The cycle-by-cycle assembly of respiratory network activity is dynamic and stochastic

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Stochastic assembly of respiratory network activity

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Abstract
Rhythmically active networks are typically composed of neurons that can be classified as silent, tonic spiking or rhythmic bursting based on their intrinsic activity patterns. Within these networks neurons are thought to discharge in distinct phase relationships with their overall network output, and it has been hypothesized that bursting pacemaker neurons may lead and potentially trigger cycle onsets. We used multielectrode recording from 72 experiments to test these ideas in rhythmically active slices containing the pre-Bötzinger complex, a region critical for breathing. Following synaptic blockade respiratory neurons exhibited a gradient of intrinsic spiking to rhythmic bursting activities, and thus defied an easy classification into bursting pacemaker and non-bursting categories. Features of their firing activity within the functional network were analyzed for correlation with subsequent rhythmic bursting in synaptic isolation. Higher firing rates through all phases of fictive respiration statistically predicted bursting pacemaker behavior. However, a cycle-by-cycle analysis indicated that respiratory neurons were stochastically activated with each burst. Intrinsically bursting pacemakers led some population bursts and followed others. This variability was not reproduced in traditional fully interconnected computational models, while sparsely connected network models reproduced these results both qualitatively and quantitatively. We hypothesize that pacemaker neurons do not act as clock-like drivers of the respiratory rhythm, but rather play a flexible and dynamic role in the initiation and stabilization of each burst. Thus, at the behavioral level each breath can be thought of as de novo assembly of a stochastic collaboration of network topology and intrinsic properties.

Keywords:
pre-Bötzinger complex
conductance-based simulation
network topology

Introduction
Common among many models of neural rhythmogenesis is the incorporation of autonomously rhythmic neurons (bursting pacemakers) as potential contributors to
network oscillations (Ramirez et al. 2011, Koch et al. 2011; Garcia et al. 2011). These pacemakers have been elemental in descriptions of a variety of rhythmic neural circuits underlying a range of motor behaviors in invertebrates (Kristan et al., 2005; Selverston, 2010; Zhao et al., 2010) and mammals (Ramirez et al. 2004; Brocard et al., 2010; Harris-Warrick, 2010) as well as both physiological and pathophysiological cortical rhythms (Chagnac-Amitai and Connors, 1989; van Drongelen et al., 2006; de Kock and Sakmann, 2008; Higgs and Spain, 2009; Marcuccilli et al., 2010; Wang, 2010). In the pre-Bötzinger complex (preBötC), a rhythmically active network which is essential for breathing (Smith et al., 1991, Tan et al. 2008; Ramirez et al. 1998), pacemakers have figured prominently, though often controversially, in causal explanations for the generation of the breathing rhythm.

While it has been suggested that pacemaker behavior in individual neurons might be a discrete epiphenomenon of a continuous distribution of intrinsic currents (Butera et al., 1999a; Del Negro et al., 2002a), the distinction between pacemakers and non-pacemakers has typically been treated as unproblematically categorical, though conditional and weak pacemaking has sometimes been invoked for borderline cases (Smith et al., 1991; Stil et al., 2009; Viemari and Tryba, 2009). The hypothesis that pacemakers are critical for rhythmogenesis has led to two interrelated predictions about the cycle-by-cycle behavior of these cells within the network: (1) The capability of such neurons to exhibit ectopic bursts might allow them to kindle global activity on some cycles (Ramirez et al., 2004), or their intrinsically generated ramping pre-inspiratory activity may trigger individual population events (Rekling et al., 1996); (2) Their firing sequence within each population burst cycle is invariably determined by each cell’s endowment of intrinsic currents as illustrated in some modeling studies (Butera et al., 1999a; Rybak et al., 2003).

Nonetheless, a precise characterization of how these neurons participate in ongoing activity under baseline conditions has not been published. In this study, we used multi-channel simultaneous extracellular recordings to analyze combined single-spike and population activity in the in vitro respiratory slice including the preBötC. We find that synaptically isolated neurons exhibit a range of rhythmic behavior that defies easy classification into pacemaker and non-pacemaker categories. In addition, though some
features of average firing behavior in control conditions are predictive of such intrinsic rhythmicity, other hypothesized features were not found to correlate with pacemaker behavior. Analysis of the cycle-by-cycle firing pattern of cells within the network did show some tendency for intrinsically rhythmic cells to lead some burst cycles, but variability in burst onset was dramatic. Network models using full cell-to-cell connectivity were unable to reproduce this variability under reasonable physiological assumptions, but sparsely connected networks were. In light of these results, we propose a novel model of rhythmogenesis in which intrinsically bursting neurons play a stochastic, dynamic and flexible role in the assembly of respiratory rhythms on a cycle-by-cycle basis within a sparse network topology.

Materials and Methods

Transverse medullary brain stem slices were taken from CD1 mice, P6-9, as described previously (Ramirez et al., 1996; Viemari and Ramirez, 2006). In accordance with protocols approved by the Institutional Animal Care and Use Committee at The University of Chicago, animals were deeply anesthetized with ether and rapidly decapitated. The brainstem was isolated in ice-cold, oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (aCSF) containing (in mM): 128 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 24 NaHCO₃, 0.5 NaH₂PO₄, and 30 D-glucose (pH 7.4). The brainstem was then glued to an agar block on the mounting plate of a Leica Vibratome with the rostral end up and the ventral face toward the blade. Serial slices proceeded until visual landmarks became clear. The cut of the rostral face of the experimental slice was typically 530 μm caudal to the opening of the 4th ventricle. From this position slices of between 600 and 620 μm were taken, and presumed to contain the preBötC. This slice was transferred directly to the recording chamber, where it was superfused with aCSF at a typical rate of 10 ml/minute, and allowed to equilibrate to experimental temperature (33-35°C). Robust population activity was initiated by raising the extracellular potassium concentration from 3 mM to 8 mM in two steps over 60 minutes.

