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Abstract:
In the mammalian respiratory central pattern generator, the preBötzing complex (preBötC) produces rhythmic bursts that drive inspiratory motor output. Cellular mechanisms initiated by each burst are hypothesized to be necessary to determine the timing of the subsequent burst, playing a critical role in rhythmogenesis. To explore mechanisms relating inspiratory burst generation to rhythmogenesis, we compared preBötC and hypoglossal (XII) nerve motor activity in medullary slices from neonatal mice in conditions where periods between successive inspiratory XII bursts were highly variable and distributed multimodally. This pattern resulted from rhythmic
preBötC neural population activity that consisted of bursts, concurrent with XII bursts, intermingled with significantly smaller “burstlets”. Burstlets occurred at regular intervals during significantly longer XII interburst intervals, at times when a XII burst was expected. When a preBötC burst occurred, its high amplitude inspiratory component (I-burst) was preceded by a preinspiratory component that closely resembled the rising phase of burstlets. Cadmium (8μM) eliminated preBötC and XII bursts, but rhythmic preBötC burstlets persisted. Burstlets and preinspiratory activity were observed in μ90% of preBötC neurons that were active during I-bursts. When preBötC excitability was raised significantly, burstlets could leak through to motor output in medullary slices and in vivo in adult anesthetized rats. Thus, rhythmic bursting, a fundamental mode of nervous system activity and an essential element of breathing, can be deconstructed into a rhythmogenic process producing low amplitude burstlets and preinspiratory activity that determine timing, and a pattern-generating process producing suprathreshold I-bursts essential for motor output. © 2013 the authors.

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Distinct Inspiratory Rhythm and Pattern Generating Mechanisms in the preBötzinger Complex

Kaiwen Kam,* Jason W. Worrell,* Wiktor A. Janczewski, Yan Cui, and Jack L. Feldman

Introduction

In vertebrate rhythmic motor behaviors, such as breathing, chewing and locomotion, central pattern generators (CPGs) produce near synchronous bursts of action potentials in populations of neurons that determine the timing and patterning of motoneuronal activity driving participating muscles (Gossard et al., 2010). For invertebrate CPGs, mechanisms underlying generation of rhythm and pattern are closely intertwined (Selverston, 2010). In contrast, distinct rhythm and pattern generating networks are hypothesized for mammalian rhythmic motor behaviors (Feldman, 1986; McCrea and Rybak, 2008), but how, or even if, rhythm and pattern generating functions are partitioned in mammalian CPGs has not yet been determined.

In mammals, breathing is a vital, rhythmic motor behavior controlled by a CPG that operates continuously to exchange gas, yet adapts rapidly and appropriately to changing physiological or environmental conditions. In the respiratory CPG in rodents, the preBötzinger complex (preBötC) is necessary for inspiratory rhythmogenesis in vitro (Smith et al., 1991) and for normal respiratory movements in vivo (Gray et al., 2001; Tan et al., 2008). Near synchronous inspiratory bursting in preBötC neurons drives activity in bulbospinal (Dobbins and Feldman, 1994) and parahypoglossal (Dobbins and Feldman, 1995; Chamberlin et al., 2007; Koizumi et al., 2008) premotoneurons projecting to spinal and XII motoneurons to produce and modulate inspiratory airflow.

preBötC bursts are critically involved in generation of patterned inspiratory motor activity and are hypothesized to be essential for rhythmogenesis. In many computational models of preBötC, inspiratory burst-initiated activation of hyperpolarizing conductances, such as Ca2+-activated K+ channels or inactivation of depolarizing conductances, such as the persistent Na+ current, I_{NaP}, (Butera et al., 1999a; Rybak et al., 2004; Rubin et al., 2009), underlie rhythmogenesis. However, pharmacological blockade of these conductances does not abolish preBötC rhythmogenesis in vitro (Pena et al., 2004; Del Negro et al., 2005; Pace et al., 2007). Also inconsistent with preBötC burst-mediated rhythmogenesis is the strong modulation of inspiratory burst rhythm in vitro by extracellular K+ (K_{ext}) without substantial changes in inspiratory burst pattern (Del Negro et al., 2009).

Here, we test the hypothesis that preBötC rhythmogenetic mechanisms are distinct from burst generating mechanisms by comparing preBötC population and XII activity in medullary...
slices from neonatal mice in conditions where the period between successive inspiratory XII bursts are highly variable (Del Negro et al., 2009) and multimodal. This temporal pattern resulted from significantly smaller amplitude preBotC bursts (burstlets) that occurred at regular intervals when a XII burst was expected, but failed to produce XII output. We characterized these events in vitro and in vivo and compared them with high amplitude preBotC bursts that produced XII bursts and multipeaked bursts that we called doublets. In preBotC single neuron and population activity, burstlets resembled preinspiratory activity, the low-level initial component of each preBotC burst. When preBotC and XII bursts were eliminated by bath-applied cadmium, rhythmic preBotC burstlets persisted. We conclude that inspiratory rhythmogenesis is primarily determined by the common mechanisms underlying burstlets and preinspiratory activity that are distinct from suprathereshold processes required to transform preBotC burstlets into preBotC bursts and bona fide XII motor output.

Materials and Methods

In vitro slice preparation and recording. The Office for the Protection of Research Subjects (University of California Animal Research Committee) approved all protocols. We used neonatal C57BL/6 mice (P0-5) of either sex for experiments in vitro. Transverse slices (350 μm thick) were cut from the neonatal mouse brainstem, which contained the specialized rhythm-generating preBotC (Smith et al., 1991) as well as respiratory premotoneurons and XII respiratory motoneurons that discharge in phase with inspiratory rhythm (Koizumi et al., 2008). To obtain slices with the preBotC at the surface, the rostral cut was made above the first set of XII nerve rootlets at the level of the dorsomedial cell column and principal lateral loop of the inferior olive, and the caudal cut captured the obex (Ruangkittisakul et al., 2011). The medial cut was made in ACSF containing the following (in mM): 124 NaCl, 3 KCl, 1.5 CaCl2, 1 MgSO4, 25 NaHCO3, 0.5 NaH2PO4, and 30 mM glucose, equilibrated with 95% O2 and 5% CO2, 27°C, pH 7.4.

