

Effect of burst duration and sampling on ApEn. All of the above observations are based on analysis of the total inspiratory burst, the 1st half of the inspiratory burst, and the 2nd half of the inspiratory burst; however, we also wanted to evaluate how much of the inspiratory burst is required to fully capture the dynamics underlying network complexity of the central inspiratory neural controller. To assess this, we examined the effects of successive 10% reductions in T1 of the total inspiratory burst on ApEn using data obtained during both basal and DLH-induced excitation of phasic phrenic nerve discharge. For these analyses, we also compared the effects of successive 10% reductions in T1 from the beginning with those from the end of the burst (i.e., forward and backward reductions) on ApEn. The results from these analyses are provided in Fig. 7. It should be noted that although these analyses were designed to characterize the dynamics underlying network complexity, they also provided insight into the network characteristics generating early versus late portions of inspiratory bursts, which exhibit a eupneic discharge pattern as well as those exhibiting a high-amplitude, rapid rate of rise, short-duration
burst pattern. Under baseline conditions, regardless of whether the beginning or end of the burst was eliminated in successive 10% increments, at least 60% of the inspiratory burst was required to fully capture the dynamics underlying network complexity and provide an adequate index of ApEn of the central inspiratory neural controller. In contrast, during DLH-induced excitation of phasic phrenic bursts, differences between forward and backward successive 10% reductions in T_I were noted. For elimination of successive 10% increments from the beginning of the burst, at least 50% of the inspiratory burst was required to fully capture the dynamics underlying network complexity while for elimination of successive 10% increments from the end of the burst, at least 80% of the inspiratory burst was required.

We also evaluated the effects of down-sampling the original (digitally band-pass filtered) data set on ApEn of the total inspiratory burst during basal and DLH-induced excitation of phasic phrenic nerve discharge to gain insight into the appropriate sampling parameters required to adequately capture the underlying (fast) dynamics of inspiratory motor output. Although previous studies (based on heart rate variability data) have suggested that the ApEn method is fairly insensitive the number of data points in a time series as long as there are at least 100 data points, the dynamics of the central inspiratory network are much faster than those associated with heart rate variability; therefore, it is necessary to use higher sampling rates for recording inspiratory motor discharges in order to adequately capture the underlying dynamics. Thus, 100 data points from an inspiratory burst recorded in the anesthetized adult cat (based on a sampling rate in accordance with the Nyquist theorem, i.e., twice the highest frequency contained within the signal) would correspond to only a short duration of the inspiratory burst (i.e., ~5-10% T_I). Therefore, we used a down-sampling procedure to examine the effects on ApEn of reducing the
number of data points in the inspiratory burst. Results from these analyses are shown in Fig. 8, and they correspond to down-sampling the original (2 kHz sampled) data by a factor of 2 (to obtain 1 kHz), 4 (to obtain 500 Hz), and 8 (to obtain 250 Hz). Under baseline conditions, the highest value of ApEn is observed when the data are down-sampled to 500 Hz, and lowest value of ApEn is observed when the data are not down-sampled from the original 2 kHz sampling rate. This reduction in ApEn at the 2 kHz sampling rate most likely reflects the fact that sampling in excess of the *Nyquist theorem* adds no new information, and thus, it serves to dilute the underlying dynamics. In contrast to the effects of down-sampling on baseline data, during DLH-induced excitation of phasic phrenic bursts, the highest value of ApEn is observed when the data are down-sampled to 1 kHz, and lowest value of ApEn is observed when the data are down-sampled to 250 Hz. In fact, during DLH-induced excitation of phasic phrenic bursts, ApEn is significantly lower for the only 250 Hz down-sampled data, presumably due to the inability to adequately capture the underlying dynamics as a result of under-sampling the signal at this sampling rate.
DISCUSSION

