Title

Patterns of inspiratory phase-dependent activity in the in vitro respiratory network

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Phase-dependent inspiratory activity

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Abstract

Mechanistic descriptions of rhythmogenic neural networks have often relied on ball-and-stick diagrams which define interactions between functional classes of cells assumed to be reasonably homogenous. Application of this formalism to networks underlying respiratory rhythm generation in mammals has produced increasingly intricate models that have generated significant insight, but the underlying assumption that individual cells within these network fall into distinct functional classes has not been rigorously tested. Here we use multiunit extracellular recording in the in vitro pre-Bötzinger complex (preBötC) to identify and characterize the rhythmic activity of cells. Inspiratory phase-dependent activity was estimated for all cells and the data set as a whole was analyzed with principal component analysis, non-linear dimensionality reduction and hierarchical clustering techniques. None of these techniques revealed categorically distinct functional cell classes, indicating instead that the behavior of these cells within the network falls along several continua of spiking behavior.
Rhythmic activity is ubiquitous in neural systems, from the slow rhythm generated by the suprachiasmatic nuclei that synchronizes mammalian circadian cycles (Dibner et al. 2010), to the broad frequency spectra covered by various cortical oscillations generated within cortical networks (Buzsaki and Draguhn 2004; Tort et al. 2010; Wang 2010). Many of the underlying rules of rhythm generation have been established in neural circuits that generate rhythmic motor behaviors (Goulding 2009; Grillner 2006; Marder and Calabrese 1996). These motor activities are typically reciprocal in nature (e.g. expiration and inspiration, extension and flexion, protraction and retraction), and are often described by ball-and-stick\(^1\) schematics that assume specific connectivity (\textit{sticks}) between different types of cells or cell populations (\textit{balls}). In these models each cell possesses functionally discrete properties firing in distinct phases with respect to a given global activity pattern (Guertin 2009; Selverston 2010). Ball-and-stick representations have been particularly powerful in invertebrate neuronal networks (Antonsen and Edwards 2003), because the firing and connectivity properties of physiologically and anatomically identified individual neurons could be reproducibly characterized across different individuals of the same species (White and Nusbaum, 2011; Katz et al. 2010; Newcomb et al. 2012; Jing et al. 2010; Marder and Calabrese 1996; Ramirez, 1998). One of the many important lessons learned from these small invertebrate networks is that even in case of a truly identified neuron, electrophysiological measures of single cell properties do not suffice to identify a neuron (Brookings et al. 2012). This has very important implications for mammalian rhythm generating networks. Despite the increased availability of molecularly identified
mammalian neurons (Bouvier et al. 2010; Gray et al. 2010; Grossmann et al. 2010; Goulding, 2009; Crone et al. 2012) it continues to be very difficult to reproducibly identify individual neurons. Instead investigations have often relied on categorizing physiological cell types based on their phase-dependent firing patterns (Dougherty and Kiehn 2010a, b; Kiehn 2010, 2006; Kwan et al. 2009; Morris et al. 2010; Richter 1982), but this approach does not necessarily identify a physiologically homogeneous class of neurons, let alone identify a single neuron.

Despite these caveats ball-and-stick schematics have also been applied to mammalian networks, and it seems that these models become increasingly popular. In the respiratory and locomotor networks, catalogues of 5-20 different firing patterns and their hypothesized connections have been incorporated into increasingly complex models of rhythm generation with the idea that they can potentially explain how these networks generate rhythmicity (Ogilvie et al. 1992; Rybak et al. 2008; Rybak et al. 1997a, b, c; Sherwood et al. 2010; Molkov et al. 2010; Rubin et al. 2009; Rybak et al. 2007; Smith et al. 2007). Yet, despite the reliance of current models on the concept of different physiological categories, the quantitative basis for these categories has not been rigorously established, nor have the criteria that specify inclusion into one or another cell class been described in detail.