Slices were perfused with 27°C ACSF at 4 ml/min in a 0.5 ml chamber mounted rostral side up in a fixed-stage Axioscope (Zeiss) or DMLFS (Leica) microscope set up for Koehler illumination. Bright-field or infrared-enhanced differential interference contrast (IR-DIC) video microscopy was performed using a MATIR IR-series charge-coupled device (CCD) video camera (DAGE) coupled to a MTI analog camera controller (Leica) microscope set up for Koehler illumination. Bright-field or IR-DIC video microscopy (40 μm tip diameter) was recorded on a computer and analyzed using Chart 7 Pro (ADInstruments), a 700A or 700B MultiClamp amplifier (Molecular Devices), and the signal was filtered at 2–4 kHz and digitized at 10 kHz. Continuous data were acquired in current-clamp using an analog-to-digital converter (Digidata 1322 or 1440, Molecular Devices) and pCLAMP (Molecular Devices). Traces were analyzed offline using custom software written for IgorPro. Patch electrodes with a 2–3 MΩ tip resistance and filled with 1 M NaCl, 1 M MgCl2, 25 NaHCO3, 0.5 NaH2PO4, and 30 mM glucose, pH 7.4.

In vivo recording and injections. Male Sprague Dawley rats (Charles River Laboratories), weighing 320–420 g, were anesthetized with ketamine-xylazine (100 mg/kg–10 mg/kg, i.p.). Atropine (0.5 mg/kg, i.p.) was given to prevent bradycardia and excessive airway secretion. Isoflurane (1–2 vol% in air) was administered throughout the experiment. The level of anesthesia was assessed by the suppression of the withdrawal reflex and by the absence of changes in heart rate and breathing rate in response to noxious stimuli. The trachea was cannulated and respiratory flow was detected with a flow head connected to a transducer to measure airflow (GM Instruments). Coupled EMG wire electrodes (Cooner Wire) were inserted into diaphragm (DIA) muscles. Wires were connected to amplifiers (Grass Model P511; Grass Technologies) and activity was sampled at 2–4 kHz (Powerlab 16SP, ADInstruments). The rats were placed in a supine position in a stereotaxic instrument (Kopf Instruments). The basal aspect of the occipital bone was removed to expose the ventral aspect of the medulla. To eliminate effects induced by stimulation of vagal reflexes or laryngeal reflexes, rats were vagotomized by resecting a portion of the vagus nerve (2 mm) at midcervical level, and the larynx was denervated. Body temperature was kept constant at 37 ± 1°C with a servo-controlled heating pad (Fine Science Tools). Data were recorded on a computer and analyzed using Chart 7 Pro (ADInstruments), Excel, IgorPro, and Origin7 (OriginLab) software. The absolute value of EMG signals was digitally integrated with a time constant of 0.1 s to calculate peak amplitude and time onset. The flow signal was high-pass filtered to eliminate DC shifts and slow drifts and digitally integrated.

Bicuculline and/or strychnine (240 μM; 110 nl/side; Sigma-Aldrich) diluted in saline were pressure injected through a sharp glass electrode (40 μm tip diameter) into the preBotC, and, in some cases, also BotC. In burstlet experiments, bombesin (240 μM; 90 nl/side; AnaSpec) was then bilaterally injected into preBotC. Janczewski et al. (2012). Coordinates were as follows (in mm): −0.75, ± 2.0, 0.7 for preBotC and −0.05, ± 2.1, 0.6 for BotC. For stereotaxic coordinates (x, y, z), the zero reference points, i.e., (0,0,0), were as follows: the rostralmost rootlet of the hypoglossal and ambulocutaneous (RRXII) tracts (x-axis, in the rostral direction), the midline for mediolateral axis (y-axis), and the ventral medullary surface in dorsoventral plane (increase in a dorsal direction; Janczewski et al., 2013). A solution of fluorescent polystyrene beads (5% by volume of an injection; Invitrogen) was added to the injectate to mark injection sites.

Histological examination was used to determine the injection placement. At the end of each in vivo experiment, rats were transcardially perfused with 4% paraformaldehyde in phosphate buffer. The brains were collected, postfixed, and cryoprotected, and 40 μm brainstem sagittal sections were cut. Specific staining for preBotC neurons (Tan et al., 2008, 2012) was used to determine whether injections were placed in the preBotC and BotC. Slides were observed under an AxioCam2 fluorescent microscope connected with AxioVision acquisition software (Zeiss).

Data analysis and statistics. Event detection, peak amplitude, duration, and area of respiratory-related activity recorded in full-wave rectified XII output or preBotC population recordings were performed using custom software written in IgorPro. Semi-automated event detection was executed using custom procedures that used multiple criteria, including slope and amplitude thresholds, to select events automatically, which were then confirmed visually by the experimenter. The onset and termination of an event (burstlet, burst, doublet) or component of an event via recording to exhibit an inspiratory discharge pattern. These experiments were performed using either a 700A or 700B MultiClamp amplifier (Molecular Devices), and the signal was filtered at 2–4 kHz and digitized at 10 kHz. Continuous data were acquired in current-clamp using an analog-to-digital converter (Digidata 1322 or 1440, Molecular Devices) and pCLAMP (Molecular Devices). Traces were analyzed offline using custom software written for IgorPro. Patch electrodes with a 2–3 MΩ tip resistance and filled with 1 M NaCl, 1 M MgCl2, 25 NaHCO3, 0.5 NaH2PO4, and 30 mM glucose, pH 7.4.
(preinspiratory, 1-burst) were determined after threshold detection of the event. Event onset was determined by scanning the signal backward in time from threshold crossing (marking event detection) to identify the time point when the signal intersected with baseline; the time point following the event peak at which the signal reached baseline and remained below event detection threshold for >200 ms was considered event termination. Baseline was the average value of the preBoëtC signal over a 30 s window. I-burst onset in preBoëtC population recordings was the time at which the preBoëtC signal deviated from a line fit to preinspiratory activity and did not re-intersect the fitted line for 20 ms.

Unlike intracellular recordings, suction electrode recordings lack a scale that allows comparisons across experiments, and the value of XII and preBoëtC burst discharge signals, i.e., measured voltage, varied significantly in absolute value between experiments. Therefore, for comparisons across experiments, the baseline was subtracted and the signal scaled to the maximum peak amplitude in the control condition for each experiment, and the value of XII fitted line for 20 ms. from a line fit to preinspiratory activity and did not re-intersect the baseline recordings was the time at which the preBoëtC signal deviated from a line fit to preinspiratory activity and did not re-intersect the fitted line for 20 ms.