We have demonstrated that both chemical stimulation and focal hypoxia in the pre-BötC, which elicit a rapid series of high-amplitude, rapid rate of rise, short-duration phrenic bursts, reduce inspiratory network complexity as assessed by the measures of ApEn and MSEn. The reduction in ApEn occurred in conjunction with the shift from the augmenting pattern of phrenic nerve discharge observed under baseline conditions to the modified phasic phrenic burst pattern elicited by microinjection of either DLH or NaCN into the pre-BötC, and was observed for all of the DLH- and NaCN-induced modified phrenic bursts. Further, with recovery of phrenic nerve discharge back to the eupneic discharge pattern, ApEn returned to baseline values. Similar to these effects, MSEn was reduced during the modified phasic phrenic bursts, but the magnitude of the reduction was enhanced with a scale factor above scale one. In addition, the influence of scale factor was greater for the eupneic pattern of discharge observed under baseline and recovery conditions than for the high-amplitude, rapid rate of rise, short-duration phrenic bursts seen in response to chemical-induced activation of the pre-BötC, suggesting that the reduction in complexity during the pre-BötC-induced modified phrenic bursts is accompanied by a decrease in temporal variability. Taken together, these observations, which demonstrate that activation of the pre-BötC reduces inspiratory network complexity, provide direct evidence for a role for the pre-BötC in regulation of complex respiratory dynamics.

Basal Discharge versus pre-BötC-induced Modified Phasic Phrenic Bursts

The findings of the current investigation suggest that the central inspiratory neural network generating the high-amplitude, rapid rate of rise, short-duration phrenic bursts is a less complex network than that producing the eupneic pattern of phrenic nerve discharge. Thus, based on the
ApEn and MSEn values calculated, the eupneic pattern of discharge, which is associated with higher values of ApEn and MSEn, reflects an inspiratory neural network with a higher degree of irregularity or greater randomness while the modified inspiratory burst pattern, which is associated with lower values of ApEn and MSEn, reflects an inspiratory neural network with a higher degree of regularity and predictability. Further, based on the measure of MSEn, the lower entropy values obtained during the modified inspiratory burst pattern are accompanied by lower temporal variability than that seen during the eupneic pattern of discharge. From these observations, we suggest that DLH- and NaCN-induced activation of the pre-BötC reconfigures the inspiratory neural network, resulting in a shift from the eupneic pattern of inspiratory motor discharge to the high-amplitude, rapid rate of rise, short-duration inspiratory motor discharge pattern, and that the “less complex network”, which produces this modified inspiratory burst pattern, exhibits a higher degree of order or synchrony among the inspiratory brainstem neurons that are responsible for the patterning of inspiratory motor discharge. Consistent with this behavior are preliminary observations from our laboratory demonstrating that high frequency oscillations (HFO), which serve as an index of short-time scale inspiratory phase synchronization proposed to be generated by medullary inspiratory neurons (17,18,20,21), are enhanced during DLH- and NaCN-induced pre-BötC-mediated modified phasic phrenic bursts as compared to HFO activity observed during the eupneic pattern of discharge (51). Our current data, however, do not exclude a potential contribution from phrenic motoneurons to the observed reduction in inspiratory network complexity. In addition, it should be noted that although the patterning changes associated the DLH-induced modified inspiratory bursts were poorly correlated with ApEn, it is this “less complex network” that generates this modified burst pattern. Thus, the patterning characteristics, which are related to the variance of the time series signal, are
incorporated into the burst configuration, and therefore in the calculation of ApEn, the latter of which is invariant to the variance of the signal because it evaluates all of the underlying dynamics contained within the inspiratory burst.

In the current investigation, we set out to characterize not only the complexity of the inspiratory neural network generating the entire inspiratory motor burst but also the complexity of the inspiratory neural network generating early versus late portions of the inspiratory burst. Since early versus late portions of the inspiratory burst exhibit quite different patterning characteristics, it has become common practice to segment the inspiratory burst for evaluation and characterization of the dynamics underlying the inspiratory neural control system. Numerous studies examining the dynamics of spectral activity during the course of inspiration, for example, have compared the 1st half versus the 2nd half of the inspiratory burst or smaller segments (e.g., 100 ms or 250 ms) of the inspiratory burst (8,9,17,19,21,32,44,45,47,56) in order to better characterize differences in network activity underlying these various burst segments. In the current investigation, we compared the ApEn and MSEn of the 1st half versus 2nd half of the inspiratory burst under baseline conditions and during the modified phasic phrenic bursts to evaluate the complexity of the inspiratory neural network responsible for generation of these early versus late portions of these two inspiratory burst patterns. Based on the measure of ApEn, no differences between the early versus late portions of the inspiratory burst under either baseline conditions or during the modified phasic phrenic bursts were identified, although the ApEn values for the 1st and 2nd halves of the inspiratory burst were lower than those observed for the total inspiratory burst. This observation suggests that the inspiratory neural network generating the 1st and 2nd halves of the inspiratory burst, although not different in the degree of complexity
from each other, may be somewhat less complex than that generating the total inspiratory burst. In addition, in some cases, the magnitude of the reduction in ApEn in response to DLH- or NaCN-induced activation of the pre-BötC was slightly greater for the 1\textsuperscript{st} and 2\textsuperscript{nd} halves of the inspiratory burst as compared to the total inspiratory burst. With respect to the MSEn method, some subtle differences between the 1\textsuperscript{st} half versus the 2\textsuperscript{nd} half of the inspiratory burst were identified, as this method takes into consideration not only the entropy but also the temporal variability in the underlying dynamics (22). Thus, based on the measure of MSEn, as the scale factor increased, a slightly smaller (or delayed onset) effect for the 2\textsuperscript{nd} half of the inspiratory burst as compared to the 1\textsuperscript{st} half was observed, suggesting that the inspiratory neural network generating the inspiratory burst exhibits differential temporal variability in the underlying dynamics as the burst progresses.

