Graded Reductions in Oxygenation Evoke Graded Reconfiguration of the Isolated Respiratory Network

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Graded reductions in oxygenation evoke graded reconfiguration of the isolated respiratory network. J Neurophysiol 105: 625–639, 2011. First published November 17, 2010; doi:10.1152/jn.00237.2010. Neurons depend on aerobic metabolism, yet are very sensitive to oxidative stress and, as a consequence, typically operate in a low O2 environment. The balance between blood flow and metabolic activity, both of which can vary spatially and dynamically, suggests that local O2 availability markedly influences network output. Yet the understanding of the underlying O2-sensing mechanisms is limited. Are network responses regulated by discrete O2-sensing mechanisms or, rather, are they the consequence of inherent O2 sensitivities of mechanisms that generate the network activity? We hypothesized that a broad range of O2 tensions progressively modulates network activity of the pre-Bötzinger complex (preBötC), a neuronal network critical to the central control of breathing. Rhythmogenesis was measured from the preBötC in transverse neonatal mouse brain stem slices that were exposed to graded reductions in O2 between 0 and 95% O2, producing tissue oxygenation values ranging from 20 ± 18 (mean ± SE) to 440 ± 56 Torr at the slice surface, respectively. The response of the preBötC to graded changes in O2 is progressive for some metrics and abrupt for others, suggesting that different aspects of the respiratory network have different sensitivities to O2.

INTRODUCTION

Although it is generally accepted that blood oxygen (O2) levels are detected and regulated by peripheral chemoreceptors such as the carotid bodies (Bissonnette 2000), it is much less appreciated that peripheral chemosensors are probably not sufficient to guarantee adequate regulation of O2 supply to the CNS (for review of O2 sensing in the CNS see Neubauer and Sunderram 2004). In the CNS, it is essential that the partial pressure of O2 (PO2) remains tightly regulated over a fine range because hyperoxia can be as detrimental as hypoxia (D’Agostino et al. 2007; Haddad and Jiang 1993). Tissue PO2 in the CNS is maintained in a narrow range between 10 and 34 Torr (corresponding to a range of 1 to 4% O2; Mulkey et al. 2001). Thus neurons, which are very metabolically active, exist in a microenvironment with an O2 tension only slightly higher than the threshold for aerobic metabolism (~1% O2; Clemens et al. 2001). The challenge of maintaining an optimal O2 tension is made more difficult by heterogeneous metabolic O2 demand throughout the CNS and dynamic network activity, which lead to rapid changes in local tissue oxygenation (Ndubuizi and LaManna 2007). Rapid, localized compensatory changes in blood supply are achieved by neurovascular coupling that involves communication between neurons, astrocytes, and the smooth muscle cells of arterioles (Filosa and Blanco 2007). Such findings suggest that central neuronal networks may have the ability to locally sense small changes in O2 and respond adaptively in terms of both mediating changes in local blood supply as well as network output.

It remains largely unresolved how neuronal networks are locally controlled by O2-sensing mechanisms. Are neuronal network states controlled by discrete central oxygen sensors or are the network alterations a consequence of multiple oxygen sensitivities inherent to the mechanisms that also govern the neuronal network activity itself? To address this issue we recorded from the pre-Bötzinger complex (preBötC) and characterized its response to graded reductions in O2. The preBötC is a neuronal network involved in respiratory rhythmogenesis during both well-oxygenated and hypoxic conditions (Peña 2008; Solomon et al. 2000). Previous studies of the isolated brain stem slice [postnatal day zero (P0) to P18] have shown that exposure to very low levels of O2 (i.e., 0% O2 or hypoxia) affects both the frequency of the in vitro respiratory rhythm (Ramirez 1997a, 1998; Telgkamp and Ramirez 1999) and the waveform of the extracellularly recorded population bursts (Lieske et al. 2000). The resemblance of these effects to those found in more intact experimental preparations as well as in vivo (Lieske et al. 2001) suggests that O2-mediated changes in activity within the preBötC contribute, at least in part, to the in vivo response to a reduction in O2.