Event duration, amplitude and synchrony between XII and preBoëtC activity were criteria used to categorize detected events as bursts, bursts, and doublets. Bursts were events in preBoëtC that did not temporally overlap a XII burst. Doublets were distinguished from bursts by the presence of multiple peaks of activity. Following event detection in XII recordings, if an event displayed a second peak that exceeded 60% of the amplitude of the first peak, the event was categorized as a doublet. Additionally, two closely spaced bursts were considered a doublet based on the distribution of the period of XII output. A small peak at <2 s in the distribution of periods of XII bursts was usually observed. A Gaussian was fit to this small peak and a threshold time interval was set that was its mean + 3 SDs. Two bursts separated by less than this threshold were considered a doublet. This criterion was validated by looking at preBoëtC population activity, which usually showed that preBoëtC activity did not fall to baseline during the period between doublet peaks (Fig. 4A). Average traces for XII and preBoëtC bursts and doublets were aligned to onset of the XII burst/doublet. Average burstlet traces were aligned by the event onset.

For loose patch single neuron recording experiments when preBoëtC population activity was not recorded, bursts of action potentials (APs) that did not coincide with a XII burst were considered burstlets. Bursts of APs were grouped based on the distribution of interspike intervals (ISIs) where the threshold ISI separating groups was determined by a lognormal fit of the data. APs with ISIs less than threshold were grouped, whereas APs separated by ISIs longer than the threshold were considered parts of successive events. AP amplitude and spike frequency adaptation were calculated by normalizing AP amplitude and ISI to the first AP amplitude and ISI, respectively, in the burstlet, burst, or doublet. For some analyses, firing patterns during bursts were divided into a preinspiratory and an inspiratory I-burst phase, which used the sharp onset in XII burst as the boundary between the two phases. Data are represented as mean ± SD. Statistical significance was uniformly set at a minimum of p < 0.05. Distributions were tested for normality using the Jarque–Bera test, which is a goodness-of-fit test, comparing the skewness and kurtosis of the test distribution with the normal distribution, where the null hypothesis is that the two distributions are not different (Jarque and Bera, 1987). Multimodality in distributions was ascertained using Hartigan’s dip test for unimodality, which is a goodness-of-fit test, comparing the test distribution to a unimodal distribution that minimizes the maximum difference, where the null hypothesis is that the test distribution is unimodal (Hartigan and Hartigan, 1985). For statistical comparisons of more than two groups, an ANOVA was first performed. In most cases, a two-way repeated-measures ANOVA was used for comparisons of various parameters in different conditions and for making comparisons across different events. If the null hypothesis (equal means) was rejected, post hoc pairwise tests were then used for pairwise-comparisons of interest. Individual p values are reported, but Holm–Bonferroni analysis for multiple-comparisons was conducted to correct for interactions between the multiple groups. For one-way and two-way ANOVAs, post hoc significance for pairwise-comparisons was analyzed using Tukey–Kramer analysis. Cumulative distribution functions (cdfs) were generated by sorting data and then binning them into 20 × 5% bins. Distributions were compared using the Kolmogorov–Smirnov goodness-of-fit test.

Results

Altering Kext specifically changes the period of inspiratory rhythm and its variability

In the rhythmic medullary slice preparation from perinatal rodents, the preBoëtC generates rhythmic inspiratory drive to XII motoneurons that leads to XII bursts (Smith et al., 1991; Ruangkittisakul et al., 2011). Changes in Kext powerfully modulate the period of XII bursts (TxII) and its associated variability (Del Negro et al., 2009; Ruangkittisakul et al., 2011), as measured by the coefficient of variation (CV = SD/mean) of TxII (CV(TxII)). We recorded XII output as Kext was lowered from 9 to 6 mM and then to 3 mM, with Caext fixed at 1.5 mM (Fig. 1A, B). Consistent with previous findings (Del Negro et al., 2009), CV(TxII) at 6 mM Kext was significantly greater than at either 9 or 3 mM Kext (Fig. 1A; RM ANOVA, F(2,12) = 11.7, p = 0.001; 9 vs 6 mM, p = 0.0003; 9 vs 3 mM, p = 0.4; 6 vs 3 mM, p = 0.04; n = 7). In contrast, mean TxII (T_xII) increased monotonically as Kext was lowered from 9 to 3 mM (Fig. 1A; RM ANOVA, F(2,12) = 20.8, p = 0.0001; 9 vs 6 mM, p = 0.001; 9 vs 3 mM, p = 0.001; 6 vs 3 mM, p = 0.003; n = 7), whereas XII burst shape parameters did not change significantly from 9 mM Kext (Fig. 1A; RM ANOVA; amplitude: F(2,12) = 3.4, p = 0.07; area: F(2,12) = 1.4, p = 0.3; duration: F(2,12) = 9.1, p = 0.004; 9 vs 6 mM, p = 0.2; 9 vs 3 mM, p = 0.054; n = 7). Occasionally, in both 9 and 6 mM Kext, longer XII bursts with two distinct peaks (“XII doublets”; see Materials and Methods) were observed (Fig. 1B) that constituted 28.2 ± 25.1% of XII events in 9 mM Kext and 27.8 ± 7.2% of XII events in 6 mM Kext (n = 7).

Underlying the changes in TxII and CV(TxII) was a shift from a unimodal distribution of TxII in 9 mM Kext (TxII,9; Fig. 1B, C) to a bimodal distribution in 6 mM Kext (Fig. 1B, C). These multimodal distributions were not significantly different from mixtures of Gaussians placed at integer multiples of the shortest peak (Kolmogorov–Smirnov, p = 0.08 – 0.7, n = 98 – 175 in each of 7 slices).

Multiple peaks in 6 mM Kext could arise from changes in T_xII over time (non-stationarity). This did not appear to be the case, as Poincaré maps of T_xII in 6 mM Kext (Fig. 1D) had multiple clusters away from the unity line (Fig. 1D), reflecting sequential short and long T_xII,6, and not a drift in the rhythm. As the modes in the distribution of T_xII,6 fell at roughly integer multiples of the lowest T_xII,6 peak (Figs. 1C, D), we hypothesized that an underlying faster rhythm originating in the preBoëtC was determining the longer T_xII.
preBo\"tC burstlets mediate an underlying higher frequency rhythm with lower variability