**Dynamics Underlying Inspiratory Network Complexity**

In addition to the above observations, we have also demonstrated that to adequately capture the dynamics underlying the complexity of the central inspiratory neural controller, it is necessary to consider the extent or duration of the burst to be evaluated and the sampling rate used for data acquisition. Previous studies (focusing predominantly on heart rate variability) have suggested that in order to estimate the complexity or regularity of a signal, as few as 100 data points (range = 100-5000) are adequate, as the ApEn method is fairly robust (10,40). Thus, most studies have relied on a specified number of data points (i.e., at least n=100) for the assessment of ApEn of the signal of interest. As some signals, such as heart rate variability, exhibit relatively slow dynamics (7,16,29-31,35), this approach may be adequate; however, for signals where the underlying dynamics are considerably faster and the sampling rates are therefore considerably
higher, this approach may be limited. For our inspiratory burst data, for example, 100 data points corresponds to ~5-10% of the inspiratory burst; thus, it is unlikely that the ApEn value associated with this segment of the burst would accurately reflect the complexity of the inspiratory neural network. In fact, segmenting the inspiratory burst by successive 10% reductions in T₁, revealed that at least 50-60% of the inspiratory burst must be included in the evaluation of ApEn in order to adequately capture the dynamics underlying inspiratory motor output and characterize the complexity of the inspiratory neural network. These successive 10% reductions in T₁, which were performed from both the beginning and end of the burst also revealed differences in the network dynamics underlying the early versus later portions of the DLH-induced, but not basal, phrenic bursts, suggesting that in order to adequately capture the dynamics of the DLH-induced modified inspiratory neural network, it may be necessary to include even a greater portion of the modified inspiratory burst in the evaluation of ApEn although the duration to be included will depend upon whether the early versus late portions of the burst are included. Thus, based on the above observations, it appears that a more appropriate index to evaluate ApEn would be to utilize a specified burst duration (i.e., minimum of 60% of T₁) instead of a specified number of data points in order to adequately capture the dynamics underlying the inspiratory burst, assuming adherence to the Nyquist theorem.

Further, to adequately capture the dynamics underlying the inspiratory burst, it appears that the data must be sampled in accordance with the Nyquist theorem, as both over-sampling and under-sampling appear to negatively affect the measure of ApEn. It should be noted, however, that in our experiments, the parameters m and r were held constant for ApEn analyses, and as a result, they may not have been optimal for all sampling rates evaluated. Sampling in excess of the
Nyquist theorem appears to be detrimental to the assessment of ApEn, presumably because it adds no “new” information in the additional data points. Although over-sampling does not appear to be detrimental to other aspects of temporal or spectral analyses, in the current investigation, we found that over-sampling may mask the real dynamics of the time series by diluting the calculated dynamics. In our experiments evaluating the effects of down-sampling on ApEn, we found that down-sampling the inspiratory burst data obtained using the original 2 kHz sampling rate to 1 kHz or 500 Hz resulted in an increase in ApEn, and that this effect was greater for the more complex signal seen during the basal eupneic pattern of inspiratory motor, as would be expected if the above explanation is correct. Additional support for this detrimental influence of over-sampling on ApEn comes from our recent computer simulation experiments, in which we demonstrated that adding data points which contain no information (e.g., zero-padding) reduces the ApEn value (unpublished observations). It should also be noted that under-sampling is also detrimental to the assessment of ApEn; however, this affect is presumably due to the inability to adequately capture the underlying dynamics because the low sampling rate does not accurately represent the underlying signal. Thus, the sampling parameters used should be appropriate to adequately capture the underlying (fast) dynamics of inspiratory motor output, without over- or under-sampling the signal. These observations in conjunction with those described above for burst duration suggest that it may be most appropriate to select a specified inspiratory burst duration, with the signal acquired at sampling rate in accordance with the Nyquist theorem, as opposed to selecting a specified number of data points (which is the general practice), for the evaluation of ApEn and network complexity in the inspiratory neural control system.
**ApEn versus MSEn**

