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Perturbations of Respiratory Rhythm and Pattern by Disrupting Synaptic Inhibition within Pre-Bötzinger and Bötzinger Complexes

Role of synaptic inhibition in pre-BötC and BötC

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Abstract

The pre-Bötzinger (pre-BötC) and Bötzinger (BötC) complexes are the brainstem compartments containing interneurons considered to be critically involved in generating respiratory rhythm and motor pattern in mammals. Current models postulate that both generation of the rhythm and coordination of the inspiratory-expiratory pattern involve inhibitory synaptic interactions within and between these regions. Both regions contain glycinergic and GABAergic neurons, and rhythmically active neurons in these regions receive appropriately coordinated phasic inhibition necessary for generation of the normal three-phase respiratory pattern. However, recent experiments attempting to disrupt glycinergic and GABAergic postsynaptic inhibition in the pre-BötC and BötC in adult rats *in vivo* have questioned the critical role of synaptic inhibition in these regions as well as the importance of the BötC, which contradicts previous physiological and pharmacological studies. To further evaluate the roles of synaptic inhibition and the BötC, we bilaterally microinjected the $GABA_A$ receptor antagonist gabazine and glycinergic receptor antagonist strychnine into the pre-BötC or BötC in anesthetized adult rats *in vivo* and in perfused *in situ* brainstem-spinal cord preparations from juvenile rats. Muscimol was microinjected to suppress neuronal activity in the pre-BötC or BötC. In both preparations, disrupting inhibition within pre-BötC or BötC caused major site-specific perturbations of the rhythm and disrupted the three-phase motor pattern, in some experiments terminating rhythmic motor output. Suppressing BötC activity also potently disturbed the rhythm and motor pattern. We conclude that inhibitory circuit interactions within and between the pre-BötC and BötC critically regulate rhythmogenesis and are required for normal respiratory motor pattern generation.

Significance Statement

Defining functional roles of postsynaptic inhibition in respiratory and other mammalian central pattern generation circuits is a longstanding problem. Inhibitory circuit interactions within and between the brainstem respiratory pre-BötC and BötC have been proposed to be critically involved in normal rhythm and motor pattern generation. A fundamental role of postsynaptic inhibition in these regions has been questioned in recent experiments attempting to pharmacologically disrupt this inhibition. To resolve this contradiction, we applied similar approaches of microinjecting selective pharmacological antagonists of $GABA_A$ergic and glycinergic receptor-mediated inhibition in the pre-BötC and BötC in rats. Our results demonstrate large, site-specific perturbations of respiratory rhythm and motor pattern including disruption of rhythmic motor output and thus confirm the critical role of inhibitory circuit interactions.

Introduction

Rhythmic movements such as breathing and locomotion are produced by central pattern generator (CPG) networks containing interacting excitatory and inhibitory circuits. These circuits are the neural substrates for producing motor behavior (Grillner, 2006) and defining their specific roles in rhythmic motor pattern generation is key to understanding the functional operation of CPGs. Here we have addressed the longstanding problem of defining roles of inhibitory circuits in core structures of the mammalian brainstem respiratory CPG.

The respiratory neural pattern under normal conditions includes three phases: inspiration, post-inspiration and late expiration (Richter, 1996; Richter and Smith, 2014). The kernel of the respiratory CPG located in the ventral respiratory column (VRC) of the medulla includes two key compartments, the pre-Bötzinger (pre-BötC) and Bötzinger (BötC) complexes (Alheid and McCrimmon, 2008; Smith et al., 2009, 2013). The pre-BötC contains a heterogeneous population of excitatory neurons, including cells with intrinsic bursting properties, and excitatory synaptic interconnections that generate rhythmic inspiratory activity and drive inspiratory motor output. This excitatory population, when isolated in slices *in vitro*, generates inspiratory bursting activity (Smith et al., 1991; Koshiya and Smith, 1999) that persists after disrupting synaptic inhibition (Johnson et al., 2001). In addition, the pre-BötC contains GABAergic and glycinergic neuron populations (Kuwana et al., 2006; Winter et al., 2009; Morgado-Valle et al., 2010; Koizumi et al., 2013) providing phasic inspiratory inhibition widely distributed in the brainstem including inhibition of BötC expiratory neurons. In turn, BötC post-inspiratory and expiratory neurons provide phasic expiratory inhibition (Jiang and Lipski, 1990; Tian et al., 1999a,b; Ezure et al., 2003a,b) including to pre-BötC inspiratory neurons to coordinate generation of expiratory and inspiratory phases.

The specific contributions of the intrinsic excitatory bursting mechanisms in the pre-BötC, and inhibitory network interactions between pre-BötC and BötC, to respiratory rhythm and motor pattern generation are not clearly understood and this issue is continuously debated. Early theoretical models, based entirely on network inhibitory interactions, could not explain the maintenance of rhythm after blockade of synaptic inhibition *in vitro*. Alternatively, the pure autorhythmic excitatory network models, developed to explain the *in vitro* data, could not account for many behaviors observed *in vivo*, such as the Hering-Breuer inspiratory inhibitory and other respiratory reflexes and the coordinated generation of multiple respiratory phases. Also, these models could not reproduce apneusis, a breathing pattern characterized by a significantly prolonged inspiration alternating with short expiratory intervals (Lindsay et al., 2012).

To resolve this problem, more complicated models have been developed (Rybak et al., 2004, 2007; Smith et al., 2007, 2009, 2013) hypothesizing that: (i) the pre-BötC, while capable of autonomous generation of rhythmic bursting when isolated *in vitro*, is embedded in the larger respiratory network where its activity is controlled by interactions with other brainstem compartments, including inputs from excitatory RTN/pFRG neurons, and from the inhibitory neuron populations in BötC, and (ii) both the intrinsic bursting of pre-BötC inspiratory neurons and inhibitory interactions between the neural populations in pre-BötC and BötC are fundamentally involved in generating the normal rhythmic respiratory pattern. Disruption of inhibition in these circuits would lead either to switching to the intrinsic rhythmic activity originating within the pre-BötC, or to sustained or apneustic-like activity.

This concept was challenged by a recent study in the anesthetized rat employing targeted pharmacological blockade of fast inhibitory neurotransmission (Janczewski et al., 2013) from

which it was concluded that (i) the BötC does not play a role in respiratory rhythm/pattern generation, and (ii) inhibition within the pre-BötC and BötC is not required for generating a normal breathing rhythm and pattern.

The present study was focused on resolving this contradiction and further evaluating roles of inhibitory interactions in pre-BötC and BötC in rhythm generation and shaping respiratory pattern. Our experiments were performed using two distinct preparations, the anesthetized, vagotomized adult rat and the arterially perfused *in situ* brainstem-spinal cord preparation of juvenile rat. Specific pharmacological blockers of glycinergic (strychnine) and $GABA_A$ergic (gabazine) receptor-mediated inhibition were selectively microinjected into the pre-BötC or BötC and perturbations of the respiratory frequency and phases were evaluated. In addition, microinjections of the $GABA_A$ receptor agonist muscimol were used to inhibit neural activity in each compartment. Our results were not consistent with those reported by Janczewski et al. (2013). Disrupting inhibition within pre-BötC or BötC as well as inhibiting BötC activity caused major perturbations of the respiratory frequency and three-phase pattern. Moreover, blocking inhibition within the BötC lead to apnea confirming the critical role of inhibitory interactions between pre-BötC and BötC. Our results are consistent with previous proposals of the important role of inhibitory interactions within and between pre-BötC and BötC in generating and shaping the respiratory pattern.

Materials and Methods

Animal procedures

All experimental procedures used in this study were approved by either the NINDS Animal Care and Use Committee, or the Drexel University Institutional Animal Care and Use Committee, which oversees Drexel University's AAALAC International-accredited animal program. All electrophysiological recording and pharmacological microinjection experiments were performed via a surgically exposed ventral brainstem for access to the ventro-lateral medullary pre-BötC and BötC regions (**Figure 1**).

Surgical procedures in adult rats in vivo

Spontaneously breathing, adult male Sprague-Dawley rats (340–380 g) were anesthetized with isoflurane vaporized in O_2 (Matrix; 4–5% induction, 1.75–2.0% maintenance) via a snout mask. Anesthetic depth was maintained at a level at which withdrawal reflexes as well as changes in heart rate and blood pressure (BP) in response to pinching the distal hind limbs were absent. After tracheotomy with a glass tube, animals were artificially ventilated with the same gas mixture (60 min^{-1}, 2.5–3.0 ml tidal volume; Columbus Apparatus rodent ventilator). Electrocardiogram (EKG) was measured via three small subcutaneous electrodes using conventional amplification and filtering (Neurolog; Digitimer) and monitored using an audio amplifier (model AM10; Grass Instruments) and oscilloscope (Tektronix). One femoral artery and vein were cannulated for measurement of arterial pressure and infusion of drugs/saline, respectively. During all surgical procedures, rectal temperature was maintained at 37.0 ± 0.1°C via a servo-controlled heating blanket coupled to a rectal thermometer (Harvard Apparatus). The phrenic (PN) nerve was prepared for recording by dissecting the nerves free from the

surrounding tissue. Ventral neck muscles (cleidomastoideus, sternomastoideus, sternohyoideus, omohypides and digastricus), infrathyroid portions of the trachea and esophagus were removed. The body of the 1st neck vertebra (atlas) and base portion of occipital bone were removed to expose the ventral medulla and the axo-occipital membrane was cut. The dura was then opened using iridectomy scissors and residual bleeding from lateral epidural sinus was arrested by applying small pieces of gelfoam (USP; Pharmacia) soaked with thrombin solution (50 $U*ml^{-1}$ USP, Biopharm Lab) dissolved in artificial cerebrospinal fluid (aCSF).