To examine whether rhythmic preBo\"tC activity could explain the multimodal TXII in 6 mM K_{ext}, we recorded simultaneously from the preBo\"tC and the XII nerve in medullary slices (Fig. 2A,B). Inspiratory-modulated bursts of activity in slices can be recorded directly from the preBo\"tC as neuronal population activity, which in 9 mM K_{ext} is expected to be correlated (nearly) one-to-one with XII bursts (Lieske et al., 2000; Ruangkittisakul et al., 2008). We defined preBo\"tC population activity that was concurrent with XII bursts as preBo\"tC bursts (Lieske et al., 2000; Ruangkittisakul et al., 2008). The distribution of periods of all preBo\"tC events in 9 mM K_{ext} (T_{preBo\"tC,6}) normalized to T_{XII,9} was similar to the distribution of T_{XII,9} (Fig. 2C; Kolmogorov–Smirnov, p = 0.3, n = 7 slices). When K_{ext} was lowered from 9 to 6 mM, T_{XII,6} increased, but mean T_{preBo\"tC} (T_{preBo\"tC}) for all preBo\"tC events did not change (paired t test, t_{6} = 0.04, p = 0.97, n = 7; Table 1). In contrast to the synchrony between preBo\"tC and XII activity in 9 mM K_{ext}, a large number of smaller preBo\"tC population events in 6 mM K_{ext} (Table 1) were not associated with XII bursts, and instead occurred during the silent XII interburst interval (IBI; Fig. 2A,†). We dubbed these preBo\"tC events burstlets to distinguish them from preBo\"tC bursts, which were accompanied by XII bursts. In triple recordings where preBo\"tC activity from both sides and XII activity were simultaneously measured, 99.3% of preBo\"tC burstlets were bilaterally synchronous (n = 6; Fig. 2E).

The distribution of T_{preBo\"tC} in 6 mM K_{ext} (T_{preBo\"tC,6}) for all preBo\"tC events, normalized to T_{XII,6} for each slice, was unimodal (Hartigan’s dip, p = 0.2–1, n = 7 slices) and significantly different from the multimodal distribution of T_{XII,6} (Fig. 2C; Kolmogorov–Smirnov, p = 10^{-16}, n = 7 slices), but not significantly different from the distribution of T_{preBo\"tC,9} (Fig. 2C; Kolmogorov–Smirnov, p = 0.8, n = 7 slices). The preBo\"tC burstlet/burst rhythm at 6 mM K_{ext} was significantly more regular [CV (T_{preBo\"tC,6}) = 0.41 ± 0.04] than that of XII bursts [CV (T_{XII,9}) = 0.60 ± 0.06] and not significantly different from CV (T_{XII,9}) (0.34 ± 0.06), CV (T_{XII,6}) (0.34 ± 0.2), or CV (T_{preBo\"tC,6}) (0.38 ± 0.07; one-way ANOVA, F_{(3,30)} = 7.3, p = 0.0003; CV (T_{preBo\"tC,6}) vs CV (T_{preBo\"tC,9}), p = 0.02; CV (T_{preBo\"tC,6}) vs CV (T_{preBo\"tC,3}), p = 0.7; CV (T_{preBo\"tC,6}) vs CV (T_{XII,9}), p = 0.7; CV (T_{preBo\"tC,6}) vs CV (T_{preBo\"tC,3}), p = 1; n = 7). Further, T_{XII,6} increased monotonically with the number of burstlets during the IBI. T_{XII,6} when 0, 1, 2, or 3 burstlets were observed in preBo\"tC were not significantly different from the corresponding integer multiple, e.g., 1, 2, 3, or 4, respectively, of T_{preBo\"tC,6} (Fig. 2D; Student’s t test; 1 burstlet: t_{6} = 0.007, p = 1; 2 burstlets: t_{6} = 2.1, p = 0.08; 3 burstlets: t_{6} = 1.5, p = 0.2; n = 7). Burstlet/burst patterns persisted when K_{ext} was lowered to 3 mM (Fig. 2A), as well as under more physiological cation concentrations when K_{ext} and Ca_{ext} were lowered to 3 and 1 mM, respectively (Fig. 3E; Table 1; Ruangkittisakul et al., 2011). Thus, preBo\"tC burstlets and bursts together constituted a regular rhythm underlying the multimodal distribution of T_{XII,6}.

Preinspiratory activity links burstlets and bursts, constituting an obligatory burstlet-like rhythmogenic process that triggers pattern generating activity

To examine whether a burstlet-like component could be observed in preBo\"tC bursts, we compared the shape of preBo\"tC bursts and burstlets in 6 mM K_{ext}. The peak amplitudes of preBo\"tC bursts were significantly larger than that of burstlets (Figs. 2A, 3A–C; paired t test, t_{6} = 7.5, p = 0.0003, n = 7), whereas...
Figure 2. Higher frequency burstlets in preBötC underlie the multimodal distribution of $T_{\text{preBötC}}$. 

A. Representative traces of simultaneous XII output (gray) and preBötC (black) recordings in 9, 6, and 3 mM $K_{\text{ext}}$ show bursts (large amplitude preBötC events that generate XII output) and burstlets (smaller amplitude preBötC events that do not result in XII activity; indicated with green asterisk). XII and preBötC doublets, e.g., fifth burst in 6 mM $K_{\text{ext}}$, were also observed. B. Representative time course of XII output and preBötC population recording in 9, 6, and 3 mM $K_{\text{ext}}$ showing $T_{\text{preBötC}}$ (top), $T_{\text{preBötC}}$ (middle), and amplitudes of preBötC events, i.e., burstlets and bursts (amplitude of preBötC burstlet) in a.u. $T_{\text{preBötC}}$ shows lower variability compared with $T_{\text{preBötC}}$ and $T_{\text{preBötC}}$. C. Top: Distributions of burstlet—next event (burstlet, burst, or doublet; green) and burst—next event (maroon) $T_{\text{preBötC}}$ and $T_{\text{preBötC}}$ (gray), all normalized to $T_{\text{preBötC}}$ for individual slices. Dotted line represents average value of shortest peak in $T_{\text{preBötC}}$ distribution. C, Bottom: Cdfs of $T_{\text{preBötC}}$ (gray) and $T_{\text{preBötC}}$ (black) in 9 and 6 mM $K_{\text{ext}}$ show significant overlap in 9 mM $K_{\text{ext}}$, whereas the distribution of $T_{\text{preBötC}}$ is significantly different from that of $T_{\text{preBötC}}$ in 6 mM $K_{\text{ext}}$. Data are mean ± SD ($n = 7$). D, Top: Representative traces of preBötC (black) and XII (gray) recordings with different numbers of burstlets during the IBI show that $T_{\text{preBötC}}$ increased monotonically with the number of burstlets. Gray bars indicate when the next burstlet was expected (green asterisk indicate burstlets). D, Bottom: $T_{\text{preBötC}}$ and $T_{\text{preBötC}}$ as a function of the number of burstlets in the IBI demonstrate that $T_{\text{preBötC}}$ increases monotonically with the number of burstlets ($n = 7$) bottom. Means for each slice are depicted as gray circles. E, Simultaneous recording of XII (gray) and ipsi- and contralateral preBötC (black) population activity show that burstlets (green asterisk) are bilaterally synchronous. Doublets, e.g., fourth burst, were also observed bilaterally in preBötC.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$T_{\text{preBötC}}$ (s)</th>
<th>Burstlets (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 mM $K_{\text{ext}}$</td>
<td>3.46 ± 0.81</td>
<td>18.4 ± 10.2</td>
</tr>
<tr>
<td>6 mM $K_{\text{ext}}$</td>
<td>3.46 ± 0.46</td>
<td>51.6 ± 9.8</td>
</tr>
<tr>
<td>3 mM $K_{\text{ext}}$</td>
<td>5.96 ± 1.39</td>
<td>70.0 ± 8.7</td>
</tr>
<tr>
<td>3 mM $K_{\text{ext}}$</td>
<td>4.13 ± 1.11</td>
<td>64.0 ± 6.3</td>
</tr>
<tr>
<td>8 μM Cd$_{\text{ext}}$</td>
<td>2.38 ± 1.30</td>
<td>100 ± 0</td>
</tr>
</tbody>
</table>