In some experiments, fast chemical synaptic transmission was blocked using: 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) for non-N-Methyl-D-aspartic acid-
mediated glutamatergic synapses; 10 μM 4-(3-phosphonopropyl)piperazine-2-carboxylic acid (CPP) for N-Methyl-D-aspartic acid-mediated transmission; 1 μM strychnine for glycineric synapses; and 50 μM picrotoxin for γ-aminobutyric acid-mediated synapses.

**Extracellular Recording**

Extracellular neural activity from the transverse medullary slice was recorded on up to 16 channels using custom-made microwire gold-plated multi-electrodes (25 micron inner diameter, Formvar-insulated Nichrome; A-M Systems). Microwire bundles were twisted together using an electric drill, then sealed into a solid shank with epoxy and clear nail polish. The bundle was then cut flush with microscissors, producing an arbitrary, but roughly circular configuration of wires within the electrode tip, typically about 300 microns in diameter. Neural signals were amplified 1000X and band-pass filtered from 250 to 7500 Hz with a custom analog amplifier. The signals were then sampled at 20kHz, digitized and saved using a Digidata 1322A and AxoScope (Molecular Devices). To reduce superimposition of waveforms from presumptive single units on multiple channels, resulting signals were preprocessed using independent component analysis (FastICA, http://www.cis.hut.fi/projects/ica/fastica/). After preprocessing, action potential waveforms from distinct neurons were typically present on from 1 to 3 channels. These waveforms were detected and sorted using Offline Sorter (Plexon) with manual cluster cutting in tetrode-based feature spaces. Care was taken to follow non-stationarities in waveform shapes in assigning spikes to separate units, and auto- and cross-correlation histograms were examined as a check on sorting results (Lewicki, 1998). To generate an integrated population activity waveform, signals from multiple channels were averaged, rectified and digitally low-pass filtered at 30 Hz. With the exception of spike detection and sorting, all signal processing and statistical analyses were done in MATLAB (The MathWorks) using custom applications.

**Quantitative Analysis**

Some of the key methods for assessing the relationship between single cell firing behavior within the intact respiratory network and after synaptic isolation are schematized in Figure 1. Simultaneously recorded single-cell activity (Fig 1A) was
associated with spiking activity after synaptic blockade (Fig 1B). The integrated population activity waveform (Fig 1A, upper panel) for each experiment was characterized by a combination of four metrics of burst shape and timing variability: variance of interburst intervals; variance of burst peak amplitudes; burst shape variability; and signal-to-noise ratio. Slow baseline nonstationarity was eliminated by subtracting off a highly smoothed version of the signal, estimated by low-order polynomial regression. A threshold was set interactively for each recording based on the signal noise power during intraburst intervals. The resulting detected bursts were screened for artifacts and false detections, and the burst onset time was defined from the remaining waveforms as the time of threshold crossing. For the purposes of this study, no effort was made to distinguish high amplitude bursts (fictive sighs; Lieske et al. 2000) from other bursts, though such bursts were observed in many recordings.

The relationship between the activity of individual neurons and the phase of the fictive inspiratory rhythm was determined by various analyses of the firing times of action potentials relative to the onset of each population burst (as defined above). Peri-burst firing rate histograms, for example, were generated by counting the number of occurrences of spikes from a given cell within small time bins relative to burst onset. These counts were then normalized by the bin width and the number of bursts recorded to provide a measure of the average firing rate for a given cell relative to the fictive inspiratory phase. These histograms were categorized according to their most prominent feature of phase-dependent firing (as described in the companion paper, [Carroll et al. 2012]) into: non-respiratory; inspiratory; expiratory; and post-inspiratory (one cell was silent during control conditions). Manual scoring was done blind to subsequent behavior of the cell under isolated conditions. For other types of analysis, the behavior of each cell relative to the population activity was characterized by the estimation of instantaneous firing rate functions. A single vector of burst-relative spike times was created and the instantaneous firing rate was generated by a summation of Gaussian kernel functions, each centered on the time of one action potential. Compared to histogram estimation, which imposes arbitrary boundaries based on the selection of the number of time bins and produces a discontinuous function, kernel rate estimation can generate a function of arbitrarily fine smoothness avoiding the imposition of ad hoc boundaries within the data.
Instead, kernel estimation requires the specification of a smoothing parameter (in the Gaussian case, the sigma width parameter of the kernel function), which determines the level of temporal detail discernable in the rate estimate. The nominal smoothing width for most analyses was 0.03 seconds.

The phase dependent firing rate functions and histograms were estimated under baseline conditions and parameterized for waveform features expected to predict subsequent behavior after synaptic isolation. Spike rate parameters included: overall spike rate (Mean Spike Rate), spike rate during expiration (between population bursts; Exp. Spike Count), mean spike rate during inspiratory burst phases (Insp. Spike Count), spike rate during the post-inspiratory phase (Post Spike Count), and spike rate modulation by the inspiratory burst (the difference in spike rate between the peak/trough of inspiration and that during the expiratory phase; Insp. Modulation). Spike rate variability (across fictive respiration cycles) was assessed as spike count standard deviation during expiratory, inspiratory and post-inspiratory phases (Exp./Insp./Post Count Std.). Spike rate change over time was evaluated as the slope of the estimated firing rate function during the pre-inspiratory phase, inspiratory onset, and offset (Pre-insp./Insp./Post Slope). Additionally, timing of inspiratory burst onset and offset was evaluated at two threshold-crossing events (quarter-amplitude and half-amplitude; Quart. Over Time and Half Over Time). Phase-dependent firing rate functions were also parameterized by principal component analysis (as described in the companion paper [Carroll et al. 2012]) by projecting the dataset into the vector space defined to capture the most variance in a single dimension.