All animals were vagotomized and baro- and chemoreceptor denervated via bilateral transection of the carotid sinus nerves to prevent cardiorespiratory reflex influences on motor nerve outputs (Richter and Seller, 1975; Grundy et al., 1986; Hopp and Seagar, 1998; Virkkia et al., 2007; Baekey et al., 2010). A bilateral pneumothorax was performed before electrophysiological recording to eliminate lung inflation-related movement artifacts and chest wall mechanoreceptor feedback. A positive end-expiratory pressure of 1.0 cm H_2O was maintained to prevent lung atelectasis during expiration. Animals were paralyzed by an intravenous bolus injection (2 mg/kg), followed by continuous infusion (3–4 mg/kg/h), of vecuronium bromide (Abbott Laboratories) dissolved in Ringer-Locke solution. End-tidal CO_2 was maintained between 5.0% and 5.5% (Capstar, CWE Inc.) by adjusting frequency of ventilation. If necessary, animals were continuously infused with Ringer-Locke solution (1.0–1.25% body weight or 10.0–12.5 ml/kg/h) to maintain a stable mean arterial pressure of 85–95 mmHg.

In situ *arterially perfused brainstem-spinal cord preparation*

Experiments were also performed with the *in situ* arterially perfused brain-stem spinal cord preparations from juvenile rats (3–5 weeks old) as previously described (Paton, 1996; Smith et

199 al., 2007). These preparations were studied because they provide the opportunity to investigate
200 roles of synaptic inhibition in an unanesthetized preparation generating the three-phase
201 respiratory pattern that could be clearly identified from simultaneous recordings of spinal and
202 cranial nerves, including prominent post-inspiratory discharge recorded from the central vagus
203 nerve (cVN). We note that cVN recordings from the normocapnic, vagotomized, carotid body
204 denervated, and isoflurane anesthetized adult rats *in vivo* in our experiments do not routinely
205 exhibit post-I discharge although neuronal recording in the BötC always shows post-I activity
206 (e.g., see Figure 3B), which is an important feature of medullary respiratory circuit activity.
207 Furthermore, a number of theoretical models (e.g., Rybak et al., 2007; Smith et al., 2007; Rubin
208 et al., 2009; Shevstova et al., 2011, 2014; Richter and Smith, 2014) postulating roles of pre-BötC
209 and BötC inhibitory circuits in respiratory rhythm and pattern generation have been based in part
210 on experimental results obtained from these *in situ* preparations. It is therefore critical to test
211 their roles in this preparation, and to compare results from the *in vivo* anesthetized adult rat
212 preparations utilizing a similar strategy for targeted disruption of synaptic inhibition in the pre-
213 BötC and BötC.

214 Preheparinized (1,000 units, given intraperitoneally) juvenile rats (Sprague-Dawley, 45–
215 90 g; male) were anaesthetized deeply with 5% isoflurane and the portion of the body caudal to
216 the diaphragm was removed. The head and thorax were immersed in ice-chilled carbogenated
217 aCSF solution (1.25 mM $MgSO_4$, 1.25 mM KH_2PO_4, 5.0 mM KCl, 25 mM $NaHCO_3$, 125 mM
218 NaCl, 2.5 mM $CaCl_2$, 10 mM dextrose, 0.1785 mM polyethylene glycol) and the rat was
219 decerebrated at a precollicular level. The descending aorta, PN and cervical vagus nerve (cVN)
220 were surgically isolated. As with the *in vivo* preparations, ventral neck muscles, infrathyroid
221 portions of the trachea, the esophagus, and then the 1st neck vertebra and base portion of the

occipital bone were removed to expose the ventral medulla. The axo-occipital membrane was cut, and the dura was then cut open. The preparation was transferred to a recording chamber and secured in a stereotaxic head frame ventral side up. The descending aorta was cannulated with a double lumen catheter for perfusion and recording of perfusion pressure with a pressure transducer (Micron Instruments). Vecuronium bromide was added to the perfusate to block neuromuscular transmission (4 μg/ml; SUN Pharmaceutical Industries). The perfusate was gassed with 95% O_2 / 5% CO_2 and maintained at 31 ^{0}C. Vasopressin (200–400 pM as required; APP Pharmaceuticals) was added to the perfusate to raise and maintain perfusion pressure between 70 and 80 mmHg (Paton et al., 2006). Unless stated, all chemicals were from Sigma.

Electrophysiological recording **in vivo**

With the rat in the supine position, the central ends of the cut PN were placed on bipolar silver hook electrodes for recording (10–5,000 Hz bandpass; Neurolog, Digitimer) and immersed in a mineral oil pool formed by skin flaps. To identify precise locations of the BötC and pre-BötC the activity of medullary expiratory and (pre)inspiratory neurons was recorded (200–3,000 Hz bandpass; Neurolog) by a ventral approach and glass (WPI) microelectrodes (tip outer diameter of 2–3 μm, 5–10 MΩ) filled with 0.5 M NaCl and 2% pontamine sky blue. The pre-BötC is readily identified by a characteristic pattern of pre-inspiratory-inspiratory (pre-I/I) activity (see **Figure 3A**) and the BötC has a characteristic profile of post-inspiratory (post-I) and augmenting expiratory (aug-E) activities (see **Figure 3B**). The microelectrode was held in a 3-dimensional stepper motor assembly (DC-3K, Märzhäuser), attached to the rail of the stereotaxic frame, and advanced in steps of 2.5–5.0 μm. After mapping neuronal activity, the medullary surface was marked bilaterally by ionophoresis for 15 min. of the pontamine sky blue dye (-25 nA, 0.125 Hz, 4 s; Axoclamp 2A) as a guide spot for insertion of drug microinjection pipettes. All

electrophysiological signals were recorded simultaneously with expiratory CO_2 level, arterial blood pressure, and lung inflation pressure, on the hard disk of a personal computer via a 16-bit analog-to-digital converter (PowerLab, AD Instruments, 10 kHz sampling rate) and displayed continuously with software (Chart, AD Instruments).

Electrophysiological recording **in situ**

To monitor respiratory network activity and motor output in the *in situ* perfused brainstem-spinal cord preparations, we recorded with fire-polished glass suction electrodes inspiratory activity from PN, and cVN inspiratory and post-inspiratory activity. Signals were amplified (50,000 – 100,000X, CyberAmp 380, Molecular Devices), band-pass filtered (0.3 – 2 kHz), digitized (10 kHz sampling rate) with an AD converter [Cambridge Electronics Design (CED)], and then rectified and integrated digitally with Spike 2 software (CED). Extracellular population activity from pre-BötC or BötC respiratory neurons in the perfused *in situ* preparations was also recorded with a fine glass electrode (3–5 MΩ) filled with 0.5 M Na^+ acetate.

Targeting the pre-BötC and BötC regions with microinjections **in vivo**

To block fast inhibitory transmission a cocktail of $GABA_A$ (gabazine, Sigma) and glycine (strychnine, Sigma) receptor antagonists (in aCSF) was pressure injected (250 μM, 105–115 nl) into the BötC or pre-BötC bilaterally and simultaneously (1–1.5 nl/s) with small diameter (OD = 15 μm, ID = 7.5 μm) polished micropipettes (Drummond Scientific). In another set of experiments the $GABA_A$ agonist muscimol was injected (100 μM, 25–30 nl) into the BötC or pre-BötC bilaterally and simultaneously. Control injections were made at the same sites with aCSF alone. All injection volumes were measured by microscopically observing the change in level of the meniscus within the micropipette. Microinjections were done after

electrophysiological recording to map the extracellular single unit/neuronal population activities characteristic of the pre-BötC or BötC regions as described above, and also in some *in vivo* experiments after pharmacological probing by microinjection of 5 nl of 10 mM L-Glutamate (L-Glu) to further confirm locations of the pre-BötC or BötC regions, which have site-specific responses to brief (500 msec) local L-Glu microinjections (i.e., transient apnea with increasing arterial blood pressure (ABP) for the BötC region, and tachypnea with decreasing ABP for the pre-BötC region) (see **Figure 3C,D**). These blood pressure responses are typical because the pre-BötC partially overlaps with the depressor caudal ventrolateral medulla (CVLM) and the BötC with the pressor rostral ventrolateral medulla (RVLM) (Kanjhan et al., 1995; Lipski et al., 1996; Moraes et al., 2012). According to our mapping by extracellular recordings, and consistent with characteristic perturbations obtained by our targeted L-Glu microinjections based on the neuronal activity maps, BötC and pre-BötC in isoflurane anesthetized adult male rats (340–380 g) occupy restricted areas: 1.8–2.1 mm lateral, 550–850 µm depth from the ventral surface and extending ~400 µm in the rostro-caudal dimension (i.e., ~800–1200 µm from the caudal pole of facial nucleus or ~1.6–2.0 mm rostral to obex) for pre-BötC, and for BötC 1.9–2.2 mm lateral, 450–750 µm depth, and 600–700 µm in the rostro-caudal dimension (~100–750 µm from the caudal pole of facial nucleus or ~2.0–2.75 mm rostral to obex). At the end of each experiment fluorescent marker yellow-green microbeads (0.1 µm diameter, 2% solution, Invitrogen) were injected via a pipette (OD = 30 µm, ID = 15 µm, Drummond) into the site of microinjections for *post hoc* morphological verification (**Figures 1** and **2**).