Table 1. Effects of changing extracellular ions on preBötC rhythm and pattern

preBötC burst duration (0.72 ± 0.17 s) did not differ significantly from preBötC burstlet duration (1.15 ± 0.34 s; paired t test, $t_{(n)} = 2.3$, $p = 0.06$; $n = 7$). In 5/7 slices, the distribution of preBötC burstlet and burst peak amplitudes was bimodal (Hartigan’s dip, $p = 2 × 10^{-5}$–0.002, $n = 5$), whereas the distributions for the other two slices showed a second peak by visual inspection that was not sufficiently distinct to reach statistical significance for multimodality (Fig. 3B). This suggests that mechanisms underlying the significantly greater amplitude of preBötC bursts were distinct from those generating burstlets.

Unlike inspiratory XII bursts, preBötC bursts in 9 and 6 mM $K_{\text{ext}}$ did not arise abruptly or smoothly from baseline. Rather, we identified two components in preBötC bursts. A high amplitude inspiratory burst component ("preBötC I-burst") initially led XII bursts (9 mM $K_{\text{ext}}$: 28 ± 14 ms; 6 mM $K_{\text{ext}}$: 60 ± 17 ms; $n = 7$), but peaked almost concurrently with XII bursts (preBötC→XII peak lag, positive indicates preBötC peak precedes XII peak; 9 mM $K_{\text{ext}}$: 8 ± 12 ms; 6 mM $K_{\text{ext}}$: 3 ± 10 ms; $n = 7$). The preBötC I-burst was preceded by low-level “preinspiratory” activity lasting 337 ± 121 ms in 6 mM $K_{\text{ext}}$, which was significantly longer than preinspiratory activity in 9 mM $K_{\text{ext}}$ (176 ± 66 ms; paired t test, $t_{(n)} = 3.2$, $p = 0.02$; $n = 7$; Fig. 3A, D).

Preinspiratory activity resembled the rising phase of burstlets. In 6 mM $K_{\text{ext}}$, the rising slope of preinspiratory activity, corresponding to the rate of increase in preBötC population activity, was not different from the rising slope of burstlets, but was significantly different from the slope of preBötC I-bursts (Fig. 3D; RM ANOVA, $F_{(2,12)} = 63.6$, $p = 4 × 10^{-7}$; burstlet vs preinspiratory, $p = 0.1$; burstlet vs I-burst, $p = 0.0004$; preinspiratory vs I-burst, $p = 5 × 10^{-7}$; $n = 7$). The peak burstlet amplitude [0.19 ± 0.10 arbitrary units (a.u.)] was also not significantly different from peak preinspiratory amplitude (Fig. 3D; 0.19 ± 0.13 a.u.; paired t test, $t_{(n)} = 0.04$, $p = 1$; $n = 7$). The congruence between burstlets and preinspiratory activity in initial slope and peak amplitude, along with the unimodal distribution of $T_{\text{preBötC}}$ suggests a common rhythmogenic process underlying both events. Furthermore, these data imply that preBötC bursts were not unitary events, but arose from a rhythmogenic burstlet/preinspiratory component that always preceded a mechanically distinct preBötC I-burst component.

To determine whether preBötC I-burst generation constituted a process distinct from rhythmic burstlet activity, we attempted to selectively block I-bursts. Bath-applied cadmium (Cd$_{\text{ext}}$; 8 μM) abolished preBötC and XII bursts in 3 mM $K_{\text{ext}}$ and 1 mM Ca$_{\text{ext}}$ (Fig. 3E, F; paired t test; preBötC/XII burst frequency: $t_{(n)} = 3.1$, $p = 0.04$; preBötC burst amplitude: $t_{(n)} = 41.1$, $p = 2 × 10^{-4}$; $n = 5$), while preBötC bursts continued, with no change in frequency or burstlet amplitude (Fig. 3E, F; Table 1; paired t test; frequency: $t_{(n)} = 0.9$, $p = 0.4$; amplitude: $t_{(n)} = 0.5$, $p = 0.6$; $n = 5$). CV($T_{\text{preBötC}}$) in control conditions (0.41 ± 0.11) and in Ca$_{\text{ext}}$ (0.25 ± 0.13) also were not significantly different (paired t test, $t_{(n)} = 2.3$, $p = 0.08$; $n = 5$). preBötC bursts appeared to be the
result of a state where preBo¨tC I-burst generation failed, either sporadically, when K_{ext} was lowered, or completely, when Cd_{ext} was added, whereas an underlying rhythmogenic mechanism continued relatively unperturbed.

An apparent threshold for transmission of activity to XII output filters preBo¨tC population activity

Low-level preBo¨tC activity, i.e., burstlets, did not generate XII output, suggesting that preBo¨tC population activity below a certain threshold was unable to propagate to premotoneurons and motoneurons to trigger XII activity. Consistent with a transmission threshold, preBo¨tC activity concurrent with XII doublets ("preBo¨tC doublets", Fig. 2A, E) usually displayed longer plateaus or multiple peaks that maintained activity during the XII interpeak pause (Fig. 4A), although with sufficiently long pauses, preBo¨tC activity could also return to baseline. Doublet→next event (burstlet, burst, or doublet) T_{preBo¨tC} were significantly longer than burst→next event and burstlet→next event T_{preBo¨tC} in 6 mM K_{ext} (Fig. 4B; RM ANOVA, F_{(2,12)} = 18.5, p = 0.0002; burstlet vs doublet, p = 0.003; burst vs doublet, p = 0.006; n = 7) and longer than burst→next event T_{preBo¨tC}, in 9 mM K_{ext} (Fig. 4B; paired t test, t_{(6)} = 3.3, p = 0.02, n = 7). Thus, following a doublet there was a longer delay to the next preBo¨tC burstlet, burst, or doublet.