The behavior of the recorded cells after synaptic isolation was assessed by two methods designed to characterize intrinsic rhythmicity. Based on the idea that bursting behavior consists of relatively long interspike intervals (ISIs) interspersed with groups of relatively short intervals (Fig 1D), producing a bimodal ISI histogram (often more easily visualized on a logarithmic scale (Fig 1E), a burstiness metric was calculated using the set of ISIs from each cell during synaptic isolation (Fig 1E). This activity was also characterized by manual inspection of each spike raster and log_{10} transformed ISI histogram, assigning each cell heuristically to one of five burstiness classes: weakly active or with regular tonic activity (Weak/Tonic); irregularly tonic activity and no high-
frequency bursts (*Tonic/Irregular*); irregularly tonic activity with some bursts (*Tonic w/Bursts*); frequent bursts over tonic background activity (*Bursting*); and high frequency bursts with little interburst activity (*Strongly Bursting*). These categories were chosen to reflect the largely descriptive classification of intrinsic behavior that is common in previous studies.

**Simulations**

Networks of point-conductance neurons was simulated using single-cell parameters taken without modification from a series of influential papers by Butera et al. (1999a) and Rybak et al. (2001; 2003b). As in those papers, each cell is represented by a single-compartment conductance model with the standard fast sodium and delayed rectifier potassium currents for spike generation as well as a persistent sodium current and a passive leak current. Synaptic connections (all excitatory in this model) are generated by a single exponential time course current source against a zero mV reversal potential. Details of the single-cell model are presented in the Appendix. Sparse random networks were generated by iterating through all possible neuron pairs in either direction and creating a synaptic connection if a uniform pseudo-random number was generated which was less than or equal to a threshold value representing the mean proportion of connectedness for each cell. Neither the spatial location nor the specific size of individual neurons was modeled in this study. All the cells within this network are assumed to lie within a roughly 350-micron spheroid where all cells are at least theoretically mutually accessible with comparatively short axonal projections; therefore, axo-dendritic conduction delays were also not a part of this model.

The network simulation was custom-written in C++ with standard libraries (GNU gcc 3.4.4 on a Pentium class laptop running Windows XP). Integration was performed with a custom implementation of the 4th order Runge-Kutta method with a fixed time step of 0.01 ms. Typical simulations included 300 cells and ran for 90 seconds of model time, of which the first 30 seconds of activity was discarded as reflecting transient initial dynamics. The C++ code was neither particularly inefficient nor specifically optimized
for execution speed, and computational demands were rather high, with typical run times
for a single simulation of around 24 hours.

### Statistical Methods

The distributions of study variables were often non-Gaussian and therefore
characterized by median (for central tendency) and half the interquartile range (for
variability). Linear relationships between raw variables were typically assessed with
Kendall’s tau, a non-parametric measure of correlation. This statistic was considered
significant for $p<0.001$ (without any further correction for multiple comparisons). The
Kruskal-Wallis test was used for a non-parametric comparison of group medians with a
Bonferroni correction for post-hoc analysis (significant for an uncorrected $p<0.05$).

### Results

#### Intrinsic Rhythmicity and Burst-triggered Firing Rate Functions

The ability to generate intrinsic bursting was assessed in 892 cells from 72
experiments by bath application of a cocktail of antagonists that block chemical
glutamatergic and GABAergic synaptic transmission (CNQX, CPP, strychnine and
picrotoxin, for more details on synaptically isolating respiratory neurons, see Tryba et al.
2003, 2008; Tryba and Ramirez, 2006, 2004). Intrinsic rhythmic bursting under these
conditions was evaluated using two distinct methods: calculation of a metric based on the
interspike interval (ISI) distribution, and by manual inspection of the ISI histogram and
spike raster plots.

The ISI metric stratified cocktail spiking behavior along a unimodal distribution
with no clear divisions (Fig 2Aiii). Classifications of the phase-dependent behavior of
individual cells in control conditions did not clearly predict subsequent burstiness during
synaptic isolation (Fig 2Ai), though those identified as non-respiratory had a significantly
lower median burstiness score than those classified as inspiratory (3.6±0.4 vs. 3.9±0.4;
median±half interquartile range). Further, no clear relationship was seen between phase-
dependent control behavior, as parameterized by principal component analysis (Fig 2Aii), and the ISI burstiness metric.

Manual scoring separated spiking activity into five categories: weak or tonic firing (20.0 %), irregular tonic (with no high frequency spiking; 28.8%), tonic with some high frequency bursts (30.9%), bursting with tonic spiking between bursts (16.7%), strongly bursting without spiking between bursts (3.5%) as shown in Fig 2Biii. There was no clear relationship between manually classified control behavior and manually scored behavior in synaptic isolation (Fig 2Bi). Similarly, these categories did not correlate with control behavior defined by the first principle component of the set of firing patterns (Fig 2Bii).