Because the locations of pre-BötC and BötC in adult rats relative to the ventral surface are shallow (550–850 and 450–750 µm, respectively), we performed tests for back leakage of injected solution along the microinjection pipette track. Pipettes with different outer diameters

(12.5–50 µm) were filled with 2% of pontamine sky blue and only large diameter pipettes (>30 µm) showed substantial leak of dye back to the ventral surface during microinjections. Accordingly for drug microinjections *in vivo* we only used pipettes with 15 µm OD that never exhibited back leak.

***Microinjections in arterially perfused brainstem-spinal cord preparations* in situ**

As *in vivo*, we bilaterally microinjected a cocktail of gabazine and strychnine and in some experiments muscimol (all drugs dissolved in the perfusate aCSF) with micropipettes (ID = 20 µm) positioned in the pre-BötC or BötC through the ventral medullary surface with microdrives (Marzhauser) after mapping locations of these regions with extracellular recording of pre-inspiratory (pre-BötC) or expiratory (BötC) neuronal population activities. Slow, continuous microinjection was accomplished by applying low pressure (30 mm Hg) to the pipettes with a pressure control and measurement system. In preliminary experiments we determined that, compared to the *in vivo* experiments, lower concentrations of drugs (30 µM of gabazine and strychnine or 10 µM muscimol) bilaterally injected into either of these regions caused rapid and large disturbances of respiratory motor output and hence all experiments with the perfused brainstem-spinal cord preparations were conducted at these lower drug concentrations. In some experiments, extracellular recordings of neuronal population activity in pre-BötC (during injections in BötC) or BötC (during injections in pre-BötC) regions were made during drug microinjections. Fluorescent microbeads were microinjected to mark the sites of drug microinjection (**Figures 1, 2**) as described above for the *in vivo* experiments.

Histological verification of microinjection sites

Immediately after marking locations of drug microinjection sites, animals *in vivo* were transcardially perfused with 400 ml of saline (10–12°C, pH 7.4) with heparin (1,000 Units/ml) followed by 500 ml of 4% paraformaldehyde (wt/vol) in 0.1 M phosphate buffered saline (PBS, 10–12°C, pH 7.4). A similar procedure was followed (but with smaller volumes of saline and paraformalydehyde perfusates) for perfusion fixation of the *in situ* brainstem-spinal cord preparations. For both preparations the brainstem was removed, post-fixed in the same fixative for 24 h at 4°C, and subsequently cryoprotected by sequential incubation in 15% and 30% sucrose in PBS and stored overnight at 4°C in 30% sucrose, 0.1 M PBS solution. The medulla oblongata was sectioned parasagittally or coronally at 30 or 50 µm thick sections with a freezing microtome. For fluorescence immunohistochemistry, floating sections were incubated with 10% donkey serum in PBS with Triton X-100 (0.3%) and subsequently incubated for 48–72 hours at room temperature with primary antibodies for choline acetyltransferase (ChAT) (goat anti-ChAT, Millipore, 1:200) to label motoneurons. Sections were then rinsed with PBS and incubated for 2 hours with secondary antibodies for ChAT (donkey anti-goat-Dylight 488, 1:500). Individual sections were mounted on slides and covered with an anti-fading medium (Fluoro-Gel; Electron Microscopy Sciences). Fluorescent labeling was visualized with a laser-scanning confocal imaging system (Zeiss LSM 510).

Signal analyses of respiratory parameters

All automated analyses of respiratory parameters from digitized nerve or neuronal population activities were performed with IDL software (Exelis VIS). Inspiratory events were detected from digitally rectified and integrated phrenic nerve signals via a 200 ms window moving average and peak detection algorithm that calculated a threshold-based zero derivative (positive peak) point. Following peak detection, inter-burst interval (IBI; inverse of burst frequency), inspiratory time

(T_I) and expiratory time (T_E) were measured. T_I was measured as the original integrated burst width at 20% of the peak height above baseline; T_E was calculated as IBI – T_I. Inspiratory amplitude (amp.) was calculated by subtracting the local baseline from the peak value. The endpoint of the parameter quantification was defined when the perturbation during or after microinjections reached it maximum or the signals declined to noise level and the program started missing peak detections (which could appear as a quantum jump in IBI).

The time courses of the changes in the above parameters for a given experimental group were variable, which we assume resulted from variability in the times required for drug diffusion to affect a sufficient number of neurons to produce the perturbations. To represent group data, we computed the mean time courses of the parameter values by the following procedures. For each experiment, the time courses of the parameters were extracted by a 30 s window moving median up to the defined endpoint, and the parameter values for each time series were normalized to the computed mean values during the control period (from 120 to 0 s before start of microinjection). Each time course was divided into 100 time points representing 1% increments from the start of microinjection (time 0) to the endpoint. We then computed the group mean time, and also mean values of the normalized parameters, at each of these points, which were plotted and connected by lines to represent the mean time series. The mean endpoint was plotted with its ± SEM's represented by crossbars (see **Figures 4B-E, 6A-D, 7B-E, 9A-D, 13B-E, and 14D-G**) and the SEM's of the preceding normalized parameter values for the group time series were represented by a gray band. To determine statistical significance, the control values were compared with the endpoint values for each experiment within a group using a two-sided Wilcoxon Signed-Rank Test (significant p-value: <0.05).

In addition to quantifying the respiratory parameters indicated above, we analyzed perturbations of amplitudes and durations of individual phases of the respiratory cycle (e.g., post-I activity in cVN recordings *in situ*) from integrated, cycle phase-triggered (peri-event) neurograms aligned at the onset of the inspiratory phase defined by PN activity. Successive cycle-triggered traces were either overlaid, or represented as a colored raster plot to depict temporal profiles of activity intensity before and during/after drug injection periods (see **Figures 10A,B and 14B**).

Results

Perturbations of respiratory rhythm and pattern by targeted microinjection of L-Glutamate in the pre-BötC or BötC in vivo

As described in Methods, we microinjected L-Glu in pre-BötC and BötC regions (n = 15, 10 mM, 5 nl) to establish characteristic regional excitatory perturbations. These *in vivo* experiments demonstrate that activation of pre-BötC (n = 5) by brief (500 msec) L-Glu microinjections always produced a transient increase of respiratory frequency (from 28.9 ± 4.4 to 44.55 ± 6.1 bursts/min, $p < 0.0001$) due to a significant reduction of expiratory phase duration (T_E) (**Figure 3C)**. In contrast, the same L-Glu microinjections into BötC (n = 10) always transiently prolonged the respiratory period (by 5.4 to 21 s range; mean value = 10.1 ± 4.03 s) (**Figure 3D**), documenting site-specificity of the perturbations.

Perturbations of respiratory rhythm and pattern by microinjection of gabazine and strychnine in the pre-BötC in vivo *and* in situ

Microinjections of the gabazine-strychnine cocktail were performed after initially identifying the pre-BötC region electrophysiologically by recording pre-I/I neuronal population activity and/or also after microinjected L-Glu (*in vivo*) to identify the pre-BötC region (**Figure 3C**). Perturbations of neural activity with bilateral microinjections of 110 nl of 250 μM of gabazine and strychnine in the anesthetized adult rat *in vivo* were stereotypical. A representative example is shown in **Figure 4A.** Drug microinjections in pre-BötC produced an increase in the respiratory frequency (f_R), primarily due to shortening of T_E, as well as a decrease of the amplitude of integrated PN activity. The group (n = 6) *in vivo* data are summarized in **Figure 4B-E**, which shows the mean time courses of the developing perturbations of all parameters (normalized to

386 control values) during and after the microinjections. Perturbations developed rapidly, within 10 s
387 following the onset of microinjection in all cases. The respiratory frequency increased to 184.8 ±
388 5.6% ($p = 0.03$), T_I decreased to 71.3 ± 4.5% ($p = 0.03$), and T_E was reduced to 52.6 ± 3.2% ($p \leq$
389 0.03) of control values. The amplitude of integrated PN activity decreased to 32.1 ± 3.9% ($p \leq$
390 0.03) of pre-injection control values. These parameter values, all of which are statistically
391 significant, were obtained at 95.83 ± 10.03 s after onset of the microinjections when the
392 perturbations approached maximum values. The average injection time for the group was 57.66 ±
393 22.09 s. After these perturbations the time required for recovery of inspiratory activity with a
394 pattern resembling the control activity was variable, but usually a period of minimally 40 min.
395 was required *in vivo*.