We hypothesized that increasing excitability in premotoneurons and/or motoneurons could lower this transmission threshold to allow preBo¨tC activity to appear in motor output. As increases in K_{ext} reduced the occurrence of burstlets, we tested whether we could generate burstlet activity in XII output by lowering Ca_{2+}^{ext} to increase excitability (Ruangkittisakul et al., 2011). When K_{ext} and Ca_{2+}^{ext} were lowered to 3 and 1 mM, respectively (Fig. 3E; Ruangkittisakul et al., 2011), rhythmic activity recorded in XII output and preBo¨tC consistently showed a preBo¨tC burstlet/burst pattern similar to that seen in 6 mM K_{ext}/1.5 mM Ca_{2+}^{ext} (Table 1). Increasing excitability by further lowering Ca_{2+}^{ext} to 0.6–0.8 mM produced smaller events in XII output ("XII burstlets") that were synchronous with preBo¨tC activity below a certain threshold was unable to propagate to premotoneurons and motoneurons to trigger XII activity. Consistent with a transmission threshold, preBo¨tC activity concurrent with XII doublets ("preBo¨tC doublets", Fig. 2A, E) usually displayed longer plateaus or multiple peaks that maintained activity during the XII interpeak pause (Fig. 4A), although with sufficiently long pauses, preBo¨tC activity could also return to baseline. Doublet→next event (burstlet, burst, or doublet) T_{preBo¨tC} were significantly longer than burst→next event and burstlet→next event T_{preBo¨tC} in 6 mM K_{ext} (Fig. 4B; RM ANOVA, F_{(2,12)} = 18.5, p = 0.0002; burstlet vs doublet, p = 0.003; burst vs doublet, p = 0.006; n = 7) and longer than burst→next event T_{preBo¨tC}, in 9 mM K_{ext} (Fig. 4B; paired t test, t_{(6)} = 3.3, p = 0.02, n = 7). Thus, following a doublet there was a longer delay to the next preBo¨tC burstlet, burst, or doublet.

Most preBo¨tC inspiratory-modulated neurons fire during both burstlets and bursts

To determine how the activity of single neurons could sum to produce preBo¨tC population activity, inspiratory-modulated preBo¨tC neurons were recorded in loose patch configuration in 3 mM K_{ext}/1 mM Ca_{2+}^{ext} (n = 18) while monitoring XII output and/or contralateral preBo¨tC population activity. Most, but not all, of these neurons generated APs during both bursts and burst-
lets (n = 16/18, 89%; Fig. 5A). In 2/18 (11%) neurons, APs were generated only during bursts (Fig. 5B). No neurons (0/18) fired only during burstlets. To test for pacemaker properties, neurons that fired during both bursts and burstlets were synchronically isolated with blockers of fast excitatory and inhibitory transmission (10 μM CPP, 10 μM NBQX, 1 μM strychnine, 100 μM picrotoxin). With fast synaptic transmission blocked, a small percentage of these neurons continued to burst (2/16, 13%; Fig. 5C). This percentage is similar to that of all inspiratory-modulated neurons identified as having pacemaker properties (Thoby-Brisson and Ramirez, 2001; Del Negro et al., 2005).

For neurons that fired during both bursts and burstlets, the duration of AP firing during burstlets was significantly shorter than during bursts (Figs. 5, 6; burstlets: 462 ± 43 ms; bursts: 665 ± 64 ms; paired t test, t(15) = 5.5, p = 0.0002; n = 16). There was a trend toward a reduction in AP amplitude during burstlets to 63 ± 4% of the amplitude of the first AP (Figs. 5, 6). During bursts, however, AP amplitude was significantly attenuated to 39 ± 4% of the mean of the first AP (Figs. 5, 6; paired t test, t(15) = 7.3, p = 4 × 10^-5; n = 16). In some cases, APs decreased in amplitude or even transiently disappeared during the middle of a burst before returning (Figs. 5). These events often corresponded to doublets, as seen in both (contralateral) preBo¨tC and XII output (Figs. 5, 6). For each neuron, the number of APs varied widely from burstlet to burstlet (Fig. 6A; range: 2–37 spikes; CV: 0.58 ± 0.05; n = 16) and less so from burst to burst (Fig. 6A; range: 2–43 spikes; CV: 0.37 ± 0.02; n = 16).

Because population data suggested a common process underlying burstlets and preinspiratory activity, we divided spiking behavior during preBo¨tC bursts into preinspiratory and preBo¨tC I-burst components and compared firing during these phases with burstlet spiking. The average number and maximum frequency of APs during burstlets were not significantly different from these values during the preinspiratory phase, but were significantly lower than these values during the preBo¨tC bursts (Fig. 6B; one-way ANOVA; AP number: F(3,44) = 11.9, p = 8 × 10^-5; burstlet vs preinspiratory, p = 0.6; burstlet vs I-burst, p = 0.003; preinspiratory vs I-burst, p = 9 × 10^-5; maximum AP frequency: F(3,44) = 20.1, p = 6 × 10^-7; burstlet vs preinspiratory, p = 1; burstlet vs I-burst, p = 7 × 10^-6; preinspiratory vs I-burst, p = 6 × 10^-5; n = 16). The similarities in firing pattern between burstlets and preinspiratory activity and their significant differences from preBo¨tC I-bursts are consistent with the population data.

**Behavioral correlates of XII burstlets, bursts, and doublets are observed in vivo under specific conditions**

To examine whether burstlets, bursts, and doublets were representative of breathing in vivo, the respiratory pattern [airflow and diaphragm EMG activity (DIAEMG)] were recorded in anesthetized adult rats. Most airway and pulmonary sensory feedback was eliminated by laryngeal denervation, tracheostomy below the larynx, and then later during an experiment, vagotomy. In anesthetized, vagotomized adult rats, the shape and timing of bursts in DIAEMG were uniform, and we did not see small burstlet-like events or any double-peaked events in DIAEMG (Fig. 7A, left). In vitro, increasing excitability by lowering Ca_{ext}^+ allowed burstlet activity to leak through to XII output, producing smaller events that were synchronous with preBo¨tC bursts (Fig. 4C). In an effort to evoke burstlets in vivo comparable to those seen in vitro when we increased excitability, we disinhibited the preBo¨tC and Bo¨tC by local injection of bicuculline (Janczewski et al., 2013). Excitability was further increased with bilateral injection into the preBo¨tC of bombesin (Janczewski et al., 2012), a peptide that depolarizes preBo¨tC neurons (Gray et al., 1999; Iniushkin and...
output. In this mode in vitro, preBoT C bursts are hypothesized to be rhythmic, i.e., necessary for determination of time to the next burst (Rekling et al., 1996; Feldman et al., 2013). We speculated that large safety factors and/or redundancies that may ensure robust inspiratory burst generation could make preBoT C rhythmic mechanisms difficult to unravel from preBoT C patterning mechanisms (Mellen, 2008, 2010; Marder, 2011). By lowering \( K_{ext} \) from 9 to 6 mM, we increased variability in the XII burst rhythm. This resulted, not from a concurrent change in the timing of preBoT C rhythmic activity, which did not differ across the two conditions, but from failures in preBoT C burst generation and consequent XII burst generation, revealing distinct rhythm and pattern-generating mechanisms in vitro.