Here we show for the isolated mammalian respiratory network that, while it is possible to qualitatively identify different firing patterns matching several of the traditionally described categories, these firing patterns vary along several continua which span and include the traditionally defined categories.
Methods

Experimental Procedures

Transverse medullary brain stem slices were taken from CD1 mice, P6-9, as described previously (Ramirez et al. 1996; Viemari and Ramirez 2006). This age range was chosen to reduce heterogeneity from neurodevelopmental changes known to affect respiratory rhythm generation in these mice. 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 histological characterizations confirmed that these slices contain the preBötzinger complex (not shown). 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.

Extracellular Recording
The basic recording and analysis methods are summarized in Figure 1. Extracellular neural activity from the transverse medullary slice was recorded on up to 16 channels using custom-made multi-electrodes. These were created by twisting nichrome microwire (25 micron inner diameter, Formvar-insulated Nichrome; A-M Systems) into tight bundles which were then sealed with epoxy, cut and gold-plated at the tips. The geometry and arrangement of the individual wires was therefore random within the multi-electrode probe which was roughly 200 microns in diameter. Neural signals were amplified 1000X and band-pass filtered from 250 to 7500 Hz with a custom analog amplifier. These filter settings were selected to reduce the effects of low frequency components on thresholding used for spike detection and include high frequency waveform features that could aid spike sorting. 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.

Intracellular Recordings

Inspiratory preBötC neurons (one neuron per slice) were recorded using the blind patch-clamp recording method, after first identifying in the cell-attached mode, which revealed their discharge pattern in phase with population activity (as described previously, Viemari and Ramirez 2006, Pena and Ramirez, 2002, 2004, Tryba and Ramirez, 2006; Tryba et al. 2008; Pena et al. 2004). Experiments were then performed in whole-cell configuration with the neuron recorded in current-clamp at the zero current potential. The patch electrodes were pulled from filamented borosilicate glass tubes (G150F-4; Warner Instruments, Hamden, CT) and filled with a solution containing 140 mM K-gluconic acid, 1 mM CaCl₂ * 6H₂O, 10 mM EGTA, 2 mM MgCl₂ * 6H₂O, 4 mM Na₂ATP, and 10 mM HEPES.

Quantitative Analysis

The integrated population activity waveform 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, or 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 inspiratory phase. 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. In this case, a single vector of burst-centered 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 was 0.03 seconds.
Principal component analysis (PCA) was used to inspect low-dimensional reconstructions of data sets representing firing rate functions. PCA is a method for finding orthogonal projections of a data set that maximally capture the variance in the data. These projections are ordered according to the amount of data variance captured, so that by selecting the first few projections, a faithful but reduced representation of the data can be derived. Reduction of large data sets to two or three dimensions allows visualization of structure within the full data set that may otherwise remain obscure (see Supplemental Materials, Figure 1). For this study, PCA was implemented using the singular value decomposition function in MATLAB.

Intracellular recordings from *in vitro* slice experiments in our laboratory using nearly identical experimental methods (Viemari and Ramirez 2006), were analyzed for comparison with extracellular data. Action potential and population burst times were extracted from 61 recordings (duration 290 ± 113 seconds) and used to estimate burst-triggered firing rate functions using the same computational methods described above for extracellular recordings. These estimated rate-functions were then projected into the same PCA subspace as the extracellularly estimated functions.

Several additional data dimensionality reduction techniques were also applied as an alternative to PCA. These methods—iso map, Laplacian Eigenmap, and local linear embedding—all are attempts to capture nonlinear manifolds within high dimensional data by relaxing the constraint on fitting the global data variance (Belkin and Niyogi 2003; Roweis and Saul 2000; Tenenbaum et al. 2000). In some cases these methods may find structure within the data that global, linear methods like PCA may miss. In addition to the number of dimensions used in the reduced space, these algorithms require specification a
neighborhood parameter which specifies the number of data points nearest to a given data point that are to be considered local to that point. All three of these algorithms were applied using MANI (http://www.math.ucla.edu/~wittman/mani/) for MATLAB. Each algorithm was run with a data reduction dimension of 2 or 3 and several neighborhood values between 3 and 10.