396 Similarly rapid and large perturbations were caused by bilateral microinjections of 30 μM
397 gabazine-strychnine within the pre-BötC of *in situ* perfused juvenile rat brainstem-spinal cord
398 preparations (n = 6, **Figure 5A and B**). Figure 5A shows a typical example of the large
399 disturbances of the respiratory frequency and amplitudes of activity recorded from PN and cVN,
400 including the pronounced increase in the frequency of PN inspiratory discharge accompanied by
401 reductions in discharge amplitude. In the example shown in **Figure 5B**, the augmented frequency
402 of PN inspiratory discharge culminated in tonic activity, which occurred in 1/6 experiments. For
403 the group data mean values of respiratory frequency increased to 175.9 ± 18.0% ($p = 0.03$), T_I
404 slightly increased to 110.4 ± 4.0% ($p = 0.03$), and T_E decreased to 43.7 ± 8.0% ($p = 0.03$) of
405 control values (**Figure 6A-C**). The integrated PN activity amplitude decreased to 62.3 ± 6.0% (p
406 $= 0.03$) (**Figure 6D)**. These disturbances followed a similar time course to those *in vivo* and were
407 measured at 84.1 ± 17 s after the onset of microinjections (average injection time of 178.3 s)
408 when maximum changes in parameter values obtained. Recovery of control patterns of nerve

activity typically occurred 15-20 min. after terminating the bilateral microinjections in these *in situ* experiments.

***Disruption of respiratory rhythm and pattern by microinjection of gabazine and strychnine in the BötC* in vivo *and* in situ**

Bilateral microinjections of gabazine-strychnine were performed in the BötC region after identifying this region by extracellular recording of augmenting expiratory and/or post-I neuronal population activity *in situ* and *in vivo,* and in some (n = 3/6) of the *in vivo* experiments in this group, after confirming the characteristic suppression of recorded phrenic discharge and pressor blood pressure responses by L-Glu microinjections. In contrast to the results obtained with microinjections in the pre-BötC, bilateral microinjections of 110 nl of 250 μM gabazine and strychnine into the BötC *in vivo* (**Figure 7**, n = 6), and also the microinjections in the *in situ* experiments with the lower concentrations of the inhibitory receptor antagonists (**Figures 8 and 9**, n = 6), significantly reduced the frequency of integrated PN discharge due primarily to a prolongation of T_E, accompanied by a reduction of T_I and the amplitude of integrated PN activity. For the averaged group *in vivo* data (**Figure 7B-E**), at 36.66 ± 6.4 s following the onset of microinjections, the respiratory frequency decreased to 25.8 ± 8.0% ($p \leq 0.03$), T_I was reduced to 88.4 ± 5.3% ($p \leq 0.06$), and T_E increased to 257.2 ± 48.0% ($p \leq 0.03$) of control values. The amplitude of integrated phrenic discharge decreased to 39.9 ± 13.8% ($p \leq 0.03$) of control values (**Figure 7E**). In two *in vivo* experiments, rhythmic inspiratory discharge was completely suppressed for ≥ 20 s (see example in **Figure 7A**). The average microinjection time for the group was 53.67 ± 19.32 s.

Similar perturbations occurred with bilateral microinjections of 30 μM gabazine-strychnine in the BötC *in situ* (**Figures 8** and **9**). For the averaged group data (n = 6, **Figure 9A-**

C) at 92.5 ± 16.77 s after injection onset, inspiratory frequency decreased to 22.6 ± 10.7% ($p = 0.03$), T_E increased to 430.2 ± 17.3% ($p = 0.03$), and T_I decreased to 56.2 ± 13.0%, ($p = 0.03$) of pre-injection control values. Integrated PN discharge amplitude was reduced to 15.4 ± 7.0% ($p = 0.03$) (**Figure 9D**). In three of these *in situ* experiments, rhythmic inspiratory activity was transiently suppressed for ≥ 20 s (examples shown in **Figure 8**). These perturbations were reflected in simultaneously recorded cVN inspiratory activity (**Figure 8A**) in all experiments analyzed and by recorded pre-BötC pre-I/I population activity (illustrated in **Figure 8B**) in two of these experiments, which demonstrate that the perturbations in BötC disrupt pre-BötC neuronal activity. In all experiments post-inspiratory (post-I) cVN discharge was also disrupted (below) and in one experiment tonic discharge on the cVN was recorded during the disruption of rhythmic motor output (**Figure 8A**). With the bilateral injections of the inhibitory receptor antagonists in the BötC *in situ* or *in vivo*, although nerve activity could emerge after transient suppression of motor output (e.g., Figures 7A and 8A), such activity was disturbed relative to control activity. In all of these experiments, typically 15-25 min. *in situ* and 40-60 min. *in vivo* were required to recover activity resembling control patterns of motoneuronal discharge.

***Disruption of three-phase respiratory pattern by blocking inhibition in the pre-BötC or BötC* in situ**

The microinjections of gabazine-strychnine in either the pre-BötC (n = 6) or BötC (n = 6) *in situ* disrupted the three-phase respiratory motor output pattern as analyzed from simultaneous recordings of PN and cVN activity, the latter of which always exhibited prominent post-I discharge. Cycle-triggered averages and time-series raster plots of integrated PN and cVN shown in **Figure 10A,B** illustrate that cVN post-I activity is eliminated as $GABA_A$ergic and glycinergic inhibition is attenuated in either region. This loss of post-I activity as the drug-induced

455 perturbation develops represents a transformation from a three-phase to a two-phase motor
456 output pattern (Smith et al., 2007).

457 ***Site-specific perturbations of rhythm and pattern by microinjections of muscimol in the***
458 ***pre-BötC and BötC* in vivo *and* in situ**

459 The differential perturbations of inspiratory discharge frequency with the $GABA_A$ and glycine
460 receptor antagonists in the BötC (frequency decrease) vs. the pre-BötC (frequency increase)
461 demonstrate site-specificity of the perturbations. To further test for site specific perturbations by
462 manipulating local inhibition, we regionally inhibited/attenuated neuronal activity by bilateral
463 microinjection of the $GABA_A$ receptor agonist muscimol. In these experiments, the pre-BötC and
464 BötC regions were first identified electrophysiologically by extracellular recording and/or by
465 microinjection of L-Glu *in vivo*. As illustrated in **Figure 11,** bilateral microinjection of 25–30 nl
466 of 100 μM muscimol in the pre-BötC *in vivo* progressively reduced the frequency and amplitude
467 of phrenic discharge and eliminated inspiratory motor output within 20 s in all experiments (n =
468 7). The suppression of inspiratory activity persisted for 40.1 ± 26.8 s during which rhythmic
469 inspiratory activity could be restored immediately by subsequent bilateral microinjection of 110
470 nl of 250 μM gabazine at the same site (**Figure 11**). This latter result also demonstrated the
471 efficacy of gabazine at the concentrations employed in our experiments to antagonize activation
472 of $GABA_A$ receptors in a regionally specific manner. Bilateral microinjections of lower
473 concentrations of muscimol (10 μM) in the pre-BötC in the *in situ* preparations (n = 5) also
474 rapidly suppressed phrenic inspiratory activity (**Figure 12**).

475 In experiments targeting the BötC *in vivo* (n = 7), bilateral microinjection of the same
476 volume and concentration of muscimol progressively increased discharge frequency, due
477 primarily to a reduction of T_E, and reduced the amplitude of integrated phrenic inspiratory

478 discharge (**Figure 13**). For the averaged group *in vivo* data at 486.4 ± 67.6 s after injection onset,
479 integrated phrenic discharge amplitude was reduced to 58.9 ± 8.9% ($p = 0.015$), inspiratory
480 frequency increased to 175.19 ± 8.4% ($p = 0.015$), T_E decreased to 51.4 ± 3.3% ($p = 0.015$), and
481 T_I decreased to 83.4 ± 3.3% ($p = 0.015$) of pre-injection control values (**Figure 13B-D**).
482 Comparable perturbations were obtained with bilateral microinjections of 10μM muscimol in the
483 *in situ* preparations (**Figure 14**, $n = 6$). At 139 ± 20 s following the onset of the microinjections,
484 the group averaged inspiratory frequency increased to 148.1 ± 16.88% ($p = 0.03$), T_E decreased
485 to 53.1 ± 8.0% ($p = 0.03$), and T_I was essentially unchanged (99.0 ± 15.3% of pre-injection
486 values, $p = 1.0$) (**Figure 14D-F**). The integrated phrenic discharge amplitude was reduced to 75.4
487 ± 6.4% ($p = 0.03$) (**Figure 14G**). In addition, integrated cVN post-I activity was progressively
488 reduced and eliminated by muscimol in the BötC *in situ* (**Figure 14B**).
489

Discussion

Role of synaptic inhibition within and between pre-BötC and BötC for respiratory rhythm and pattern generation

For the discussion of the role of synaptic inhibition in respiratory rhythm and pattern generation, it is necessary to clarify our definitions of the terms "rhythm" and "pattern". We define rhythm as the respiratory cycle period/frequency. Respiratory pattern is defined as all features of activity occurring within the respiratory cycle, including the presence and coordination of the different activity phases (inspiratory, post-inspiratory, late expiratory) as recorded from neuronal populations/nerve motor output activity, as well as the durations, amplitudes, and shapes of integrated activity within phases.