Rhythmogenesis in the preBötC is differentially sensitive to rituzole (RIL) and flufenamic acid (FFA). During hypoxia, rhythmogenesis (i.e., fictive gasping) is ablated in RIL, but persists in FFA. In well-oxygenated conditions neither FFA nor RIL, alone, inhibits the rhythm, but coapplication of these substances eliminates rhythmicity (Del Negro et al. 2005; Peña et al. 2004). The effects of RIL and FFA on rhythmogenesis are attributed to the antagonism of two membrane currents implicated in bursting of preBötC neurons: the persistent sodium current (INaP), which is RIL-sensitive; and the Ca2+-activated nonspecific cation current (ICaN), which is FFA-sensitive (Del Negro et al. 2005; Koizumi and Smith 2008; Peña et al. 2004). In conjunction with a hypoxia-induced reduction in synaptic inhibition (Lieske et al. 2000; Ramirez et al. 1997b), changes in these two currents contribute to network reconfiguration of the preBötC at the population level during hypoxia (Peña et al. 2004; Ramirez and Garcia...
It has been hypothesized that during hypoxia a reduction in $I_{\text{CAN}}$-dependent mechanisms renders the respiratory network dependent on $I_{\text{NaP}}$ mechanisms (Peña et al. 2004). Additionally, a reduction in synaptic inhibition contributes to the loss of expiratory activity, a switch of some neurons from postinspiratory to inspiratory activity, and a change in burst shape from an augmenting/bell-shaped inspiratory burst to a decrementing burst shape (Lieske et al. 2000; Ramirez et al. 1997b). Together these changes characterize a network state during hypoxia that is referred to as fictive gasping (Lieske et al. 2000; Peña et al. 2004).

Although much progress has been made in understanding how this neuronal network continues to function in hypoxia, several issues concerning the nature of O$_2$ sensitivity of the preBötC remain largely unanswered. For example, does the respiratory network switch abruptly from fictive eupneic to fictive gasping activity or does it make a gradual transition as O$_2$ levels decrease? We tested the hypothesis that population activity of the preBötC is modulated in a progressive manner over a broad range of O$_2$ tensions. We found that some metrics that characterize the activity of the preBötC respond to graded reductions in tissue oxygenation in a progressive manner, whereas others switch abruptly in response to a severe reduction in O$_2$. Based on these results it appears that the respiratory network does not undergo a discrete network reconfiguration in which all components switch in a concerted manner, but rather the network assumes multiple configurations as it responds to different levels of reduced oxygenation. Moreover, graded reductions in oxygenation differentially affect the pharmacological response to RIL and FFA. We therefore propose that the hypoxic response of the respiratory network is an emergent property of multiple mechanisms with differential O$_2$ sensitivities.

**METHODS**

Experiments were done with prior approval from the Animal Care and Use Committee at the University of Chicago, the Animal Care Facilities Committee at Rutgers University, and the Office of Animal Care at Seattle Children’s Research Institute. Protocols were followed that minimized the number of animals used and their suffering.

**Medullary brain stem slice preparation**

Experiments were performed using a transverse brain stem slice preparation of the medulla of mice (Fig. 1) (Ramirez et al. 1996; Smith et al. 1991). CD-1 mice bred from founders obtained from Charles River Laboratories were anesthetized with isoflurane until the absence of a withdrawal reflex from toe pinch. Neonatal mice (P7–P11) were decapitated and the brain stem and a short length of spinal cord were dissected in ice-cold artificial cerebrospinal fluid (aCSF) containing the following (in mM): 118 NaCl, 25 NaHCO$_3$, 1 NaH$_2$PO$_4$, 1 MgCl$_2$, 6H$_2$O, 3 KCl, 30 D-glucose, and 1.5 CaCl$_2$. The pH of this aCSF was 7.4 when equilibrated with a gas mixture containing 5% CO$_2$ at room temperature (~21°C). The brain stem and spinal cord were mounted with cyanoacrylate glue such that the brain stem was above the spinal cord and the dorsal side of the brain stem faced the sloping face of an agar block (23° from vertical). Serial sections (100 μm) were cut in the rostrocaudal direction using a vibrating blade microtome (Leica VT 1200). Neuroanatomical landmarks such as the compact division of the nucleus ambiguus and the rostral border of the inferior olive were used to identify the appropriate rostrocaudal level to cut the final 600 μm thick slice (Ramirez et al. 1996). Slices were allowed to recover from the slicing procedure at room temperature for 30 min in oxygenated aCSF before electrophysiological recordings were conducted. The slice was placed in a superfusion chamber (RC-29; Warner Instruments) that allowed for fluid flow both above and below the submerged slice, ensuring adequate oxygenation. The flow rate was 10 ml/min and the bath volume was about 2.5 ml. Once the slice was transferred to the recording chamber the level of extracellular KCl was increased to 8 mM and the temperature was increased to 32°C. The increase in extracellular potassium concentration leads to rhythmic population activity of the preBötC (Tryba et al. 2003). The slice was allowed to recover from these changes for 30 min before the commencement of recordings. Bath temperature was continuously regulated and monitored using a solution reservoir heating system as well as a stage and in-line heater system (TC-344B; Warner Instruments).