Hierarchical clustering of burst-triggered firing rate functions was implemented with standard MATLAB routines. First, Euclidian distances between all pairs of data vectors (constructed from kernel estimation as described above) were calculated. Next, agglomerative clustering (using the Ward method) was applied to successively combine first single vectors, then groups of vectors, into a binary tree structure in which the within-group variance is minimized at each branch in the tree. Results of this analysis were visualized with a dendrogram, a diagram in which the lengths of individual branches represent the dissimilarity between the groups those branches subsume.

Results

Classification of burst-triggered rate functions

Of a total of 951 identified cells from 77 experiments (and an equal number of slices), peri-burst histograms from 918 cells with 30 or more recorded spikes in control conditions were generated by compiling spike times relative to each burst onset as defined by the time of threshold crossing of the rising phase of the integrated population waveform. Qualitative examination of these histograms (30 time bins between 1 second before and 1 second after the burst) revealed a mix of burst-related firing signatures in the recorded cell population (Figure 2). The most common pattern (n = 738; 80.4%) was an
inspiratory discharge, where spiking in the individual cell was in phase with population
bursts. Presumptively non-respiratory cells, in which firing was not related to population
activity and which typically fired tonically throughout all fictive respiratory phases, were
also abundant (n = 100; 10.9%). 5% of the neurons (46 out of 951 neurons) had a
relatively tonic firing rate which was suppressed during the population burst,
characteristic of expiratory neurons (Ramirez et al. 1998). 3.7% of the neurons (34 out of
951 neurons) showed inhibition during fictive inspiration with a marked post-inhibitory
rebound during post-inspiration. These neurons have previously been described as post-
inspiratory neurons in vitro and in vivo (Ramirez et al. 1997). Because the purpose of this
analysis was only to provide contrast with a more quantitative approach (to be presented
below) no effort was made to further sub-divide cell firing patterns into finer categories
such as incrementing or decrementing.

Because it is possible that qualitative inspection of burst-related firing patterns
may either miss true subcategories of discharge types, or conversely, arbitrarily divide a
continuum of morphology into perceptually salient but ultimately physiologically
indistinguishable classes, we used quantitative analyses of the firing pattern data to
determine if these neural signatures could be considered as naturally separable classes.

Peri-burst spike times were used to generate estimates of a smooth instantaneous firing
rate function for each cell using Gaussian kernel smoothing (with a kernel sigma of 0.03
s). To focus further analysis on the shape rather than the amplitude of these functions,
each was demeaned and normalized by its peak-to-peak amplitude. These functions were
then sampled from 0.5 seconds before to 0.7 seconds after the population burst onset time
at a temporal resolution of 4 ms (Figure 3B illustrates a density cloud of the normalized
waveforms for all cells). In this way, each waveform was represented by a vector of 301 values, meaning that the entire population can be considered as a group of points in a 301-dimensional space. Principal component analysis was used to project this cloud of points into a low dimensional subspace preserving maximal variance (Application of this technique to synthetic data representing 50 waveforms in each of two classes illustrates how PCA can reveal structure in high-dimensional data sets; Figure 3A). This allowed visual inspection to determine if this population could be easily characterized by set of distinct subgroups or was more consistent with a continuum of firing patterns. Projection of the data onto the first 3 principal eigenvectors accounted for 85% of the total variance in the data set, indicating that this compact representation was relatively faithful to the complete data. Figure 3C illustrates the resulting point cloud in three dimensions with shadow projections on the three axis planes. There are perhaps two or three diffuse point clouds with only weak, poorly separated concentrations of points.