Brainstem respiratory networks consist of excitatory and inhibitory circuits distributed within interacting regions of the medulla and pons including the medullary pre-BötC and BötC, which are proposed to contain core circuits involved in respiratory rhythm and pattern generation. While there is general agreement that excitatory pre-BötC circuits generate rhythmic inspiratory activity transmitted to and driving spinal and cranial inspiratory motor outputs, functional roles of inhibitory pre-BötC and BötC circuits continue to be debated (e.g., Janczewski et al., 2013; Feldman et al., 2013, vs. Smith et al., 2009, 2013; Richter and Smith, 2014). It has been postulated that phasic synaptic inhibition originating in these circuits, known to contain populations of rhythmically active glycinergic and GABAergic neurons, are critically involved in generating the three-phase pattern of respiratory neuron activity during normal breathing by shaping firing patterns of active populations of neurons, orchestrating phase transitions, and controlling which populations are inactive during each phase (e.g., Rybak et al., 2004, 2007; Smith et al., 2007, 2009, 2013). Inspiratory and expiratory neurons in these regions

receive phasic volleys of inhibitory post-synaptic potentials/currents as clearly established by intracellular recordings (Schmid et al., 1996; Molkov et al., 2012; Shevtsova et al., 2014; Richter and Smith, 2014), and the inhibitory neurons in the pre-BötC and BötC are proposed to interact during the respiratory cycle via mutual inhibitory connections for dynamic control of rhythm generation, although these interactions have not been definitely established experimentally.

We have further evaluated roles of the glycinergic and GABAergic synaptic inhibition within the pre-BötC and BötC by regionally disrupting/attenuating this inhibition pharmacologically with specific receptor antagonists, which has been attempted in previous studies in several species (cat, rat, rabbit) *in vivo*, most recently in adult rats with conflicting results (Janczewski et al., 2013). In the present experiments we have established that pharmacologically attenuating $GABA_A$ and glycine receptor-mediated inhibition in the pre-BötC or BötC causes major site-specific perturbations of respiratory rhythm and pattern both in anesthetized, vagotomized adult rats *in vivo* and unanaesthetized, vagotomized juvenile rat perfused brainstem-spinal cord preparations *in situ*. The results obtained *in vivo* and *in situ* are congruent and confirm that:

(1) Blocking inhibition in the pre-BötC augmented inspiratory discharge frequency due to a shortening of T_E, reduced inspiratory discharge amplitude, and eliminated post-I activity thereby disrupting the normal three-phase respiratory pattern. In some cases rhythmic motor output was completely terminated.

(2) Blocking inhibition in the BötC reduced inspiratory discharge frequency associated with a lengthened T_E, reduced the amplitude of inspiratory discharge, disrupted the three-phase pattern due to a loss of rhythmic post-I activity, and in some cases transiently terminated inspiratory motor output accompanying a loss of pre-BötC inspiratory activity.

Therefore, we conclude that ongoing glycinergic and $GABA_A$ergic inhibition in pre-BötC and BötC circuits are fundamentally involved in dynamical regulation of respiratory rhythm generation and are required for generating the normal three-phase respiratory pattern. Our studies also confirm a fundamental role of BötC inhibitory circuits and their interactions with pre-BötC inspiratory rhythm generating circuits.

Comparisons with results from previous targeted pharmacology studies

Our results, obtained in juvenile and adult rats, are consistent with previous studies in anesthetized, non-vagotomized adult cats (Pierrefiche et al., 1998) demonstrating that bilateral pharmacological disruption of glycinergic synaptic inhibition with strychnine in the pre-BötC reduces inspiratory discharge amplitude and markedly augments inspiratory discharge frequency or totally abolishes rhythmic phrenic nerve activity. In their studies, simultaneous block of $GABA_A$ergic and glycinergic inhibition led to sustained tonic discharge on phrenic nerves, indicating disruption of rhythmic inspiratory motor output. Furthermore, results obtained in anesthetized vagotomized adult rabbits by Bongianni et al. (2010) also demonstrated that bilateral block/attenuation of glycinergic inhibition in the pre-BötC augmented inspiratory frequency and reduced inspiratory discharge amplitude. Also, blocking GABAergic inhibition with gabazine in the BötC strongly depressed inspiratory amplitude and frequency leading to apnea as we demonstrated with $GABA_A$ergic and glycinergic antagonists in several cases. Moreover, in these studies in rabbits, bilateral microinjection of muscimol in the BötC potently augmented inspiratory discharge frequency and caused loss of rhythmic inspiratory activity that was replaced by tonic discharge of phrenic nerves.

The general conclusions from these previous studies in both vagotomized and vagus-intact animals, similar to our conclusions, are that ongoing neuronal $GABA_A$ and glycine

559 receptor-mediated postsynaptic inhibition in the pre-BötC and BötC have a major role in
560 controlling the frequency and amplitude of inspiratory circuit activity during eupneic breathing
561 *in vivo*. Other results obtained by pharmacologically blocking glycinergic inhibition with
562 systemically applied strychnine in perfused rat and mouse brainstem-spinal cord preparations *in*
563 *situ*, disturbing synaptic inhibition throughout the respiratory network, also demonstrate major
564 disturbances of respiratory rhythm and disruption of the three-phase respiratory pattern at
565 cellular and circuit levels (e.g., Shevtsova et al. 2011, 2014; Richter and Smith, 2014).

566 All of the above results and conclusions contrast with those from the recent
567 pharmacological study by Janczewski et al. (2013) performed in anesthetized spontaneously
568 breathing, vagus-intact or vagotomized adult rats *in vivo*. They reported that sequential bilateral
569 microinjections of bicuculline and strychnine in the pre-BötC and then BötC via a ventral surface
570 approach does not significantly disturb inspiratory frequency in vagotomized rats, whereas in
571 vagus-intact rats inspiratory frequency was significantly reduced, accompanied by a prolongation
572 of T_I and T_E– perturbations that the authors attributed to only suppression of the Breuer-Hering
573 inspiratory inhibitory reflex (BHIR), supported by their results showing that the HBIR is blocked
574 by antagonizing synaptic inhibition in the pre-BötC. Other results presented suggested that block
575 of inhibition in the pre-BötC and BötC does not disturb generation of laryngeal post-I activity in
576 vagus-intact rats and thus they concluded that inhibitory circuit interactions do not participate in
577 normal three-phase respiratory pattern generation. This result conflicts with our present and
578 previous results (Shevtsova et al., 2011) *in situ,* where we could routinely record post-I activity
579 on cVN or post-I neuronal activity in this unanaesthetized preparation, and found that disruption
580 of synaptic inhibition in the pre-BötC or BötC consistently eliminated post-I activity. Another
581 major result by Janczewski et al. (2013) was that bilateral ablation of the BötC did not

582 significantly perturb inspiratory frequency or inspiratory-expiratory phase durations, from which
583 they concluded that the BötC is not involved at all in generating the three-phase rhythmic
584 respiratory pattern. This clearly contrasts with our results and those of Bongianni et al. (2010)
585 that disrupting inhibition in the BötC can cause apnea, whereas suppressing BötC neuronal
586 activity with muscimol augments inspiratory frequency and can lead to apneustic-like tonic
587 discharge, similar to disrupting inhibition in the pre-BötC. Janczewski et al. (2013) presented
588 data showing a similar augmentation of frequency leading to tonic discharge and disruption of
589 rhythm generation with muscimol injections in the pre-BötC, which is the opposite of ours and
590 previous results that suppressing neuronal activity with muscimol in the pre-BötC potently
591 reduces inspiratory frequency and rapidly terminates inspiratory rhythm generation. Overall
592 Janczewski et al. (2013) concluded that inhibition is not required for a normal eupneic breathing
593 rhythm *in vivo* except for mediation of the HBIR in vagus-intact animals.