In the current investigation, two measures of entropy, ApEn and MSEn, were evaluated in order to characterize the complexity of the central inspiratory neural network generating the basal eupneic pattern of phrenic nerve discharge and the high-amplitude, rapid rate of rise, short-duration phrenic burst pattern elicited by DLH- and NaCN-induced activation of the pre-BötC. The ApEn method was developed by Pincus (36), and introduced as a measure to quantify and describe the complexity of physiological signals in health and disease. This measure of complexity has been used since its introduction in numerous studies focusing on cardiac dynamics (e.g., 24,27,28,36-38,40,46), and more recently in studies evaluating respiratory function (4-6,11-13,23,57). For studies examining respiratory function, most have focused on breathing irregularity (i.e., ApEn of respiratory rate and/or tidal volume) among various patient populations (e.g., patients being weaned from mechanical ventilation, 23; patients with panic disorder, 13,57) or during waking and various sleep stages (11,12), and they have demonstrated greater breathing irregularity (i.e., higher ApEn values) among patient populations versus control subjects and more regular respiratory movements (i.e., lower ApEn values) during slow-wave (stage IV) sleep than during other stages of consciousness. Three recent studies, however, have focused on the complex dynamics underlying respiratory (phrenic nerve discharge) patterns, and have reported a reduction in respiratory network complexity (i.e., lower ApEn values) with maturation (albeit transient), severe hypoxia, and the early stages of re-oxygenation (4-6). Although some of the above studies have suggested that the observed changes in ApEn arise from alterations in the brainstem respiratory neural controller, including the rhythm-generating network (4-6,13) (although higher centers such as cortical and limbic areas which influence respiration could also exert an effect; 13, but see 3), none of these studies directly examined the
influence of specific respiratory regions in the brainstem on respiratory network complexity. In the current investigation, we have shown that during pre-BötC-mediated excitation of phasic inspiratory bursts, the ApEn of phrenic nerve bursts is reduced, suggesting a role for this region in the regulation of complex respiratory dynamics; our results, however, provide no insight into the role of this region in the modulation of ApEn observed either in the patients described above or in response to severe hypoxia and re-oxygenation during maturation.

Since complex dynamics typically exhibit feature patterns on multiple spatial and temporal scales, which are not taken into consideration by the ApEn method, Costa et al. (22) introduced the MSEn analysis method to overcome the limitations of conventional entropy (e.g., ApEn) calculations. They proposed that the MSEn method would provide a more meaningful assessment of the complexity of physiological dynamics than other entropy approaches because it could be used to quantify the regularity of the physiological signals of interest over multiple time scales. To demonstrate the robustness of the MSEn algorithm, they used this approach to identify differences in heartbeat intervals that were not revealed by the ApEn approach (presumably because it is based on single-scale analysis) between healthy subjects and congestive heart failure (CHF) and atrial fibrillation (AF) patients. Based on application of the MSEn method, they found that the AF patients exhibited the highest entropy values for a scale of one (which corresponds to ApEn), and that the lower entropy values corresponding to the healthy and CHF patients overlapped. By increasing the scale factor, the entropy value for the healthy subjects increased while that of the AF patients fell to the level seen for the CHF patients, resulting in a significantly higher MSEn value for the healthy subjects as compared to MSEn values for the AF and CHF patients. The differences in the MSEn values observed at the larger
scale factors are consistent with the expected levels of complexity in the underlying physiological dynamics associated with cardiac interbeat intervals (22), as many pathological conditions lead to a loss of complexity in cardiac dynamics (22,24,37,38,40).