To evaluate potential relationships between phasic activity patterns under control conditions (characterized by burst-triggered firing rate functions), several dimensions of control activity were parameterized for all active cells (i.e. more than 50 spikes in both control and cocktail conditions: n = 683) and these variables were compared with the two intrinsic bursting metrics by non-parametric correlation analysis (Kendall’s tau). Measured correlations were positive and significant against both burstiness metrics (p<0.001) for all variables except those related to burst timing (Fig 3A and B). The uniformity of correlations suggested a high degree of collinearity in the data, probably dominated by the influence of overall spike rate in the metrics of control and cocktail spiking behavior. Indeed, mean spike rate during control epochs (log_{10} transformed) was significantly correlated with mean spike rate during cocktail epochs (Kendall’s tau = 0.32; p <0.0001; data not shown).

As an alternate evaluation of the relationship between burst-triggered firing rate functions and subsequent intrinsic bursting (during synaptic blockade), firing rate functions corresponding to the most and least intrinsically rhythmic quartile (in the ISI metric) were extracted and averaged, producing an illustration of the characteristic firing rate function of the control behavior for those groups of cells. Though variability is high, the higher mean firing rates in most rhythmic cells is apparent through all phases (Fig 3C). To highlight possible differences in shape and timing of these average waveforms independent of mean and peak rate differences, the rate functions were normalized to unit amplitude and zero mean before being averaged again (Fig 3D). While the mean and
interquartile bounds are nearly overlapping in these two populations, a sharper and more pronounced burst onset is evident in the group that proves later to have intrinsic rhythm capability (Fig 3D).

**Cycle-by-Cycle Variability**

While the mean phase-dependent spiking behavior of cells during control conditions did not show a tendency for intrinsically bursting neurons to fire earlier in the inspiratory phase than other cells (see above), it is possible that this analysis of mean spiking behavior obscured important timing relationships between population bursts and the firing of cells found subsequently to be intrinsically rhythmic in synaptic blockade. To address this possibility, multicellular data was analyzed cycle-by-cycle to determine the identity of units which anticipate the population burst. As illustrated in a representative raster plot (Fig 4B), in which the firing of 18 simultaneously recording neurons is indicated with an instantaneous frequency color code, considerable variability in the cycle-by-cycle firing pattern of each cell is obvious. Some bursts (indicated by the population activity shown in gray) seem to be led by spiking in cell #17 while in other cycles, cell #14 or #13 leads. To quantify these timing relationships, a score representing the probability of a cell anticipating a population burst based on a range of definitions of “anticipate” (i.e. firing the earliest 1st, 2nd, 3rd etc. spike). These probabilities (Fig 4A) indicate that while some cells (#14 and #17, for example) often anticipate the population burst, none is invariantly ahead of all others. By sorting the cells in ascending order of their tendency to burst in a subsequent epoch of synaptic block, it can be seen that neurons that prove to have intrinsic bursting capabilities do not necessarily lead the population rhythm. Cell #18, for example, despite its score as the most intrinsically rhythmic, always follows the spiking of other cells, while cell #3 occasionally leads despite a lack of rhythmicity during synaptic block. Since the lead probability calculation is at least partially dependent on the mean spike rate, the relationship between these variables was assessed with correlation analysis (Fig 4C). Burst lead probability is strongly correlated with mean spike rate during control epochs (Kendall tau = 0.60,
p<0.0001) and more weakly correlated with rhythmicity in synaptic block (Fig 4D; Kendall tau = 0.24, p<0.0001).

An alternative perspective on the variability in the identity of cells which spike in anticipation of the population burst is to look at the variability (jitter) of the mean spike time of each cell over all cycles. The variance in this timing (log10 s^2) showed a wide range of values and was very weakly (though significantly) and negatively correlated with the tendency of the cell to show intrinsic rhythmicity in subsequent synaptic block (Fig 5A; Kendall tau = -0.16; p<0.0001). As suggested above (Fig 4B), even those cells which prove to be intrinsically rhythmic can show significant variability on a cycle-by-cycle basis. Figure 5B shows frequency histograms for periburst spike times for a randomly selected set of 10 cells which subsequently proved to be rhythmic in synaptic block. The first row shows the first spike times relative to the population burst onset, while the 3rd and 5th spike times are shown in subsequent rows (Fig 5B). From this figure, it can be seen that even intrinsically rhythmic cells may fire their first spike in a cycle 200-300 ms after the onset of the population burst.

Possible Sources of Cycle-by-Cycle Variability: Simulation Experiments

Conductance-based network simulations were conducted to determine if connectivity profiles might account for the cycle-by-cycle variability seen in the in vitro experiments. Network simulations using a standard model with full connectivity failed to reproduce variability in population activity seen experimentally (Fig 6A), but by increasing the size of the network while decreasing the probability of connections between cells (with a proportional increase in synaptic weight), population activity which mimicked experimental variability on several metrics could be generated (Fig 6B). These sparsely connected networks also generated cycle-by-cycle variability at the single cell level that was similar in scale to that recorded in vitro (Fig 6C). Other plausible sources of variability such as weak synaptic connections or membrane noise were simulated, but either failed to generate the observed statistics or did so only at the expense of realistic constraints on other parameters (data not shown).
Discussion

In characterizing the role of intrinsically bursting pacemaker neurons in network oscillations, the simplistic opposition between network and pacemaker theories of rhythmogenesis has given way to more nuanced models that incorporate essential elements from both camps (Ramirez et al., 2004; Selverston, 2010; Wang, 2010). These models are well exemplified in the preBötC, a neuronal network critical for breathing (Ramirez et al., 2004; Feldman and Del Negro, 2006). The data presented here indicate that the intrinsic rhythmic bursting of neurons in the preBötC can be predicted by the behavior of these cells during baseline conditions. Specifically, cells with higher mean firing rates in all phases of fictive respiration, with higher spike count variability across cycles and with more rapid burst onsets are also likely to spike more in synaptic block conditions. However, other commonly assumed aspects of burst timing or shape were not predictive of pacemaker activity. Thus, contrary to the most straight-forward model of pacemaker rhythmogenesis, the data presented here do not support an invariant role for pre-inspiratory activity on average or on a cycle-by-cycle basis in initiation network bursts (Rekling et al., 1996). Instead, pre-inspiratory spiking activity is profoundly variable for all neurons within the network, including those that prove to be capable of generating bursting pacemaker activity.