594 These major discrepancies are not readily resolved. The results obtained with these
595 targeted pharmacological approaches depend critically on accurate site-directed delivery of the
596 inhibitory agonists/antagonists, the ability to antagonize inhibitory post-synaptic receptors on a
597 sufficient number of neurons by regional spread of the antagonists/agonists to ultimately perturb
598 motor outputs, and also on regionally confining the pharmacological perturbations. In our
599 experiments, we verified locations of injection sites histologically and we consistently obtained
600 differential, regionally specific perturbations of inspiratory rhythm: slowing of the rhythm in all
601 cases by inhibitory antagonists in BötC, and contrasting augmented inspiratory frequency with
602 antagonists in pre-BötC. Our reconstructed microinjection sites appear to be identical to the sites
603 reconstructed by Janczewski et al. (compare our **Figure 1** with Figure 1 in Janczewski et al.
604 2013). In our *in vivo* experiments employing a similar ventral surface approach in adult

anesthetized rats, we used identical concentrations and ejection volumes of antagonists, which we also showed at least for gabazine had pharmacologically effective $GABA_A$ receptor antagonist-agonist interactions (e.g., **Figure 11**). With our targeting procedures, which included mapping of characteristic neuronal activity profiles as well as differential respiratory and blood pressure responses to local L-Glu microinjections, we also consistently obtained regionally specific perturbations with muscimol microinjections at targeted sites: reduced inspiratory frequency and apnea in the pre-BötC, but augmented inspiratory frequency in the BötC. This specificity also suggests that it was possible to confine actions of the agonists/antagonists to the targeted region. Differences in the numbers of neurons and inhibitory synapses affected by local diffusion of the antagonists might contribute, although assuming accuracy of targeting, we would expect similar perturbations since ejection volumes and antagonist concentrations employed *in vivo* were identical.

Relation to studies employing selective targeting of inhibitory neurons

In a recent study employing optogenetic approaches to selectively stimulate or inhibit pre-BötC glycinergic neurons by virally-transduced expression of photosensitive opsins channelrhodopsin (ChR2) or archaerhodopsin in spontaneously breathing adult mice *in vivo*, Sherman et al. (2015) recently found that optical stimulation of this subset of inhibitory neurons in pre-BötC *in vivo* can terminate inspiration, delay the onset of inspiration with photoactivation during the expiratory phase, and produce long-lasting apnea with prolonged photostimulation. Conversely prolonged photoinhibition augmented the amplitude and frequency of inspiratory activity and could reverse reflex-induced apneas. This approach can provide important information on dynamic perturbations resulting from augmentation or loss of inhibitory neuron function (Abdala et al., 2015) and their results demonstrate that at least pre-BötC glycinergic neuron activity can

strongly modulate ongoing inspiratory rhythm and pattern generation. The authors concluded, however, that glycinergic inhibition is not essentially involved in rhythmogenesis despite these perturbations since inspiratory rhythm persisted after photoinhibition, although as the authors discuss it is unclear if sufficient numbers of glycinergic neurons were optically silenced to reveal full affects of pre-BötC glycinergic neuron activity on rhythm generation.

Another important consideration for interpreting the significance of these results is that silencing pre-BötC glycinergic neurons may not be analogous to pharmacologically blocking even postsynaptic glycinergic receptors, particularly since relevant glycinergic synapses may originate from neurons outside of the pre-BötC such as BötC neurons not transduced in sufficient numbers with viral vectors targeted to the pre-BötC. These optogenetic results also cannot be readily compared with the effects of pharmacological blockade of both glycinergic and $GABA_A$ergic postsynaptic inhibition. As known, pre-BötC contains inhibitory neurons with each type of transmission, as well as neurons co-expressing both GABA and glycine (Koizumi et al. 2013), so all these neuron types would have to be optically inhibited to fully evaluate the functional role of local inhibitory circuits in the pre-BötC. The augmented inspiratory frequency that we observed with disrupting inhibition in the pre-BötC, which has also been observed by pharmacologically attenuating only glycinergic inhibition with strychnine (e.g., Pierrefiche et al. 1998) as discussed above, is a common result of both pharmacological and optical approaches. However, inspiratory amplitude was always markedly reduced, not augmented, by pharmacologically perturbing postsynaptic inhibition in the pre-BötC, suggesting that more complex inhibitory interactions in pre-BötC inspiratory rhythm generation circuits are revealed by the pharmacological approaches. Experiments were not presented by Sherman et al. (2015) on photostimulation/inhibition of glycinergic neurons in the BötC. Optical stimulation of BötC

651 neurons transduced with ChR2, although not selectively in GABAergic and glycinergic neurons
652 but likely including these neurons in these studies, strongly suppresses inspiratory activity in
653 contrast to the powerful augmentation of frequency with photostimulation of pre-BötC neurons
654 in anesthetized rats *in vivo* (Alsahafi et al., 2015). These observations are analogous to our
655 results with local glutamate microinjections, which also do not selectively stimulate specific
656 neuronal phenotypes in these regions.

657 ***Normal eupneic rhythm generation in the absence of synaptic inhibition* in vivo?**

658 A continuing debate is whether a normal eupneic inspiratory rhythm and respiratory pattern can
659 be generated without synaptic inhibition in the pre-BötC in the intact system where the pre-BötC
660 interacts with the BötC and other sources of phasic/tonic synaptic inhibition. We show that
661 disrupting pre-BötC synaptic inhibition leads to either higher frequency oscillations accompanied
662 by a large reduction in the amplitude of pre-BötC/phrenic inspiratory activity, or to tonic phrenic
663 nerve activity in some causes, indicating that a normal eupneic inspiratory rhythm generally does
664 not occur in the absence of synaptic inhibition. The variable persistence of inspiratory rhythmic
665 activity in other cases, albeit with large disturbances of the inspiratory rhythm and inspiratory-
666 expiratory pattern, indicates that either we did not sufficiently block postsynaptic inhibition in
667 the pre-BötC with our targeted pharmacological approach in these cases, or some form of
668 inspiratory rhythm generation occurs after disruption of $GABA_A$ergic and glycinergic inhibition
669 in the intact system. The former is a technical problem that is difficult to solve. This would
670 require extensive local intracellular recordings to analyze endogenous postsynaptic inhibitory
671 potentials simultaneously with the pharmacological perturbations. In our approach, we limited
672 the microinjection volumes of the pipette solution containing inhibitory antagonists, in an
673 attempt to achieve site-specificity of the perturbations. This may have precluded sufficient

674 inhibitory receptor block throughout the local circuits to cause complete disruption of inspiratory
675 rhythm generation in these cases. On the other hand, pre-BötC excitatory neurons and circuits
676 have intrinsic rhythmogeneic properties (Smith et al., 2007; St. John et al., 2009; Richter and
677 Smith, 2014), so it is possible that some form of inspiratory rhythm generation, albeit abnormal,
678 can persist in the absence of synaptic inhibition in the intact juvenile/adult system, as occurs in
679 the neonatal rat/mouse pre-BötC isolated in slices *in vitro*.

680 Our models of excitatory and inhibitory pre-BötC circuits and their interactions with
681 BötC inhibitory neurons in the intact system incorporate intrinsic rhythmogenic properties in the
682 excitatory pre-BötC population (Rybak et al., 2004, 2007; Smith et al., 2007; Rubin et al., 2009).
683 These models indicate that depending on the initial excitation state of the excitatory kernel
684 neurons, the endpoint after complete block of postsynaptic inhibition can either be tonic activity
685 of pre-BötC neurons at high levels of excitation, or rhythmic activity at lower excitation levels. It
686 is clear that to answer the question of whether any form of inspiratory rhythm generation can
687 occur, intracellular recordings will have to be employed to monitor the level of membrane
688 potential and activity patterns during and after local block of postsynaptic inhibition.

689 ***Disorganization of the three-phase respiratory pattern after disrupting postsynaptic***
690 ***inhibition***

691 The three-phase organization of the respiratory pattern at least *in situ* was disrupted
692 accompanying the large disturbances of inspiratory rhythm by blocking inhibition in the pre-
693 BötC or BötC. This confirms that the normal eupneic breathing pattern with appropriately
694 coordinated spinal and cranial motoneuron activity relies on synaptic inhibition in BötC and pre-
695 BötC circuits. These inhibitory circuits normally operate with pontine and other excitatory inputs
696 (Rybak et al., 2004; Smith et al., 2007; Dutschmann and Dick, 2012). The pontine Kölliker-Fuse

nucleus projects heavily to both the pre-BötC and BötC affecting their inhibitory interactions. Specifically, pontine input is required to generate post-I activity (Rybak et al., 2004; Smith et al., 2007; Dutschmann and Dick, 2012) so the observed loss of this activity in part reflects disruption of the balance of excitatory and inhibitory interactions. Recent experiments in a transected perfused rat brainstem preparation have confirmed the critical role of the pons in three phase respiratory pattern generation (Jones and Dutschmann, 2016). Previously it has been demonstrated that blocking glycinergic inhibition systemically causes a shift of post-I neuron activity into the inspiratory phase, due to phasic excitation of post-I neurons that is normally shunted by inspiratory phase inhibition (see Richter and Smith, 2014) and this shift causes abnormal glottal constriction during inspiration (Dutschmann and Paton, 2002). This also implies that the shifted post-I inhibitory neurons would provide abnormally timed inhibition during the inspiratory phase, possibly including to inspiratory neurons in bulbospinal circuits downstream from the BötC and pre-BötC, which could contribute to the large reductions of inspiratory amplitude and attenuation of PN ramping inspiratory discharge observed after disrupting inhibition. The loss of the three-phase pattern is predicted from models (e.g., Rybak et al., 2004, 2007; Smith et al., 2007; Shevtsova et al., 2011, 2014) after inhibitory block in the BötC, where post-I inhibitory neuronal activity is presumed generated. Our results that the three-phase pattern is also disrupted after blocking inhibition in the pre-BötC may reflect the known presence of post-I neurons in the pre-BötC (Schwarzacher et al., 1995; Alheid and McCrimmon, 2008), and shows that the pre-BötC also plays a role in three-phase pattern generation, conceivably by either interactions with the BötC and/or by local inhibitory circuit interactions, which need to be delineated.