For the current investigation, comparison of the results obtained from the ApEn and MSEn approaches revealed that both entropy measures identified the primary change (\textit{i.e.}, reduction) in inspiratory neural network complexity in response to DLH- and NaCN-induced activation of the pre-BötC, which shifted the augmenting pattern of phrenic nerve discharge to the high-amplitude, rapid rate of rise, short-duration phrenic burst pattern. This is not surprising since the measure of MSEn used in the current investigation was based on a simple modification of the ApEn method; however, the results from the MSEn approach, which were generally similar to those obtained using the ApEn method, did reveal a slightly greater reduction in MSEn between the basal discharge and the DLH- and NaCN-induced modified burst pattern as well as a reduction in temporal variability during the modified bursts and a slightly smaller (or delayed onset) effect of scale factor for the 2\textsuperscript{nd} half of the inspiratory burst as compared to the 1\textsuperscript{st} half. These differences, however, do not appear to be substantial enough to warrant the additional computation time required for the determination of MSEn; thus, we believe that our results indicate that evaluation of ApEn alone may have been sufficient to characterize the effects of the pre-BötC-mediated modulation of inspiratory motor discharge on the complexity of the central inspiratory neural network.
Summary and Conclusions

In summary, we have demonstrated that both DLH- and NaCN-induced activation of the pre-BötC reduce ApEn and MSEn, two statistical indices used to represent the complexity of the central neural network generating inspiratory motor output. This reduction in network complexity was associated with the shift from the augmenting pattern of phrenic nerve discharge observed under baseline conditions to the high-amplitude, rapid rate of rise, short-duration phrenic burst pattern elicited by these focal perturbations in the pre-BötC. Our observations further suggest that to adequately capture the dynamics underlying inspiratory motor discharge and the complexity of the inspiratory neural network, it is necessary to evaluate a sufficient duration of the inspiratory burst (e.g., minimum of 50-60% T_i) using appropriate sampling parameters instead of relying on a pre-set number of data points (e.g., n=100-5000) as suggested in previous studies (focusing predominantly cardiac dynamics). Thus, our findings indicate that the pre-BötC, which is the primary locus for respiratory rhythm generation, may participate in the regulation of complex respiratory dynamics.
ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

**Figure 1.** A: Example showing a rapid series of high-amplitude, short-duration phasic phrenic bursts elicited by microinjection of DLH into the pre-BötC. B: Patterning and timing characteristics for phrenic burst data associated with the example in panel A. Data provided correspond to burst-to-burst (B1) amplitude, (B2) rate of rise, (B3) inspiratory duration (T_I), and (B4) expiratory duration (T_E) along with the coefficient of variation (CV) for baseline (BL), DLH-induced, and recovery (Rec) conditions. Note: This trace corresponds to data presented in Figs. 2 and 5.

**Figure 2.** Example demonstrating the effects of DLH-induced activation of the pre-BötC on ApEn of the phasic phrenic bursts. Microinjection of DLH into the pre-BötC elicited a reduction (~54%) in (A) ApEn_{total} and (B) ApEn\_1st (circles) and ApEn\_2nd (squares) during the rapid series of DLH-induced high-amplitude, rapid rate of rise, short-duration phasic phrenic bursts.

**Figure 3.** Example demonstrating the effects of NaCN-induced activation of the pre-BötC on ApEn of the phasic phrenic bursts. Microinjection of NaCN into the pre-BötC elicited a reduction (~44%) in (A) ApEn_{total} and (B) ApEn\_1st (circles) and ApEn\_2nd (squares) during the rapid series of DLH-induced high-amplitude, rapid rate of rise, short-duration phasic phrenic bursts.

**Figure 4.** Summary data showing the effects of DLH-induced activation of the pre-BötC on ApEn of the phasic phrenic bursts. During the series of DLH-induced high-amplitude, rapid rate of rise, short-duration phasic phrenic bursts, (A) ApEn_{total} was reduced by ~35% and (B) ApEn\_1st and ApEn\_2nd were reduced by ~39%. Asterisks represent a statistically significant difference (P <
between the DLH-induced effect versus the pre-injection baseline (BL) and recovery values (Rec).

Figure 5. Examples demonstrating the effects of DLH- and NaCN-induced activation of the pre-BötC on MSEn of the phasic phrenic bursts. A,B: Microinjection of DLH (A) and NaCN (B) into the pre-BötC elicited a marked reduction in (A1,B1) MSEn\textsubscript{tot}, (A2, B2) MSEn\textsubscript{1st}, and (A3, B3) MSEn\textsubscript{2nd}; however, the values of MSEn were dependent on the scale factor, with the greatest effect being observed for baseline and recovery data. Further, the effect of increasing scale factor, which was observed for MSEn\textsubscript{tot}, MSEn\textsubscript{1st}, and MSEn\textsubscript{2nd}, appeared to be slightly smaller (or delayed in onset) for MSEn\textsubscript{2nd}. Note. these data correspond to the same data shown in Figs. 2 and 3 for ApEn. Symbols: circles, DLH/NaCN; squares, baseline; diamonds, recovery.