**Electrophysiological recording**

Bursts of rhythmic extracellular activity were observed by placing a saline-filled pipette on the surface of a 600 μm thick slice in an area of the ventral respiratory group (VRG) called the preBötC (Fig. 1). Differential extracellular recordings were made of population activity of preBötC neurons (Fig. 1) (Telgkamp and Ramirez 1999), using a 100-fold gain extracellular preamplifier (JFIE 1626) made by the James Franck Institute Electronics Laboratory, University of Chicago, in series with a variable gain amplifier (Model 410; Brownlee Precision). The preBötC was identified anatomically based on its proximity to neuroanatomical landmarks such as the nucleus ambiguus and the inferior olive and functionally based on the presence of strong rhythmic bursts of extracellular activity that occur in synchrony and one-to-one correspondence with bursts recorded from the hypoglossal nucleus (Telgkamp and Ramirez 1999; Tryba et al. 2003). In a few experiments (data not shown) the bursting population activity of the ventral respiratory group was confirmed to be inspiratory-related by simultaneously recording from the surface of the slice in the area of the hypoglossal nucleus, which generates activity during the inspiratory phase of respiration (Tryba et al. 2003).

**Signal processing, data acquisition, and data analysis**

Raw extracellular recordings were band-pass filtered (100 Hz to 1 kHz) and rectified and integrated with a time constant of 50 ms using an analog Dual Channel Integrator (JFIE 1620A; James Franck Institute Electronics Laboratory, University of Chicago). Analog signals were digitized using a Digidata 1440A interface by
Molecular Devices. In vitro inspiratory bursts recorded from the preBöC were characterized by measuring changes in the rectified and integrated form of the raw extracellular signal. The rectified and integrated voltage signal shows a positive deflection in phase with each population burst, which consists of the near-simultaneous bursting activity of many respiratory interneurons (Fig. 1). Burst metrics such as frequency and half-width were determined using scripts written by the authors for MATLAB (a programming language by The MathWorks). The calculation of burst frequency was done using all bursts. The calculation of shape metrics excluded fictive sighs because the waveform is complex, often consisting of two bursts joined together: a small eupneic-like burst followed by a larger burst (Fig. 4B) (Lieske et al. 2000). The mean values of various metrics of the fictive respiratory bursts were calculated using two different sized time bins: 30 and 100 s. The 30-s time bins were necessary to capture the dynamics of rapidly changing phenomenon, whereas 100-s time bins were appropriate for slowly changing phenomena and allowed for data from a larger number of bursts to be averaged. Data from different preparations were combined by temporally aligning them based on the transition to low O₂. Because we were interested in quantifying the relative changes in a particular metric in response to a reduction in O₂ tension and because of the large range of values of some metrics under control conditions, data were normalized to the mean value of the control bin, which immediately preceded the transition to reduced O₂. The mean values, unless otherwise stated, are presented as means of normalized data ± SE.