Indeed, approaching the question of burst onset from the point of view of simultaneously recorded neurons revealed a surprising degree of cycle-by-cycle variability both in the identity of neurons leading successive population bursts, and in the timing jitter of spike patterns of individual neurons followed across many cycles. Together with the results of the above analysis of average burst-triggered firing rate functions, several conclusions are suggested. Neurons that fire robustly under control conditions are more likely to fire robustly during synaptic isolation. Neurons with more robust firing under these conditions will also be more likely to exhibit spiking behavior that is clustered into bursts, though this distinction is quantitative rather than categorical. Though it is impossible to make mechanistic claims based on this extracellular evidence, this behavior is consistent with the hypothesis that neurons in this network are endowed with generally continuous distributions of membrane conductances that result in
qualitatively graded firing behavior (Del Negro et al., 2002b; Purvis and Butera, 2005; Koizumi and Smith, 2008). The question of how conductance ratios are established is of considerable interest not only for the respiratory network, but for neuronal networks in general (Hudson and Prinz, 2010; Zhao et al. 2010; Temporal et al. 2012; Khorkova and Golowasch, 2007; Brookings et al. 2012). Although particular combinations of conductances may interact in ways that produce dramatically discontinuous behavioral phenotypes, such discontinuities were not detected in our data. This may be a result of the limitations of the experimental approach used here, as other electrophysiological, immunohistochemical and anatomical evidence may be more sensitive to such distinctions. Intracellular recording, for example, can reveal the membrane potential trajectory that underlies spiking behavior, revealing depolarization envelopes that help distinguish phasic bursting behavior from clusters of spikes riding on more placid membrane potential waveforms. On the other hand, intracellular recordings can be characterized by a strong selection bias (as discussed for example in Peña et al., 2004), perhaps leading to overly rigid categorical distinctions. Therefore, the detail of an intracellular recording comes at the expense of limiting the number of cells that can be recorded simultaneously (especially for long periods) as well as the types of neurons that can be directly compared in a simultaneous recording. Additionally, intracellular whole-cell recording may also reduce the diversity of cellular behavior through dialysis of second messenger molecules and clamping of the intracellular calcium concentration.

The present finding of a high degree of burst onset timing variability, even among those cells subsequently confirmed as pacemakers, was in fact confirmed with intracellular data, suggesting that this phenomenon is not an artifact of the experimental methods used here. These data are inconsistent with models that have suggested that the onset timing of individual cells in each cycle is determined by their complement of intrinsic conductances and synaptic contacts, functioning as literal “pacemakers” on a cycle-by-cycle basis in rhythmically active networks. In contrast to that model, the stochastic assembly of rhythms from only sparsely synchronized individual neurons has also been recently emphasized as a more robust model for rhythmogenesis in the cortex (Wang, 2010). The considerable cycle-by-cycle variability found in the preBötC is also consistent with the notion that excitatory synaptic interactions play a critical role in
establishing the respiratory rhythm (Feldman and Del Negro, 2006; Del Negro et al., 2010).

It must be emphasized that our findings are also consistent with recent results in invertebrate neuronal networks, in which neurons are well identified. A large cycle-to-cycle variability was described, for example, in the well-defined Aplysia feeding network (Brezina et al. 2006; Horn et al. 2004; Lum et al. 2005). Indeed this variability caused a mismatch in some cycles between the animal’s movement and the requirements of the feeding behavior, but it was found that this strategy was optimal in an uncertain and changing feeding environment (Brezina et al. 2006). Clearly, mammalian breathing is very similar, as it needs to have the flexibility to adjust to changes in metabolic, environmental and behavioral conditions, including even the extremely complex demands of vocalization. Nonetheless, the Aplysia studies characterized variability at the behavioral level while our results point to variability at the level of component neurons in a network with a relatively regular output. Thus, we conclude that it is the rhythm generating mechanism that functions with a large onset-variability, which may or may not result in an irregular behavioral output. As for breathing *per se* we would expect that this behavior will not only be determined by the preBötC itself, but in addition also by other central and peripheral components of the wider respiratory network.