In slices bathed in 6 mM \( K_{ext} \), XII activity displayed skipped bursts (Fig. 1), similar to “quantal slowing” seen in breathing rhythms following opiate administration in vitro and in vivo (Mellen et al., 2003; Janczewski and Feldman, 2006) and “deletions” in motor nerves during in vitro locomotion (Zhong et al., 2012). Such behavior is postulated to arise from the persistence of rhythmic activity in one (or more) rhythmic population(s) of neurons that drive nonrhythmic downstream premo-toneuronal and motoneuronal pattern-generating networks (Mellen et al., 2003; Janczewski and Feldman, 2006; McCrea and Rybak, 2008). Here, rhythmic preBoT C burstlet activity maintained timing information during skipped XII bursts (Fig. 2). Moreover, \( T_{XII} \) increased monotonically with the number of burstlets and were distributed multimodally with longer \( T_{XII} \) approximately equivalent to integer multiples of the interburstlet interval (Fig. 2D).

preBoT C activity, however, was not just rhythmic. preBoT C bursts, which were closely associated with XII bursts, occurred in phase with the burstlet rhythm yet were significantly larger than burstlets. The markedly lower amplitude of burstlets was not due to small, isolated clusters of active neurons. More than 93% of burstlets were bilaterally synchronous in population recordings and in simultaneous recordings of individual neurons and contralateral population activity (Figs. 2E, 5A). Burstlets observed unilaterally could result from failure to detect smaller amplitude burstlets due to noise. Burstlets, together with preBoT C bursts, produced \( T_{preBoT C} \) that were significantly more regular than \( T_{XII} \). Burstlet→next event and burst→next event \( T_{preBoT C} \) did not differ, suggesting that substantial additional activity during a preBoT C I-burst did not significantly affect timing. With sufficient suprathreshold activity, the period defined by burstlet-mediated rhythmogenesis can be modified, as with the significantly longer doublet→next event \( T_{preBoT C} \) (Fig. 4B) and the occasional longer burst→next event \( T_{preBoT C} \) (Fig. 2A,E). The preBoT C, therefore, generates rhythmic activity and initiates its transformation into XII bursts to participate in pattern generation.

We suggest that preBoT C bursts result from a low amplitude rhythmic preinspiratory component, resembling burstlets in rise time, duration, and peak amplitude, and a high amplitude pattern-generating I-burst. When preBoT C bursts occurred, the rapidly rising preBoT C I-burst obscured the falling phase of burstlets, so the burstlet appeared as preinspiratory activity. Conversely, the failure of rhythmic burstlets to produce XII output during skipped bursts resulted from the inability to engage preBoT C I-burst-generating, i.e., patterning, mechanisms.

This two-stage model of preBoT C burst generation asserts that burstlet/preinspiratory activity is obligatory for I-bursts and, consequently, XII bursts. Indeed, triggering XII bursts with patterned photostimulation of small numbers (4–9) of

Glazkova, 2007). These injections produced small bursts of \( DA_{EMG} \) activity during the IBI (Fig. 7A); burstlet normalized to burst amplitude: 0.16 ± 0.05 a.u., \( n = 4 \) rats, similar to the burstlets recorded in vitro that leaked through to XII output in conditions of high excitability.

In addition to in vivo burstlets, we observed double-peaked bursts of \( DA_{EMG} \) activity following bombesin and bicuculline injections that resembled doublets observed in vitro in XII motor output (Fig. 7A; Janczewski et al., 2012). These in vivo doublets were more reliably produced by blocking inhibition with local injection of bicuculline and the glycine receptor antagonist strychnine into the preBoT C (Fig. 7B; Janczewski et al., 2012). This resulted in bursts with two peaks where the second peak was not significantly different from the first peak in \( DA_{EMG} \) (Fig. 7B); doublet second peak normalized to first peak: 0.74 ± 0.09 a.u.; paired t test, \( t_{(3)} = 2.6, p = 0.08; n = 4 \). The first peak of these doublets did not differ significantly from the amplitude of the single-peaked “eupneic” bursts (Fig. 7B); doublet first peak normalized to burst: 1.02 ± 0.08 a.u.; paired t test, \( t_{(3)} = 0.23, p = 0.8; n = 4 \). These in vivo burst shapes, with two equal peaks, seen in \( DA_{EMG} \) after vagotomy and blockade of the inhibitory neurotransmitters GABA and glycine (Fig. 7B), closely resembled XII doublet activity in vitro (Fig. 4A).

Discussion

In high excitability conditions, such as 9 mM \( K_{ext} \)/1.5 mM \( Ca_{ext} \) in the slice preparation and presumably the resting state in vivo, the mammalian respiratory CPG generates a stable and robust motor

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inspiratory-modulated preBo\textsuperscript{T}C neurons elicits ectopic XII bursts, resembling endogenous bursts, with an average latency of 255 ms (Kam et al., 2013), similar to the 100–400 ms duration of preinspiratory activity observed here and previously (Rekling et al., 1996). Increasing the number of targeted neurons decreases the latency between stimulation and XII burst initiation (Kam et al., 2013), just as increasing excitability decreased preinspiratory duration. We propose that onset of activity in a few preBo\textsuperscript{T}C neurons during the IBI is the key rhythmonic event that seeds both burstlet and preinspiratory activity, which percolates through the network to trigger an I-burst by a distinct mechanism.