Principal component analysis can provide a parsimonious description of high dimensional data, but the resulting dimensions are linear combinations of the original data vectors, meaning they are difficult to interpret in the context used here. In order to illustrate the variation in waveforms captured by PCA, Figure 3D shows the subsets of rate functions represented by discrete regions of the first two dimensions of the PCA space. The dense point cloud at the left of the scatter plot represents most of what would be considered inspiratory waveforms with a transition from bottom to top along the second PC dimension from narrow waveforms (3Div) to more linear decrementing shapes (3Diii) and more rounded bell-shaped waveforms with somewhat later onset latency (3Dii). Along the primary axis from left to right, weakly modulated (3Dv), non-
respiratory (3Dvi), and finally a somewhat distinct cloud of expiratory (3Di) waveforms is apparent. Because it seeks projections that capture maximum data variance, PCA is susceptible to the influence of outliers within the sample. It is also possible that relatively distinct clusters of points are obscured by intermediate points from high variance waveforms generated by poorly estimated firing rate functions or from burst-triggered firing rate functions which are poorly defined because of timing jitter in the detection of the burst onset in the population activity. To address these possibilities, three subsamples of the full dataset were taken using successively more stringent standards for selecting waveforms for inclusion in the PCA. For Figure 3Ei, the experiments in which the quality of the integrated population activity waveform was in the lowest 25% for all experiments were eliminated. In addition, no cells were included in this subset from which there were fewer than 100 action potentials used to estimate the firing rate function. This left 688 of the original set of rate functions from which the first two PCA projections are shown. For Figure 3Eii, only the experiments with the top 50% quality population waveforms were included, and the 437 cells from those experiments with 300 or more spikes were used in the PCA. For Figure 3Eiii, only the experiments within the highest quartile of population regularity were selected from which cells that fired more than 500 spikes are shown in PCA coordinates. Spatial clustering in the projections of these more exclusive data subsets remained weak, suggesting that the smear of data points in the initial analysis is not the result of experimental variability.

To confirm that findings from extracellular multi-electrode experiments were not the result of artifacts of the recording or spike sorting process, intracellular data from 61
recordings done in our laboratory were analyzed using only spike-timing and population
burst timing information to estimate burst-triggered firing rate functions (Figure 4A-C
left; sample inspiratory bursts, right). These estimated rate-functions were then projected
into the same principal component subspace as the extracellularly-estimated functions
(Figure 4D; red crosses). The rate functions from these intracellular recordings showed a
similar spatial distribution, perhaps with a bias toward more robustly inspiratory units (a
selection bias of the intracellular recordings, where non-respiratory cells were discarded
before achieving a whole-cell patch configuration after monitoring on-going spiking
activity in cell-attached mode).

The lack of clear clustering in the principal component analyses may also be a
result of the approach itself. Since PCA works to maximize the variance of linear
projections of the data in a global way, it may fail to capture local regularities in the data
that occur on non-linear manifolds. In an effort to detect such regularities, a topologically
local approach which is capable, in principle at least, of detecting such regularities was
applied to the same firing rate data as used for PCA. The full dataset was reduced to 3
dimensions using the isomap algorithm (Tenenbaum et al. 2000) with a neighborhood
parameter of 10. The first two resulting dimensions are shown in Figure 3F, where only
weak and indistinct concentrations of points are visible. The callouts of representative
waveform sets from the 2D space show that despite its drastically different assumptions
the isomap algorithm identifies axes of variability which are similar to those produced by
PCA. Analyses with different parameter values and other nonlinear manifold methods
(local linear embedding and Laplacian eigenmaps) produced similar results, as did PCA
repeated with rate functions estimated with histogram methods produced similar results (data not shown).

In order to clarify potential structure in the burst-triggered firing rate function data further, we applied a hierarchical clustering method to the same data set. This method attempts to find subsets in which data vectors are as similar as possible while making the groups as distinct from each other as possible. The results of hierarchical clustering algorithms can be displayed in a dendrogram, a diagram in which similar data points/groups are linked by vertically short branches, and more distinct clusters are indicated by long vertical lines (Figure 5A-B). Thus long vertical branches indicate relatively natural groupings within the data, and the number of such appropriate groups is suggested by a threshold behavior from long vertical branches to indistinguishable clusters of short branches (though from a theoretical point of view there is nothing to prevent successively splitting resulting groupings until only individual data points remain). Figure 5C illustrates the dendrogram produced by hierarchical clustering of the full burst-triggered firing rate function data set used for the basic principal component analysis above. The result of the first-order split in the data is shown in Figure 5Ci-ii, where a division between waveforms with inspiratory patterns (i) and those with expiratory or non-respiratory waveforms is evident. The second-order division of the respiratory group (iii and iv) produces a group of waveforms with a sharp onset and a group with more rounded shapes. The non-inspiratory group is split at the second-order branch into a variable and non-respiratory set (v) and a class with clear expiratory (or possibly post-inspiratory) waveforms. Further splitting of these classes is possible, but not as compelling as these initial divisions.
Characterization of Simultaneously Recorded Firing Patterns