Our data suggest that each population burst is a dynamic and stochastic assembly of diverse neurons, rather than an orderly recruitment beginning with the most intrinsically excitable and finally arousing the most recalcitrant. These data in fact suggest a more fluid role even for pacemakers, which here have been shown to anticipate (perhaps kindle) some population bursts, but also follow the onset of many others, possibly contributing to stability or providing nonlinear amplification. This is not to suggest that some cells might not participate more frequently in one of these roles than the other based on intrinsic or synaptic properties. In fact, previous research has established the existence in this network of two classes of pacemakers with distinct conductances and responses to neuromodulators that differentially affect the frequency, stability and amplitude of network activity (Tryba and Ramirez, 2003; Viemari and Ramirez, 2006; Thoby-Brisson and Ramirez, 2001; Peña et al. 2004). It is important to note that, in contrast to these earlier studies, the current experiments were performed at
slightly higher (and presumably more physiological temperatures). The effect of
temperature on the respiratory network containing the preBöC has been well
characterized in two studies (Tryba and Ramirez 2003; 2004), where the network was
found to be very sensitive to changes in temperature but not to differences in absolute
steady-state temperature (Tryba and Ramirez 2003). This is very similar to the situation
in response to hypoxia, where the network responds very sensitively to changes in
oxygen levels, but less robustly to absolute differences in the steady-state oxygen levels
(Hill et al. 2011). Thus, it is not very likely that the difference in steady state temperature
of 3-5 degrees had a dramatic effect on the frequency of the overall network output.
Interestingly, at the cellular level it was also demonstrated that transient increases in
temperature resulted in increased burst frequency of bursting pacemaker neurons, while
the discharge of tonically firing neurons was not much affected (Tryba and Ramirez,
2003; 2004). These studies also demonstrated that at more physiological temperatures
some non-bursting, tonically spiking neurons turned into bursting neurons. Thus, while it
is conceivable that the number of bursting pacemaker neurons may be somewhat higher
in our study compared to in vitro studies performed at lower temperature, it must be
emphasized that none of these differences would be considered to be less physiological
than our prior in vitro studies. Also, while a 3-5 degree difference in steady state
temperature may have some minor network affects, it seems very unlikely that our
principle findings would be different. We thus conclude that the emerging picture of
respiratory rhythm generation indicated by our data is suggestive of the dynamic and
flexible role bursting neurons are thought to also play in cortical networks (Wang, 2010).
Indeed, the single-cell and network variability seen in these in vitro data is
difficult to reconcile with previous computational models of this network (Rybak et al.,
1997a, b, c; Butera et al., 1999a, b; Del Negro et al., 2001; Shevtsova et al., 2003). While
these models have generated population behavior with a high fidelity to many
experimental results, as well as generating a rich diversity of new hypotheses, they have
tended to produce activity which is much more rigidly deterministic and regular than that
from the multicellular in vitro recordings presented here. We hypothesized that activity
with more physiological variability could be generated with such models if the
connectivity profile were modified. Thus while previous models have typically used
networks with full cell-to-cell connectivity (though some have mentioned similar findings in more sparsely coupled network, these have not been presented in detail), we simulated networks with decidedly sparse interconnectivity, where each cell only had a 1% chance of connecting with another cell in the network. In order to generate stable global rhythms, larger populations of cells were required for these networks, and by comparing the variability of population activity seen in simulated sparse networks of varied sizes with that of in vitro networks, a Goldilocks network size of 300 cells was found to produce population rhythms with a just right level of variability by comparison with in vitro data. This population size is surprisingly consistent with estimates of the population size of the rhythmogenic network in situ (Rekling et al., 2000; Gray et al., 2001; Hayes and Del Negro, 2007). Indeed a recent study demonstrated that not only the frequency, but also the regularity of the respiratory rhythm was directly related to the number of active respiratory neurons within the preBötC (Hayes et al. 2012). This study estimated that the preBötC was comprised of 650 neurons. The authors also assumed that half of these neurons were inhibitory which would suggest 325 excitatory neurons, which is very consistent with our computer model. Furthermore, simulations that attempt to reproduce the observed variability in fully connected networks fail to do so under physiologically reasonable assumptions.

Our simulation results therefore suggest that sparse network connectivity is a plausible architecture to produce the dynamic and stochastic assemblies of cells seen in the multicellular data presented here. These networks not only qualitatively reproduced the patterns seen in spike rasters from in vitro networks, quantitative analysis of burst onset variability in these networks show that variability increases monotonically with increased sparseness until it reaches experimental levels at 1% connectivity. The degree of sparseness necessary in our simulations is indeed dramatically smaller than that reported (3/23; 13%) in what we believe is the only published paired whole-cell data from the preBötC (Rekling et al., 2000). However, there is some evidence that, in our hands at least, the preBötC is far less densely interconnected. Cross-correlation analysis from pairs of 10778 cells in the multicellular experiments described here produced a connectivity probability of only 1.0%. The reason for the discrepancy between this and previous findings remains unclear; but considering the distinctness of the methods and
preparations, is not all that surprising. It may also be that more moderate degrees of 
sparsity could exist in vivo but interact with other intrinsic or topological features to 
produce the variability described here. Alternatively, recent anatomical evidence from a 
slice culture preparation has been presented suggesting that the preBötC may be 
connected in a relatively complex topology that combines clusters of more densely 
coupled neurons with sparse inter-cluster connections (Hartelt et al., 2008). It remains 
unclear whether such a topology is functionally active in other preparations, or whether 
such a connectivity structure could produce the features of population activity identified 
in the current study.

The suggestion that the dynamics of rhythmical active networks might be non-
trivially influenced by network topology will not come as a surprise to those familiar with 
recent interest in a broad range of complex networks (Watts and Strogatz, 1998; Barabási 
and Albert, 1999). The findings presented here indicate a view of breath-to-breath 
respiratory rhythm generation that is dynamic and stochastic, involving the interaction of 
neurons with active membrane conductances embedded within networks of neurons 
connected by non-trivial topologies. Additional research will be required to determine 
whether these characteristics are present in more diverse rhythm generating circuits and 
how such characteristics may contribute to network robustness and flexibility.
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Conflict of Interest

Neither of the authors has any conflict of interest.