Control of O₂ tension

The partial pressure of oxygen (Pₒ₂) was controlled by changing the fractional value of oxygen (Fₒ₂) that was bubbled at a rate of 50 ml/min into a heated 40 ml reservoir of aCSF, using an aeration stone. Premixed tanks of O₂, CO₂, and N₂ were used with various Fₒ₂ values (0, 21, 50, 75, and 95%). In all tanks Fₒ₂ was 5% and the balance consisted of N₂. Pₒ₂ was changed rapidly within the superfusion bath by switching the flow of aCSF between two reservoirs: one saturated with a control gas (Pₒ₂ = 95%) and another saturated with a test gas (Pₒ₂ = 0–75%). We found that, due to the use of a recirculating system, the aCSF within each reservoir was not fully saturated with the equilibrating gas; thus the measured partial pressure differs from what one would expect by calculation (Pₒ₂ = Fₒ₂ × atmospheric pressure). For example, the expected Pₒ₂ in the superfusion chamber when the reservoir is bubbled with an Fₒ₂ of 0% is 0 Torr, yet the actual measured Pₒ₂ was about 38 Torr. We found that the main reason that the aCSF within each reservoir was not fully saturated with the equilibrating gas was because media was exposed to ambient air in the tubes returning solution from the bath to the reservoir. As this media returns to the reservoir it mixes with media that is already in the reservoir, resulting in incomplete saturation with the equilibrating gas. In addition, gas diffusion at the media–ambient atmosphere interface in the superfusion chamber may also contribute to the deviation of the measured Pₒ₂ in the media in the superfusion chamber from the theoretical value. Throughout this study we refer to the Pₒ₂ of gases used to saturate the aCSF as simply percentage O₂ (e.g., 95% O₂). Measured values of O₂ tension in the aCSF and tissue are expressed as Pₒ₂ in units of Torr. Many plots (e.g., Fig. 4D) use the mean Pₒ₂ at the slice surface, as determined empirically (Fig. 2; Table 1), rather than the corresponding Pₒ₂ values of the gas mixtures used to equilibrate the reservoir.

Measurement of bath and tissue O₂ tension

An amperometric system consisting of custom constructed platinum wire electrodes and a polarographic amplifier (Model 1900; A-M Systems) was used to measure Pₒ₂ in the superfusion bath and in the slice. Flush-tip electrodes were made by cutting 30- to 50-μm-diameter Tellon-insulated or Iselon-insulated platinum wire (A-M Systems). The electrode was polarized to a voltage of between −675 and −700 mV with respect to an Ag/AgCl reference electrode placed in the bath (Fatt 1982). In O₂-depth profile experiments, the electrode was calibrated prior to and following each experiment. Calibrations were performed in the superfusion bath using aCSF equilibrated with 0% O₂ (supplemented with the oxygen scavenger, Na₂SO₃, to produce anoxia) and at least three to five other points (between 10% and 95% O₂). A second-degree polynomial was used to define the calibration curve to convert amperometric measurements to Pₒ₂. Pₒ₂ was corrected for vapor pressure. Measurements of the tissue Pₒ₂ within a slice were started 400 to 500 μm above the slice and the electrode was moved in 100-μm steps, downward to the upper surface of the slice, to the core, and, in most cases, beyond the lower slice surface (±400 μm below the lower slice surface). Contact with the upper surface of the slice was identified visually using a stereoscope. On touching the upper surface with the electrode tip, dimpling of the tissue was apparent. At a given depth, the measured current was allowed to stabilize for 60–180 s before the next step.

Statistical methods

All differences (P < 0.05) between three or more means were determined using one-way ANOVA. In analyzing attributes of the rhythm the one-way measures ANOVA was followed by multiple comparisons testing using Dunnett’s comparisons (control was defined as values measured at 95% O₂). This test was done at three different time bins: early within the time of exposure to low O₂ (augmentation phase), late during the exposure to low O₂ (depression phase), and at an appropriate time during the return to 95% O₂ to highlight differences in the recovery between test and control experiments (recovery phase). In contrast, in O₂-depth profile experiments, differences (P < 0.05) between three or more means were determined using one-way ANOVA followed by Tukey’s comparisons test. Paired t-tests were used when appropriate for other data comparisons.

Linear regression analysis was used to identify linear relationships between metrics of rhythmic population activity and slice surface Pₒ₂. Although the r² value provides a relative index for the “goodness of fit” of a given data set to the calculated linear regression, the absolute value of the slope was used as an index to determine the relative strength of the correlations (the absolute value of the slope is used so that negative and positive slopes could be easily compared). Absolute slope values near or equal to 0 indicated that the predictive value of the linear model was low (i.e., progressive changes in O₂ do not affect the given metric). Conversely, the greater the divergence of the absolute slopes from 0, the greater the dependence of a given metric on tissue O₂ tension. All statistical tests were done using either KyPlot Data and Visualization software package (Kynoslab, Tokyo) or Graphpad InStat (GraphPad Software, La Jolla, CA).