While the previous analysis investigated the variability in the shapes of firing rate functions as a population across experiments, it did not establish the potential temporal relationships between these functions within single experiments. Figure 6A characterizes the population burst-related activity of 11 cells recorded simultaneously through 150 population bursts in 275 seconds of baseline recording. The central panel is a spike raster plot where individual ticks represent individual spike times from different cells (in 11 separate blocks) relative to the onset times of each population burst (designated as $t=0$, with each successive population burst cycle plotted along the ordinate axis within each block). Each tick is also color coded for instantaneous spike rate (defined as the inverse of the interspike interval ending with that spike) on a heat map scale from 0 to 55 Hz. As illustrated in this figure, a variety of firing rate patterns is present within this experiment. Weakly modulated cells with low baseline activity rates (e.g. cells #1 and #2) are present, as are those with strong expiratory phase activity as well as more robust inspiratory bursting. Estimated firing rate functions shown in panel B illustrate some of these differences. Also evident in the raster plots, and in the comparison plots in panel C, are onset timing differences between the average burst-triggered firing rates. In this experiment, for example, cell #9 tends to fire before cell #8 and cell #5 typically precedes #7. In panel D the timing differences between cells are characterized statistically by a non-parametric comparison of median spike times for all cells during the period from 0.4 seconds before to 0.6 second after the burst onset. In a point process series, the median (or mean) characterizes the center of mass of the spike time density. Within this panel,
statistically significant different median spike times ($\alpha = 0.05$; Kruskal-Wallis test corrected for multiple comparisons by the Bonferroni method) between all possible pairs of cells are shown, with significance indicated by an asterisk within the grid (statistically significant onset differences for cell #6 are also depicted to the left of panel A). In this experiment 68% of possible pairs have distinguishable onset times. Thus, statistically significant differences in the median firing times in the early part of the population burst are present between most pairs of cells. The histogram in panel F shows the significance percentage distribution for all 77 experiments, indicating that onset timing differences are common between simultaneously recorded cells. Figure 6E summarizes the simultaneous spiking behavior from 10 experiments in cycle-triggered rasters. The presence of firing patterns with a variety of shapes is evident in the texture of these panels (percentages of statistically distinguishable pairs are listed below each raster, and a histogram of percentages from all 77 experiments is also shown in Fig 6F).

Discussion

Characterization of the typical firing behavior of single neurons with respect to the respiratory cycle has been central to models of respiratory rhythm generation (Molkov et al. 2010; Rubin et al. 2009; Rybak et al. 2007; Smith et al. 2007). Categorization of this behavior into different types has provided the substrate for understanding rhythmogenesis as arising from antagonistic pools of neurons with characteristic firing patterns. The simplest of these models is the half-center oscillator, where two mutually inhibitory populations can produce a two-phase rhythm (Brown, 1911, Getting, 1989; Sharp et al. 1996). However, it must be emphasized that the original
half-center concept only works with neurons that possess certain intrinsic membrane properties that facilitate transitions from one to the other phase, otherwise such a network configuration will not generate rhythmic activity based on reciprocal inhibitory connectivity alone (Sharp et al. 1996; Skinner et al. 1994). Moreover, this network configuration can not only generate rhythmicity, but many different activity states including synchrony (Sharp et al. 1996; Wang and Rinzel, 1994; Jalil, Belykh & Shilnikov, 2010, 2012; Belykh and Shilnikov, 2008; ). Subsequent classification systems in a variety of animal models and preparation types have grown more and more diverse, as have the complexity of the interactions proposed between these physiological classes (Rybak et al. 2007, Smith et al. 2007; Abdala et al. 2009). Undoubtedly, neurons with varying patterns of respiratory modulation do exist, and decades of experimental evidence suggests that these prototypical classes correspond to physiologically relevant functional differences within the network (Bianchi et al. 1995, Richter 1982; Duffin, 2004). However, little effort has been made so far to establish a mathematical foundation for these categories that might provide a more nuanced substrate for further functional and modeling studies.