Role of BötC circuits and synaptic inhibition in respiratory pattern generation

A fundamental role for BötC neurons in respiratory pattern generation has been questioned by Janczewski et al. (2013) as noted above. Their conclusion that this region plays no role is incompatible with the present and previous results showing that augmenting BötC neuron activity (by glutamate microinjections) suppresses pre-BötC inspiratory activity whereas suppressing BötC activity (by muscimol) augments pre-BötC inspiratory rhythm. The BötC is known to contain major populations of active excitatory and inhibitory expiratory post-I and aug-E neurons, and this region is critical for generating post-I activity (Burke et al., 2010). Excitatory BötC neurons are thought to be a major source of expiratory activity in the respiratory network including for generation of post-I premotoneuron activity. BötC glycinergic and GABAergic expiratory interneurons are proposed to provide widely distributed network synaptic inhibition during expiration (Jiang and Lipski, 1990; Tian et al., 1999a,b; Ezure et al., 2003a,b). These postulated inhibitory connections include to pre-BötC excitatory and inhibitory inspiratory neurons to provide phasic synaptic inhibition orchestrating rhythmic alternation between expiratory and inspiratory activity in the network. Mutual inhibitory synaptic interactions between BötC inhibitory (post-I and aug-E) expiratory neurons and inhibitory inspiratory (e.g., early-I) pre-BötC neurons have been proposed to coordinate generation of the three-phase pattern of neuronal activity during eupneic breathing (Rybak et al., 2004, 2007; Smith et al., 2007; Rubin et al., 2009; Shevstova et al., 2011, 2014; Richter and Smith, 2014). Based on these proposed interactions, augmenting BötC activity or disrupting postsynaptic inhibition within the BötC should suppress rhythmic activity in the pre-BötC, assuming in each case that BötC neurons shift from phasic to tonic activity. Correspondingly, suppressing BötC activity should augment inspiratory discharge frequency and, depending on the level of ongoing pre-BötC

743 excitation, this can lead to tonic inspiratory activity. Both of the above are features of the present
744 experimental results.

745 We note that the projections and postsynaptic targets of BötC and pre-BötC inhibitory
746 interneurons, particularly mutual inhibitory connections between these neurons, have not been
747 mapped structurally. Establishing these connections remains an important problem. Nevertheless,
748 our results are consistent with mutual interactions and a fundamental role of the BötC and its
749 expiratory inhibitory neurons in three-phase respiratory pattern generation and control of
750 inspiratory rhythm.

751

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884 **Figure Legends**

885 **Figure 1**. Ventral view of the adult rat medulla and histology illustrating targeted sites for
886 pharmacology experiments *in vivo*. **A**, photograph of adult rat brainstem ventral surface as
887 exposed in the *in vivo* experimental preparations with an overview of targeted locations (caudal
888 to facial motor nucleus, VII) of BötC and pre-BötC as routinely identified by
889 electrophysiological mapping of neuronal activity profiles in the present experiments. **B**,
890 photomicrograph of ventral medullary surface and bilaterally arranged pipettes (blue dye-filled
891 for visualization) as typically configured for near perpendicular penetrations of the ventral
892 surface for simultaneous microinjections. **C, D,** confocal microscopic images of parasagittal
893 histological sections (50 μm thick) showing, respectively, examples of targeted sites for
894 microinjection of inhibitory antagonists in the pre-BötC ventral to the semi-compact subdivision
895 of nucleus ambiguous (NAsc), and in the BötC ventral to the compact subdivision of nucleus
896 ambiguus (NAc). Targeted sites are marked by microinjectioned solution of fluorescent
897 microspheres (green). NA and VII motoneurons are immuno-labeled by ChAT antibody (red).
898 Rostro-caudal spatial extent of the pre-BötC and BotC compartments are indicated. Other
899 abbreviations: vs– ventral surface.
900
901 **Figure 2**. Confocal microscopic images of histological sections illustrating *post-hoc* validation
902 of microinjection sites marked by fluorescent microspheres (green) in the pre-BötC or BötC
903 regions in fixed sections from anesthetized adult rat *in vivo* preparations and juvenile rat *in situ*
904 brainstem-spinal cord preparations. **A**, **B**, coronal sections (30 μm thick) of fixed tissue at the
905 level of pre-BötC (**A**) and BötC (**B**) from *in vivo* preparations. **C, D,** coronal sections (30 μm) of
906 fixed tissue at pre-BötC (**C**) and BötC (**D**) levels from juvenile rat *in situ* brainstem-spinal cord
907 preparations. Subdivisions of nucleus ambiguus (NAc, compact subdivision, and NAsc, semi-
908 compact subdivision), labeled with ChAT antibody (red), provide regional landmarks for pre-

BötC (ventral to NAsc) and BötC (ventral to NAc) levels of the medulla. Each image is taken from serial coronal histological sections obtained from experiments targeting these regions. Labeling with microinjected solution of fluorescent microbeads, with varying extent of local spread, indicates the approximate center of the drug microinjection sites in these examples. Other abbreviations: vs– ventral surface.

Figure 3. Characteristic profiles of extracellularly recorded neuronal population activity and examples of perturbations of inspiratory motor output activity and blood pressure produced by pharmacological excitation of neurons within the pre-BötC or BötC regions in the adult rat *in vivo*. **A**, a typical example of pre-inspiratory/inspiratory (pre-I/I) population activity used for the identification of pre-BötC. **B**, an example of post-inspiratory (post-I) and augmenting expiratory (aug-E) population activity (post-I/aug-E, simultaneously recorded in this example) used for the identification of BötC. In both **A** and **B**, the raw recording from the phrenic nerve (PN) and PN integrated activity (∫PN**)** are shown at the bottom. **C**, a 500 msec duration microinjection of L-Glutamate (Glutamate) in the pre-BötC produced an increase in the ∫PN burst frequency (see the trace for integrated PN activity, ∫PN), and a transient decrease in the arterial blood pressure (ABP). The respiratory frequency (f_R, bottom trace, green) increased primarily due to reduction in expiratory phase duration (T_E trace, blue) at a relatively unchanged inspiratory duration (T_I trace, red). **D**, a microinjection of L-Glutamate in the BötC caused a rapid suppression of PN activity (see traces for ∫PN and inspiratory, T_I, and expiratory, T_E, durations) accompanied by an increase of ABP. In **C** and **D**, L-Glutamate (10 mM, 5 nl) was microinjected bilaterally during the brief (500 msec) pulse; the moments of injections are indicated by brown arrows. Traces for T_I, T_E, and f_R, represent corresponding running time intervals of these parameters before, during, and recovery from L-Glutamate microinjection.

Figure 4. Perturbations of respiratory activity by pharmacologically disrupting $GABA_A$ergic and glycinergic inhibition in the pre-BötC of anesthetized adult rat *in vivo*. **A**, simultaneous bilateral microinjections of gabazine and strychnine (both 250 μM delivered by slow microinjections of 110 nl during the time interval indicated by the brown bar at the top and blue rectangle) caused an increase of respiratory frequency and a reduction in the integrated phrenic nerve activity amplitude (see ∫PN trace, top, black). The increase of respiratory frequency (f_R, green trace at the bottom) was mainly due to reductions of expiratory phase duration (T_E, blue trace) accompanied by only a small increase of inspiratory phase duration (T_I, red trace). **B-E**, group mean time series showing developing changes of normalized T_I (panel **B**, red curve), T_E (panel **C**, blue curve), respiratory frequency, f_R (panel **D**, green curve), and ∫PN amplitude (amp., panel **E**, magenta curve) computed over the time window shown from the start (time = 0) of microinjection. Data were computed from ∫PN for this representative experimental group (n = 6). Solid colored curves are group mean time and mean normalized parameter values; gray shaded bands are ± 1 SEM for the mean parameter values. Endpoints shown are mean time and normalized parameter values ± 1 SEM for both at the maximal perturbation for the injection periods employed.