By recording extracellular activity from single preBo\textsuperscript{T}C neurons (Figs. 5, 6), we determined that low amplitude preBo\textsuperscript{T}C burstlet activity resulted from low-frequency firing in ~90% of preBo\textsuperscript{T}C inspiratory-modulated neurons that also fired during bursts. No neurons fired only during burstlets. The increase in population activity during preBo\textsuperscript{T}C I-bursts resulted from significantly higher firing frequency in the same neurons generating burstlet activity, which percolates through the network to trigger an I-burst by a distinct mechanism.

preBo\textsuperscript{T}C rhythm (burstlet) and pattern (I-burst) generation could be disassociated with Cd_{ext}^2\textsuperscript{+}, Cd_{ext}^2\textsuperscript{+} abolished preBo\textsuperscript{T}C bursts and all XII output, while leaving rhythmic burstlets intact (Fig. 3 E, F). Cd_{ext}^2\textsuperscript{+}, a broad spectrum blocker of voltage-gated Ca\textsuperscript{2+} channels that can reduce Ca\textsuperscript{2+}-activated conductances, could affect preBo\textsuperscript{T}C I-burst generation by inhibiting activation of I_{CAN} (Thoby-Brisson and Ramirez, 2001) or reducing the efficacy of synaptic transmission between preBo\textsuperscript{T}C neurons (Reid et al., 1997).

Alternatively, several models of preBo\textsuperscript{T}C rhythmogenesis show rhythmic patterns similar to burstlet/burst rhythms, e.g., “irregular” or “intermittent” bursting in (Butera et al., 1999b; Rybak et al., 2004; Purvis et al., 2007), that suggest other mechanisms mediating preBo\textsuperscript{T}C I-burst generation. The regimes where “irregular” rhythms appear can be functions of the distribution of I_{NaP} conductance (Rybak et al., 2004; Purvis et al., 2007) or of coupling strength (Butera et al., 1999b). Thus, lowering excitability may increase variability in I_{NaP} across neurons or decrease coupling strength to produce a mixed burstlet/burst preBo\textsuperscript{T}C rhythm, suggesting that I-burst generation is sensitive to manipulations that decrease synchrony across the network.

preBo\textsuperscript{T}C bursts did not solely determine the pattern of XII activity. Increasing excitability could produce low levels of motor activity synchronous with burstlets, in vitro (XII; Fig. 4C) and in vivo (diaphragm; Fig. 7A), suggesting a tunable transmission threshold from preBo\textsuperscript{T}C to inspiratory motoneurons. This threshold is below peak preBo\textsuperscript{T}C burst activity as XII burst initiation occurred during the preBo\textsuperscript{T}C I-burst rise, and preBo\textsuperscript{T}C and XII bursts peaked almost concurrently. Additionally, some XII doubles exhibited a pause between peaks of activity that was not reflected in preBo\textsuperscript{T}C activity, which decreased, but did not reach baseline (Fig. 4A). This transmission threshold may involve preBo\textsuperscript{T}C I-burst-only neurons that, despite being a small minority, could function as an output or response-amplifying population, important in many networks (Feldt et al., 2011), or downstream premotoneurons or motoneurons, acting as high-pass amplitude filters that prevent lower levels of preBo\textsuperscript{T}C activity from reaching XII output (Chamberlin et al., 2007).

Measurement of motor output and, for in vitro preparations, acknowledgment of the absent role of sensory feedback in shaping motor pattern is critical for interpreting the role of interneuronal activity in the respiratory CPG. Solely recording preBo\textsuperscript{T}C population activity, small and large bursts observed in vitro were designated as “fictive” eupnea and sighs, respectively (Lieske et al., 2000; Ruangkittisakul et al., 2008). However, sighs, a double-peaked inspiratory effort where a eupneic-like burst is followed closely by a larger second burst of activity (Cherniack et al., 1981; Orem and Trotter, 1993), were not observed in vivo in untreated vagotomized animals, even after increasing preBo\textsuperscript{T}C excitability (Fig. 7; Cherniack et al., 1981). Thus, sighs might not be expected to appear in medullary slices where sensory feedback is also absent. Additionally, “fictive” sighs (Lieske et al., 2000; Ruangkittisakul et al., 2008) are similar to preBo\textsuperscript{T}C bursts seen here, in both shape and sensitivity to Cd_{ext}^2\textsuperscript{+}. How our burstlets and bursts map

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**Figure 6.** Characteristics of preBo\textsuperscript{T}C neuron firing patterns during burstlets and bursts. **A,** Top, APs during burstlets (left, green), bursts (middle, maroon), or doubles (right, black) of one preBo\textsuperscript{T}C inspiratory-modulated neuron. Each line of each column of the raster plot represents APs of recorded neuron during a single burstlet, burst, or doublet. **A,** Middle, Histograms of raster plots show the average AP frequency during burstlets, bursts, and doubles. **A,** Bottom, Amplitudes of APs over time during burstlets, bursts, and doubles are shown below the histograms. Time course of amplitude shows that AP waveform does not change significantly during burstlets, but decreases during bursts and doubles. After a pause in firing during some doubles, more APs are seen, corresponding to a second peak in the doublet. Raster plots, histograms, and amplitudes in a.u. are aligned to the average burstlet or XII burst/doublet waveform. Half-arrow indicates onset of preBo\textsuperscript{T}C burstlet, burst, and doublet. **B,** Comparison of firing pattern properties including AP number, frequency, and minimum amplitude normalized to the first AP in the event during burstlets (green), and the preinspiratory and I-burst components (maroon) of bursts in inspiratory-modulated neurons. Data are mean ±SD (*p < 0.05; n = 16) with means for each slice as gray circles.
to “fictive” sighs and eupnea (Lieske et al., 2000; Ruangkittisakul et al., 2008) remains to be determined since records of simultaneous XII activity were absent in these prior studies, and experimental conditions differed.

Concurrent recordings of XII activity provided motor context to preBoTc bursts and burstlets in vivo, but we also elicited burstlet-like motor activity in vivo by eliminating sensory feedback and increasing preBoTc excitability, conditions intended to mimic those in the slice (Fig. 7). Our in vivo observations suggest that burstlets reflect processes also present in the adult intact rat, but not seen in motor output under normal conditions presumably due to a high safety factor for preBoTc burstlets to ultimately trigger motor nerve bursts. Doublets were also observed in vivo. Although double breaths do not occur spontaneously in intact animals, we speculate that the two peaks of preBoTc activity are transformed by sensory feedback and downstream pattern-generating networks to produce the classic augmented breath shape of sighs (Janczewski et al., 2012).

We conclude that distinct mechanisms within the preBoTc underlie inspiratory rhythm and pattern generation. Separation of rhythm and pattern-generating mechanisms within the preBoTc network provides a functional substrate for targeted modulation of either timing or pattern by other brain areas, e.g., raphe, retrotrapezoid nucleus, locus ceruleus, necessary to alter ventilation or affect breathing movements for various reflexive, emotive, and volitional respiratory-related behaviors. Because rhythmic bursting is a fundamental mode of nervous system activity, used in the encoding of multimodal sensory information and the execution of all forms of motor behavior, these mechanisms may be relevant for burst generation in many neural circuits.

References