Results

Tissue oxygenation states when exposed to different levels of media O₂

O₂-depth profiles measured in aCSF saturated with 95, 75, 50, 21, or 0% O₂ revealed that two-sided superfusion of aCSF across a transverse medullary slice (Fig. 1) created U-shaped O₂-depth profiles in the slice where the upper and lower surfaces had similar values and the core had the lowest values (Fig. 2A). Although distinct profiles are produced at each degree of oxygenation, single point measurements of tissue Pₒ₂ could overlap at a given depth when using different Pₒ₂ values to equilibrate the circulating media. For example, at the core of slice (300 μm), equilibrating with 21% O₂ resulted in tissue Pₒ₂ values that could overlap with core
tissue Po2 measurements made in 0% O2 (Fig. 2A, inset). A comparison of Po2 values (Table 1) reveals that, whereas media Po2 is different when equilibrated with 21% versus 0% O2, tissue Po2 is not different either at the surface or the core. At 95% O2, media and slice surface Po2 values were different when compared with corresponding measurements made in O2 ≤ 75%. Core Po2 in 95% O2 is different from core Po2 values in O2 ≤ 50%. In 75% O2, Po2 values in the media and at the surface are different from corresponding Po2 values created when using gas mixtures with O2 ≤ 50%. Po2 at the core in 75% O2 is different from core Po2 values created by both 21 and 0% O2. In 50% O2, media, surface, and 0% O2. 

![Graphical representation](http://jn.physiology.org/)
and core PO2 values are different from corresponding PO2 values in either 21 or 0% O2.

Plotting media PO2 as a function of O2 in the equilibrating gas mixture demonstrates the progressive increase in bath PO2 from 0 to 95% O2 (Fig. 2B). The calculated slope of this relationship using linear regression analysis is 6.8 Torr FO2⁻¹ (r² = 0.98). Plotting surface and core PO2 (Fig. 2C) as a function of media PO2 demonstrates the progressive relationship between PO2 with respect to media PO2. The calculated slope of the surface PO2 to media relationship using linear regression analysis is 0.67 Torr FO2⁻¹ (r² = 0.99), whereas the calculated slope of the core PO2 to media relationship is 0.08 Torr FO2⁻¹ (r² = 0.98).

**Rhythmogenesis responds to progressive reductions in oxygenation**

Although the O2-depth profiles conducted at multiple FO2 values indicated that unique states of tissue oxygenation were produced, it was unknown how the rhythm generated by the preBötC responds to progressive reductions in O2 ranging between 95 and 0%. Therefore we conducted a series of electrophysiological experiments exposing individual slices to reduced levels of media O2 (Fig. 3A). Blitz and Ramirez (2002) previously reported that hypoxic exposure can condition subsequent responses to hypoxia and thus individual slices were exposed to only a single bout of reduced O2. Exposure to reduced oxygenation levels from 0 to 75% for 300 s from a baseline level of 95% produces a stereotypical biphasic frequency response: augmentation followed by depression (Fig. 3, B–E). In addition to quantifying the frequency modulation (FM) of the rhythm, metrics related to shape changes of the averaged integrated waveform were also quantified (Fig. 3F).

Although the present study is primarily concerned with the characterization of fictive respiratory bursts that resemble eupneic and gasplike bursts recorded in vivo, there are also population bursts in vitro that resemble sighs (Lieske et al. 2000). Fictive sighs are large in amplitude and have a more complex waveform than that of either fictive eupneic bursts or fictive gasps (Fig. 4, A–C). They occur spontaneously at a low baseline frequency (0.0036 ± 0.0028 Hz; n = 26) and increase in frequency at the transition to low O2 (Fig. 4A) (Lieske et al. 2000). To determine whether the increase in sigh frequency is dependent on the degree of reduction in O2, we recorded the number of sighs that occurred during the 100-s time bin immediately after the transition to reduced O2. This time bin was chosen because almost all sighs occurred within this time span on exposure to reduced O2. We found that there were significantly more sighs in response to 0, 21, and 50% O2 compared with control (95% O2) (Fig. 4D).