Abbreviations: MSEn\textsubscript{tot}, total inspiratory burst; MSEn\textsubscript{1st}, 1\textsuperscript{st} half of the inspiratory burst; MSEn\textsubscript{2nd}, 2\textsuperscript{nd} half of the inspiratory burst

Figure 6. Summary data showing the effects of DLH-induced activation of the pre-BötC on MSEn of the phasic phrenic bursts. A: Microinjection of DLH into the pre-BötC elicited a marked reduction in (A1) MSEn\textsubscript{tot}, (A2) MSEn\textsubscript{1st}, and (A3) MSEn\textsubscript{2nd}, with the values of MSEn being dependent on the scale factor. For these data, increasing the scale factor to \( \geq 2 \) significantly increased the value of MSEn (tot, 1\textsuperscript{st}, and 2\textsuperscript{nd}), with the greatest effects being observed on baseline and recovery values; significant effects for MSEn\textsubscript{2nd} following DLH microinjection into the pre-BötC were observed at scale \( \geq 6 \). Symbols: circles, DLH; squares, baseline; diamonds, recovery. * \( p < 0.001 \) for MSEn at scale factor versus scale 1. B: Summary data demonstrating the effects of DLH-induced activation of the pre-BötC on (B1) MSEn\textsubscript{tot}, (B2) MSEn\textsubscript{1st}, and (B3)
MSEn at scale factor 1 and the first significant scale factor under baseline (BL), DLH, and recovery (Rec) conditions. MSEn was reduced during DLH-induced phasic phrenic bursts at all scale factors (including scale 1), but the magnitude of this reduction was enhanced at the first significant scale factor. Symbols: circles, scale 1; triangle, scale 2; diamond, scale 6. Abbreviations same as in Fig. 5. * p < 0.001 for baseline and recovery versus DLH-induced responses

**Figure 7.** Example demonstrating the effects of successive 10% reductions in T1 of the total inspiratory burst on ApEn during basal and DLH-induced excitation of phasic phrenic nerve discharge. Under baseline conditions (squares, backward reductions in T1; diamonds, forward reductions in T1), at least 60% of the inspiratory burst is required to fully capture the dynamics underlying network complexity while during DLH-induced excitation of phasic phrenic bursts (circles, backward reductions in T1; triangles, forward reductions in T1), the extent of the burst required is dependent upon which end of the burst is eliminated. For elimination of successive 10% increments from the beginning and end of the burst, respectively, at least 50% and 80% of T1 are required to fully capture the dynamics underlying network complexity. * p < 0.05 for % of burst versus total burst for backward reductions; † p < 0.05 for % of burst versus total burst for forward reductions

**Figure 8.** Example demonstrating the effects of down-sampling the original (digitally band-pass filtered) data set on ApEn of the total inspiratory burst during basal and DLH-induced excitation of phasic phrenic nerve discharge. Down-sampling the original (2 kHz sampled) data under baseline conditions (squares) significantly increased ApEn at all sampling intervals
examined, with the highest value being observed when the data were down-sampled to 500 Hz. In contrast, during DLH-induced excitation of phasic phrenic bursts (circles), the highest value of ApEn was observed when the data were down-sampled to 1 kHz, and further down-sampling to 250 Hz significantly reduced ApEn. Further, the ApEn was significantly reduced during the DLH-induced modified phasic phrenic bursts as compared to baseline bursts at each sampling interval except the original 2 kHz sampling rate. * p < 0.001 for baseline versus DLH-induced phasic phrenic bursts at sampling interval indicated; top (baseline data) and bottom (DLH data) brackets denote p < 0.001 for sampling interval pairs indicated
A

ApEn_total

Phrenic Burst Number

DLH

B

ApEn

Phrenic Burst Number

DLH

Chen et al., Figure 2
Chen et al., Figure 3

(A) ApEn vs Phrenic Burst Number

(B) ApEn vs Phrenic Burst Number
Figure 4

A

B

Chen et al., Figure 4
Chen et al., Figure 5
Chen et al., Figure 6
Chen et al., Figure 7
Chen et al., Figure 8