Author Contributions

MSC developed and applied experimental protocols, conducted data analysis and led manuscript preparation and review. JMR assisted with experimental and analytical design, manuscript preparation and manuscript review.
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Figure 1. Schematic of multielectrode recording and analysis of \textit{in vitro} rhythmic activity. Example spike time raster of 13 neurons recorded during several inspiratory population bursts under baseline conditions (A) and after synaptic isolation (B). Average population burst phase-dependent firing activity is parameterized on multiple features (C), which are tested for correlation with endogenous activity after synaptic block. For this comparison, a burstiness metric based on a characteristic pattern of long and short interspike intervals (D), and exemplified in a bimodal interspike interval histogram (E), is comprised of a $\log_{10}$ sum of the ratio of longest to shortest intervals (F).
Figure 2. Comparison of in-network firing patterns with two characterizations of intrinsic rhythmicity derived from recordings made in synaptic isolation. An intrinsic burstiness metric produces a broad unimodal distribution (Aiii) which is uncorrelated with the first principle component decomposition of in-network firing behavior (Aii) and only weakly related to manual categorization of control activity (Ai). Manual score of intrinsic behavior also produces a unimodal distribution (Biii) that is not clearly related to in-network behavior as defined by manual classification (Bi) or PCA (Bii).
Figure 3. Correlations between features of firing patterns during control epochs and subsequent behavior during synaptic blockade. Kendall tau shows prominent correlations between an algorithmic metric of burstiness (A) and manually scored burstiness classes (B) for features related to spike rate and variability, though not for features associated with the timing of burst-triggered single unit activity. Legend for A and B: Mean Spike Rate, average spike rate across all phases of population burst activity; Exp. Spike Count, spike count during expiratory phases of population activity; Insp. Spike Count, count during inspiratory phases; Post Spike Count, count during post-inspiratory period; Insp. Modulation, difference between inspiratory and expiratory phase spike counts; Exp./Insp./Post Count Std., standard deviations of spike counts during indicated phases; Pre-insp. Slope, slope of the firing rate function during the pre-inspiratory phase; Insp. Slope, slope during the inspiratory burst onset; Post Slope, slope during the inspiratory burst offset; Quart. Over Time, relative timing of the quarter-amplitude threshold crossing during the inspiratory phase; Half Over Time, relative timing of the half-amplitude threshold crossing. C) Mean (solid lines) and quartile bounds (dashed) of firing rate function for the most (purple) and least (orange) bursty 25% of cells. Means and interquartile bounds are shown after amplitude normalization and detrending in D.
Figure 4. Cycle-by-cycle activation of individual units in relation to population activity is stochastic. Spike rasters for 18 simultaneously recorded neurons (B), ordered along the ordinate axis by degree of burstiness exhibited in synaptic block, and color-coded for instantaneous firing rate (shown against population burst activity, background line in gray), and A) Probability of anticipating population burst activity for each recorded cell calculated over entire control epoch. C) Burst initiation probability (log10 transformed) of 688 cells is strongly related (Kendall tau = 0.60; p<0.0001) to overall spike rate in control epochs and more weakly (though significantly; tau = 0.24; p<0.0001) correlated with interspike interval metric of burstiness in synaptic block (D).
Figure 5. Burst onset timing is variable in individual neurons. Burst onset timing variability (A) is weakly, though significantly, anticorrelated with subsequent endogenous bursting (Kendall tau = -0.16; p < 0.0001). B) Periburst first \( n^{th} \) (for \( n = 1, 3 \) and 5; second axis) spike times for 10 randomly selected strong pacemakers confirms that burst onset in individual bursters can follow the population burst onset on many cycles.
Figure 6: Population activity and single cell onset variability are dependent on network sparsity. A) Four measures of variability in simulated population activity from sparsely connected (1% connection probability) networks of different sizes illustrate importance of larger networks in simulating physiological variability. Networks with only 50 cells produce overly irregular population rhythms (i) while networks with 500 units are unphysiologically regular (ii). B) The same metrics applied to population recordings from 21 in vitro experiments (example in i) indicate that the simulation experiments shown in A reach similar levels of irregularity at population sizes of 200-300 cells. In 300-cell models, variability in single cell burst onset timing (C) with respect to the population burst varies with network sparsity and approaches mean levels from 21 in vitro experiments (left) only when the connection probability is near 1%.
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Appendix

Network Model Description

The preBötC network in this study was simulated using single-cell models based on the Hodgkin-Huxley formalism. Each single-compartment cell included the following conductances: fast sodium ($NaT$); persistent sodium current ($NaP$); delayed rectifier potassium current ($K$); leak ($L$); and an excitatory synaptic ($syn$) conductance. Membrane potential was integrated over time ($t$) for each cell from the standard current-balance equation:

$$C\frac{dv}{dt} = -I_{NaT} - I_{NaP} - I_{K} - I_{L} - I_{syn}$$  

(1)