Because the pre-Bötzinger complex can be physiologically defined by the presence of a variety of firing patterns within a confined area (Lieske et al. 2000; Ramirez et al. 1997; 1998) and is accessible in the slice to functional/pharmacological manipulation, we have focused this initial quantitative analysis of cellular firing patterns on this preparation. There is evidence for only weak clustering of these patterns into mathematically distinct classes. Instead, respiratory-phase related firing rate functions lay along several continua of variability, including the strength of activity during the
expiratory phase, the degree of firing rate change during the inspiratory burst, and several
features of the timing and shape (Figure 7A). Two dimensions of variation in these data
are obvious without any special statistical analysis: modulation of burst amplitude
(including inhibition of baseline firing rates; Figure 7Ai), and variation in the mean firing
rate outside of the inspiratory burst phase (7Aii). To uncover more subtle regularities in
the shapes of the estimated waveforms, these two features were normalized out of the
data for the analyses illustrated in the previous figures. The remaining variation in shape
and timing are illustrated qualitative and diagrammatically in Figure 7B using smoothed
example waveforms from the current database. The strength of spiking activity preceding
the population burst has been addressed in earlier studies, and hypothesized to indicate
cells with a role in driving the population bursts (Ramirez and Viemari 2005). Waveform
features indicative of this activity are clearly present in this data set as well (characterized
by Figure 7Bi). Firing patterns with notably earlier and later onset latency to the
population were also apparent in these data (as in Figure 7Bii), and even occurred within
the same recording. As with the pre-burst ramp onset mentioned above, earlier initiation
of a cell’s burst phase has been hypothesized to play a role in rhythmogenesis (Butera et
al. 1999; Ramirez et al. 2004; Rekling et al. 1996). A range of roundedness of burst shape
(Figure 7Biii) has also been seen in these data, and these are again consistent with some
prototypical waveforms seen before (Richter 1982). The width of the inspiratory burst
peak varied as well (Figure 7Biv), though this feature might be thought of as part of the
continuum of shapes leading to the rounded burst in the previous panel. Relative
decreases in activity can also be indicative of a neuron’s burst signature with some
exhibiting relatively little post-burst depression (Figure 7Bv). Conversely, in those
neurons presumably inhibited during the inspiration burst, a range of post-inhibitory rebound is seen (Figure 7Bvi). Clearly these features are not independent, and understanding their covariance relationships is an area for future analysis.

It is tempting to hypothesize about the physiological significance of these dimensions of variability in the context of synaptic or intrinsic cellular properties, though ultimately the observations made here are non-mechanistic. Modeling studies have routinely presupposed, for example, that heterogeneity in cell response can be generated by endowing individual cells with a statistical distribution of intrinsic currents following parameters established from current-clamp studies (e.g. Rybak et al., 2003; for an interesting exception see Golowasch et al., 2002, where mean values of measured current densities failed to reproduce the experimentally observed behavior). Large-scale parameter search studies modeled on neurons within the stomatogastric ganglion of the crab have also established that whole cell behavior derives from relatively smooth manifolds in the parameter space of intrinsic conductances (Prinz et al. 2003; Prinz et al. 2004). Within the respiratory network, some authors have proposed that intrinsic bursting behavior in some cells is the result not of a quantitatively distinct subclass of intrinsic conductance distributions, but rather stems from threshold behavior in the space of persistent sodium and leak current densities (Del Negro et al. 2002). Similarly, some have hypothesized that burst onset timing of individual cells within the respiratory network derives from the particular distribution of synaptic and intrinsic properties of each cell (Butera et al. 1999). Their model also suggests that the timing of the inspiratory burst onset for a given cell is a relatively invariant feature of that cell. The data presented here generally support the concept of a continuous distribution of intrinsic and synaptic
properties resulting in a relatively continuous distribution of cellular behavior, and the
burst timing data presented here also confirm (though obviously not for the first time) the
existence of variety of burst onset latencies in the mean behavior of cells within the same
network. The variability in burst onset timing for individual cells is addressed in a
companion paper (Carroll and Ramirez, 2012 [COMPANION PAPER]).