Figure 5. Perturbations of respiratory activity by pharmacologically disrupting $GABA_A$ergic and glycinergic inhibition in the pre-BötC of juvenile rat perfused brainstem-spinal cord *in situ*. **A**, example of experimental recordings illustrating perturbations of integrated PN (∫PN) and cVN (∫cVN) activities by simultaneous bilateral microinjections of gabazine and strychnine (both 30 μM; injection period indicated by the brown bar at the top and blue rectangle), which caused an increase of respiratory frequency (f_R) due to a reduction in T_E (blue) without significant changes in T_I, and a reduction in amplitude of ∫PN and ∫cVN. **B**, example of perturbations where the ∫PN

activity progressed to tonic activity, as indicated by the upward shift of the ∫PN signal baseline. Analysis of respiratory parameters in this case was performed up to the time point indicated by the vertical dot-dashed line. The progressive reduction in ∫cVN amplitude in both examples reflects in part a reduction and eventual loss of post-I activity (see **Figure 10** for more detailed analysis).

Figure 6. Group data summarizing changes of respiratory activity parameters by pharmacologically disrupting $GABA_A$ergic and glycinergic inhibition in the pre-BötC of juvenile rat perfused brainstem-spinal cord *in situ*. **A-D**, mean time series from the start of microinjection showing developing changes of normalized T_I (**A**, red curve), T_E (**B**, blue curve), respiratory frequency, f_R (**C**, green curve), and integrated PN discharge amplitude (**D**, amp., magenta curve), which were computed from ∫PN for a representative experimental group (n = 6). Solid colored curves are group mean time and normalized parameter values; gray bands are ± 1 SEM for the mean normalized parameter values as in **Figure 4B-E**.

Figure 7. Perturbations of respiratory activity by pharmacologically disrupting of $GABA_A$ergic and glycinergic inhibition in the BötC of adult rat *in vivo*. **A**, gabazine and strychnine cocktail (110 nl, 250 μM) slowly injected during time period indicated (by the brown bar at the top and blue rectangle), progressively reduced respiratory frequency leading to transient apnea in this example (see ∫PN trace, top, black). Changes in the inspiratory (T_I, red) and expiratory (T_E, blue) phase durations and respiratory frequency (f_R) are shown at the bottom; f_R was reduced mainly due to prolongation of T_E. **B-E**, mean time series from the start of microinjection showing developing changes of normalized T_I (**B**, red curve), T_E (**C**, blue curve), respiratory frequency, f_R (**D**, green curve), and integrated PN activity amplitude (**E**, magenta curve) for a representative

experimental group (n = 6). Solid colored curves are group mean time and normalized parameter values; gray bands are ± 1 SEM for the mean normalized parameter values.

Figure 8. Disruption of rhythmic respiratory activity by block/attenuation of $GABA_A$ergic and glycinergic inhibition in the BötC of *in situ* perfused brainstem-spinal cord preparations. **A and B**, two examples from experiments using different preparations illustrating perturbations caused by microinjections of gabazine and strychnine (30 μM slowly injected during the period indicated by the brown bars at the top and blue rectangles). As in *the vivo* experiments, respiratory frequency was reduced by a prolonged T_E, integrated phrenic nerve discharge amplitude (∫PN) was also reduced, and ultimately apnea occurred. Simultaneously recorded integrated cVN (∫cVN) in **A** shows disruption of rhythmic activity and tonic discharge (shift of integrated activity baseline) during apneic period. In **B**, simultaneously recorded integrated pre-BötC pre-I/I population activity from another experiment also reflects the reduction of inspiratory frequency and termination of rhythmic activity during bilateral microinjections of the inhibitory receptor blockers in BötC.

Figure 9. Group data (n = 6) summarizing perturbations of respiratory rhythm and motor output pattern parameters by disrupting $GABA_A$ergic and glycinergic inhibition in the BötC of *in situ* perfused brainstem-spinal cord preparations. Solid colored curves in these time series are mean time and normalized parameter values and gray bands are ± 1 SEM for the normalized values of T_I (**A**), T_E (**B**), respiratory frequency (f_R, **C**), and integrated inspiratory activity amplitude (**D**) computed from the start of microinjections from recordings of integrated PN activity.

Figure 10. Disturbances of three-phase respiratory pattern including disruption of post-inspiratory (post-I) activity by bilaterally microinjected gabazine and strychnine (30 μM each) in the pre-BötC (**A**) or BötC (**B**) of *in situ* perfused juvenile rat brainstem-spinal cord preparations. Upper traces in **A** and **B** show seven consecutive overlaid and aligned integrated PN (red traces) and cVN (blue traces) activity signals in the pre-microinjection control period (light red and light blue larger amplitude traces, respectively) and also seven overlaid traces of recorded signals during microinjections (dark red and dark blue traces with reduced amplitudes). Simultaneously recorded pre-BötC pre-I/I population activity before and during microinjections (light and dark green traces, respectively) is also shown at the top in **B** to indicate activity perturbations in this region occurring with disruption of inhibition in BötC. Signals are aligned (cycle-triggered) at the onset of PN inspiratory activity indicated by the vertical dotted line in **A** and **B**. Raster plots below overlaid traces show consecutive series of inspiratory-onset aligned respiratory cycles with integrated PN inspiratory (red) and cVN including post-I (blue) activities before and during microinjections. Arrows on the raster plots indicate time interval over which the overlaid traces above the raster plots were obtained for the pre-microinjection period. During microinjections in the pre-BötC or BötC, the amplitude of inspiratory and post-I activity is progressively reduced, and post-I discharge is eliminated as seen on the aligned (darker) ∫cVN traces above the raster plots and in the raster plots with loss of post-I discharge indicated (white arrow).

Figure 11. Suppression of rhythmic inspiratory activity by bilateral microinjection of muscimol in the pre-BötC of adult rat *in vivo* and gabazine antagonism of $GABA_A$ receptor activation. Muscimol (30 nl, 100 μM) microinjection (during yellow shaded area) rapidly reduced integrated PN activity and produced a long-lasting suppression of inspiratory activity that could be restored by bilateral microinjection of gabazine (110 nl, 250 μM) at the same site in the pre-BötC (blue shaded area). Abbreviations (∫PN, T_E, T_I, f_R) are the same as for previous figures.

Figure 12. Illustration of prolonged suppression of rhythmic inspiratory activity by bilateral microinjection of muscimol (10 μM) in the pre-BötC (yellow shaded area) of juvenile rat perfused brainstem-spinal cord *in situ*. As *in vivo*, muscimol rapidly reduces PN inspiratory discharge frequency and terminates inspiratory activity. Abbreviations (∫PN, T_E, T_I, f_R) are the same as for previous figures.

Figure 13. Perturbation of inspiratory activity by bilateral microinjection of muscimol in the BötC of anesthetized adult rat *in vivo*. **A,** muscimol (30 nl, 100 μM) slowly microinjected (brown bar at the top and yellow rectangle) in the BötC augments PN frequency due to a reduction of T_E. **B-E**, mean time series for group data (n = 7) summarizing perturbations of respiratory rhythm and motor output pattern parameters. Solid colored curves are mean time and normalized parameter values and gray bands are ± 1 SEM of T_I (**B**), T_E (**C**), respiratory frequency (f_R, **D**), and integrated inspiratory activity amplitude (**E**) computed from recordings of integrated phrenic nerve activity as in previous figures.

Figure 14. Disturbances of respiratory pattern including elimination of post-inspiratory activity by bilateral microinjection of muscimol (10 μM) in the BötC of juvenile rat perfused brainstem-spinal cord *in situ*. **A**, representative example of augmented ∫PN inspiratory discharge frequency and pronounced reduction of cVN integrated (∫cVN) activity amplitude due to suppression of post-I activity. Overlaid traces in **B** and time series raster plot in **C** of consecutive respiratory cycles aligned to inspiratory onset (indicated by vertical dotted line in **B**) illustrate perturbations of cVN post-I discharge during BötC muscimol microinjection in another experiment. In **B**, integrated PN (red traces) and cVN (blue traces) activity are shown before (lighter red and lighter blue traces, respectively) and after (dark red and dark blue traces) muscimol microinjection. Aligned traces of ∫PN (red) and ∫cVN (blue) are shown in raster plot in **C** with the loss of post-I cVN discharge indicated (white arrow), which is also clearly seen from the ∫cVN traces (dark blue) above. **D-G,** mean time series for group data (n = 6) summarizing perturbations of respiratory rhythm/pattern parameters. Solid colored curves are mean time and

parameter values and gray bands are ± 1 SEM of normalized T_I (**D**), T_E (**E**), inspiratory frequency (f_R) (**F**), and integrated inspiratory activity amplitude (**G**) computed from recordings of integrated PN activity.

Visual Abstract. See abstract