Where $I_x$, with $x \in \{NaT, NaP, K, L, syn\}$, denote the whole-cell current for each channel type, and $C$ is the cell capacitance. For these simulations $C = 36.2$ pF. These ionic currents are determined as follows:

$$I_{NaT} = \bar{g}_{NaT}m_{NaT}^3h_{NaT}(V - E_{Na})$$

$$I_{NaP} = \bar{g}_{NaP}m_{NaP}h_{NaP}(V - E_{Na})$$

$$I_{K} = \bar{g}_{K}m_{K}^4(V - E_{K})$$

$$I_{L} = g_{L}(V - E_{L})$$

$$I_{syn} = g_{syn}(V - E_{syn})$$  

(2)

where $m_x$ (with $x \in \{NaT, NaP, K\}$) are activation variables, and $h_x$ (with $x \in \{NaT, NaP\}$) are inactivation variables, and maximal conductances denoted $\bar{g}_x$ for $x \in \{NaT, NaP, K\}$, and whole-cell leak and synaptic conductances $g_L$ and $g_{syn}$. Conductance values used in the present simulations included deterministic and Gaussian distributed values (in nS; mean ± standard deviation): $\bar{g}_{NaT} = 150, \bar{g}_{NaP} = 4.0 \pm 0.4, \bar{g}_{K} = 50.0 \pm 5.0, g_{L} = 2.0 \pm 0.2$ (calculation of $g_{syn}$ is described below). The reversal potential values are (in mV): $E_{Na} = 60, E_{K} = -96, E_{L} = -76$, and $E_{syn} = 0$. The kinetics of the inactivation variables were modeled in the traditional formalism based on voltage dependent functions for the steady-state and time constant in the dynamics equations:

$$\frac{dm_x}{dt} = \frac{(m_{\infty}(V) - m_x)}{\tau_{m_x}(V)}$$

$$\frac{dh_x}{dt} = \frac{(h_{\infty}(V) - h_x)}{\tau_{h_x}(V)}$$  

(3)
Where \( m_{\alpha x}(V) \) and \( h_{\alpha x}(V) \) denote the steady-state activation functions and \( \tau_{m x}(V) \) and \( \tau_{h x}(V) \) are the voltage dependent reaction rate functions. The steady-state activation sigmoid functions are parameterized by half-activation \( (V_{1/2}) \) and slope terms \((\sigma)\) for the respective kinetic variables:

\[
\begin{align*}
m_{\alpha x}(V) &= \frac{1}{1+e^{-\frac{(V-V_{1/2})}{\sigma_{mx}}}} \\
h_{\alpha x}(V) &= \frac{1}{1+e^{\left(V-V_{1/2}\right)^{\sigma_{hx}}}}.
\end{align*}
\]

For the current simulations the fast sodium conductance defined (in mV) as:

\[
\begin{align*}
V_{1/2}m_{Na T} &= -43.8, \\
V_{1/2}h_{Na T} &= -67.5, \\
\sigma_{mNa T} &= 6.0, \\
\sigma_{hNa T} &= 10.8. \\
\end{align*}
\]

Persistent sodium was modeled as:

\[
\begin{align*}
V_{1/2}m_{Na P} &= -47.1, \\
V_{1/2}h_{Na P} &= -57.0, \\
\sigma_{mNa P} &= 3.1, \\
\sigma_{hNa P} &= 3.0. \\
\end{align*}
\]

The delayed rectifier potassium current was described by:

\[
\begin{align*}
V_{1/2}m_{K} &= -44.5, \\
\sigma_{mK} &= 5.0. \\
\end{align*}
\]

Rate functions are similarly defined:

\[
\begin{align*}
\tau_{m x}(V) &= \frac{\bar{\tau}_{m x}}{\cosh((V-V_{1/2})/\sigma_{m x})} \\
\tau_{h x}(V) &= \frac{\bar{\tau}_{h x}}{\cosh((V-V_{1/2})/\sigma_{h x})},
\end{align*}
\]

with values: fast sodium, \( \bar{\tau}_{mNa T} = 0.9 \text{ ms}, V_{1/2}m_{Na T} = -43.8 \text{ mV}, \sigma_{t m Na T} = 14.0 \text{ mV}, \) \( \bar{\tau}_{hNa T} = 35.2 \text{ ms}, V_{1/2}h_{Na T} = -67.5 \text{ mV}, \sigma_{t h Na T} = 12.8 \text{ mV}; \) persistent sodium, \( \bar{\tau}_{mNa P} = 0.9 \text{ ms}, V_{1/2}m_{Na P} = -47.1 \text{ mV}, \sigma_{t m Na P} = 6.2 \text{ mV}, \) \( \bar{\tau}_{hNa P} = 20,000 \text{ ms}, V_{1/2}h_{Na P} = -57.0 \text{ mV}, \sigma_{t h Na P} = 6.0 \text{ mV}; \) potassium, \( \bar{\tau}_{m K} = 4.0 \text{ ms}, V_{1/2}m_{K} = -44.5 \text{ mV}, \sigma_{t m K} = 10.0 \text{ mV}. \) The reversal potential for the leak current was defined as \( E_L = -76.0 \text{ mV}, \) derived from the standard Goldman equation with parameters \([Na^+]_{in} = 15 \text{ mM}, [Na^+]_{out} = 145 \text{ mM}, [K^+]_{in} = 140 \text{ mM}, [K^+]_{out} = 9 \text{ mM}, \) a \( Na^+/K^+ \) permeability ratio of 0.03, and a temperature of 308° Kelvin.

Network sizes of between 50 and 500 with either all-to-all or random sparse connectivity were simulated. The total synaptic conductance \( g_{syn} \) was calculated as a sum of a stochastically initialized tonic drive \((0.12 \pm 0.012 \text{ nS, mean } \pm \text{ standard deviation})\) with connections from other cells in the network. For fully connected 50-cell networks, these connections were modeled as a product of Gaussian distributed synaptic weights \((0.2 \pm 0.02)\) and a scaling parameter of \(0.1 \text{ nS.}\) For simulation with larger networks and sparser connectivity, synaptic parameters were adjusted to maintain the same mean synaptic conductance to each cell.