Limitations of the Current Approach

The use of the medullary slice preparation for this analysis of burst-triggered rate
function variability (including timing) may limit the scope of the analysis in the sense
that this network may not contain the full range of firing patterns that have been
documented in less reduced respiratory network preparations. While admitting the central
and autorhythmic character of the preBötC, recent models have placed it in the context of
a larger network containing pools of mutually antagonistic neurons which interact to
produce a three-phase rhythm (Rybak et al. 2008). It is entirely possible, therefore, that
analysis of recordings from a larger variety of structures along the rostro-caudal axis of
the brainstem would reveal more robust and distinct differences between cellular firing
patterns than could be shown here. However, because the preBötC does have cells with a
variety of firing patterns in an experimentally accessible preparation, it provides an
excellent starting point for a more quantitatively rigorous description of cellular behavior
within the wider respiratory network.

The process of clustering high dimensional data sets containing mixtures of
subpopulation samples drawn from potentially arbitrary probability distributions does not
yet have a strong statistical foundation. Thus the existence of natural classes within large
data sets is, outside of the most obvious cases, largely in the eye of the beholder. In this study, we have used a collection of standard dimension reduction techniques to look for evidence of discrete substructures within the data, and we have found this evidence weak. However, there is always the possibility that some other analytical approach may be more sensitive to the existence of such substructures within the population.

Conclusion

The analyses presented here have provided a reasonable quantitative outline of the range of variation in cycle-triggered firing rate waveforms within the in vitro pre-Bötzinger complex network. The finding here of a continuous distribution of firing rate behavior is consistent with an assumed graded distribution of intrinsic currents, likely determined by both developmental and activity-dependent factors. However, these data contradict a dominant model in the field of central respiratory control that views different participants in the network as possessing categorically distinct behaviors which determine each cell’s role in specific phases of the rhythm. This model likely stems from conceptual foundations inspired by studies of invertebrate rhythm generators, where anatomically and physiologically identified cell types are abundant and more consistent with ball-and-stick representations. It is our hope that this more quantitative description of physiological behavior can provide a basis for a more in-depth understanding of the relationship between these waveform features and the physiological/functional roles of the cells within the respiratory network. This will be particularly important for neurons located in different areas of the wider respiratory network.
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Conflict of Interest

None 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. JCV conducted intracellular experiments and assisted in manuscript review. JMR assisted with experimental and analytical design, manuscript preparation and manuscript review.

End Notes

1The term *ball-and-stick* has several uses in the neuroscientific literature, so it is important to specify our particular usage here. We refer to the schematic network diagrams that have driven conceptual models of rhythmogenesis for more than 100 years, and as used more recently, for example, by Antonsen and Edwards (2003) in reference to the crayfish escape circuit. More recently, computational modelers have also used *ball-and-stick model* to refer to various types of lumped parameter conductance models of neurons and neural circuits. While the phrase is descriptive and useful in that particular
context, it is not the meaning we wish to employ here, and we hope our readers will indulge us in this lexical overlap without undue confusion.
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Figure 1. Schematic of multielectrode recording and analysis of *in vitro* rhythmic activity. A) Transverse medullary slice with the localization of the ventral respiratory group (presumptive preBötC) showing the placement of the twisted multiwire electrode. Multiple signals are recorded (B) and crosstalk is reduced using independent component analysis (C). Extracellular spikes are detected and sorted in PCA feature space (D) identifying action potential times for individual neurons (E) in relation to the ongoing population activity (shown above).

Figure 2. Prevalence of four traditionally defined firing patterns from 77 *in vitro* multiunit recording experiments. For each cell type, histograms illustrate the population-burst triggered firing pattern for example cells in that class (lower), and the population of burst-triggered firing rate functions for each class is shown as a density plot (upper).

Figure 3. Analysis of cycle-triggered rate functions by dimensionality reduction techniques. A) An illustrative example of principal component analysis (PCA) applied to synthetic data with two subtly distinct waveform classes buried in high amplitude Gaussian noise. Projection of the original high-dimensional data (Ai) onto the first two PCA dimensions shows distinct clusters (Aiii) from which the original waveform classes can be perfectly recovered (Aii and Aiv). B) Density plot showing all data waveforms demeaned and normalized to unit peak-to-peak amplitude. Point cloud (C) of waveforms in 3 primary PCA dimensions with shadow projections on axis planes. The features captured by the first two dimensions of PCA are illustrated with examples from various regions in this space (Di-vi). Additional PCA analyses of more selective subset of waveforms (Ei-iii) show similar weak clustering. F) Globally nonlinear dimensionality reduction algorithm (isomap) identifies a similar feature space to PCA.

Figure 4. Intracellularly recorded burst-triggered firing patterns. Samples of firing rate functions from intracellular recordings (A-C, left; sample membrane potential traces, right) are morphologically similar to those estimated from multi-electrode extracellular recordings. D) Rate functions from 61 intracellular recordings (red crosses) shown along the primary principal component axes against the field of points from Figure 4D.
Figure 5. Hierarchical cluster analysis applied to cycle-triggered rate functions. Hierarchical groupings in synthetic data (A) can be revealed by a dendrogram (B), which illustrates relationships between groups of data points. Application of this technique to burst-triggered firing rate functions shows a primary but weak division between inspiratory and non-inspiratory waveforms (Ci and ii), and a secondary bifurcation into sharp (Ciii) vs. more rounded (Civ) inspiratory waveforms, and weakly or unmodulated firing patterns (Cv) vs. clear expiratory cells (Cvi).

Figure 6. Cycle-by-cycle spiking behavior of eleven simultaneously recorded neurons across 150 population bursts. Each block of panel shows spike times of a different neuron relative to burst onset with successive cycles stacked in the block and the instantaneous spike rate coded as a heat map from between 0 and 55 Hz. Cycle-triggered rate function estimates are shown in a column in panel B with each cell given a distinct color. Onset timing differences are illustrated in C) between cell #8 and #9 (Ci) and between #5 and #7 (Cii). Significantly different median onset times between all cell pairs are indicated in the grid in panel D where 68% of pairs were statistically distinguishable. Cycle-triggered rastergrams from 10 other experiments including percentages is statistically significant pairs (E). Panel F shows the distribution of such percentages over the entire data set of 77 slice experiments.

Figure 7. A schematic illustration of variation in modulation amplitude, baseline firing rate, and timing features of rate functions. A) Illustrates variation in modulation amplitude and baseline firing rate in unnormalized rate functions, while B shows shape and timing features that distinguish normalized rate functions: i) presence or absence of a ‘pre-inspiratory’ ramp onset; ii) pure onset timing difference; iii) sharp or rounded burst envelope; iv) burst width; v) post-inspiratory spike-rate depression; and vi) post-inhibitory rebound.
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Figure 7. A schematic representation of several continua of variability inferred from dimensionality reduction and cluster analyses. A) Illustrates variation in modulation amplitude and baseline firing rate in unnormalized rate functions, while B shows shape and timing features that distinguish normalized rate functions: i) presence or absence of a ‘pre-inspiratory’ ramp onset; ii) pure onset timing difference; iii) sharp or rounded burst envelope; iv) burst width; v) post-inspiratory spike-rate depression; and vi) post-inhibitory rebound.