![Image](http://jn.physiology.org/)

**FIG. 3.** The response of the in vitro respiratory rhythm is dependent on the degree of reduction in oxygen. Representative preparations were each subjected to a single reduction in O2. A: PO2 values measured within the superfusion chamber resulting from a switch between a control reservoir saturated with 95% O2 and a second reservoir saturated with 0 to 75% O2. During the 300-s test period there was a rapid decrease in the PO2 within the superfusion chamber, which eventually stabilized at a new O2 level. B–E: responses of the in vitro respiratory rhythm to 5 min long reductions in O2. The dots placed above certain bursts denote sigh-like bursts, which were distinguished by their large amplitude compared with that of normal respiratory bursts (Lieske et al. 2000). F: an averaged burst waveform calculated from the bursts within a 100-s time bin in 95% O2. In this example, an average waveform was calculated based on 9 individual burst waveforms. The baseline was determined by averaging the voltage immediately prior to and immediately after the averaged burst waveform. The burst amplitude was calculated as the difference between the peak of the burst and the baseline. The burst area was the area bounded by the baseline on the bottom, the averaged waveform on the top and vertical lines at the times corresponding to 20% of the peak amplitude on the rising and falling phases of the waveform. The rise time was calculated as the time interval between 20 and 80% of the peak amplitude on the rising phase of the waveform. The half-width was calculated as the time interval between 50% of the peak amplitude on the rising phase of the waveform and 50% of the peak amplitude on the falling phase.
To quantify change in burst frequency, we normalized and averaged data from different preparations. A biphasic response to a reduction in O$_2$ is evident in the change in mean frequency (Fig. 5, A–C), whereas mean burst area decreased monotonically during exposure to reduced O$_2$ (Fig. 5, D–F). To better compare dynamic changes in frequency, burst area, and other burst metrics, we measured these metrics at three important time points. The first time point is early during the drop in O$_2$ burst metrics, we measured these metrics at three important time points. The first time point is early during the drop in O$_2$ (i.e., augmentation phase), the second corresponds to the final 100-s bin during the exposure to reduced oxygenation (i.e., depression phase), and the third time point is taken following return to 95% O$_2$ (i.e., recovery phase) (Fig. 5, A and D).

**Augmentation phase**

During the initial phase of the exposure to reduced oxygenation, an augmentation in frequency was observed in response to 0 to 50% O$_2$ (Fig. 6A). Waveform analysis revealed that burst area decreases when exposed to 0 to 50% O$_2$ (Fig. 6B). Burst amplitude decreased in 0% O$_2$ (Fig. 6C), half-width was reduced in 0 to 50% O$_2$ (Fig. 6D), and rise time was reduced in 0 and 21% O$_2$ (Fig. 6E).

**Depression phase**

During the depression phase, the frequency of the rhythm decelerated in response to a drop in O$_2$ between 0 to 75% O$_2$ (Fig. 7A), whereas the burst area decreased when exposed to 50% O$_2$ and below (Fig. 7B). However, burst amplitude decreased only in 0% O$_2$ (Fig. 7C), which is similar to the response to 0% found during the augmentation phase. Similar to burst area, the half-width decreased between 0 and 50% O$_2$ (Fig. 7D). Rise time decreased in 0 and 21% O$_2$, which was similar to its response during the augmentation phase. Linear regression of the relationship of each metric to surface PO$_2$ revealed that frequency, burst area, and half-width have $R^2$ values $\geq 0.5$ (Fig. 7F). Furthermore, of these three metrics, frequency and burst area have slopes $>0.1$, indicating a relatively high O$_2$ sensitivity of these two metrics compared with that of the others. For non-normalized values under control conditions and during the depression phase see Table 2.

**Recovery phase**

To quantify the speed with which the respiratory network recommences rhythmic activity during the upswing from a reduced state of oxygenation the time to first burst (TTFB) was measured (Fig. 8). As previously reported, reoxygenation from hypoxia leads to a transient cessation of rhythmicity (Blitz and Ramirez 2002). TTFB is defined as the time from the onset of reoxygenation to the first burst that is part of a continuous train of bursts (Fig. 8A). This definition discounts single spurious bursts that are not followed by successive bursts with frequency values approaching the baseline frequency during control. The TTFB is greater after exposure to 0, 21, and 50% O$_2$. After an initial increase in the number of sighs during the first 100 s, sighs were very rare during the second 100 s and no sighs were observed in the third 100 s of a 5-min exposure, except in the control experiment in which the level of O$_2$ was maintained at 95%. Note that empirically derived mean slice surface PO$_2$ values (20, 73, 177, 341, 440 Torr; see Table 1) are reported on the x-axis rather than the corresponding media PO$_2$ values (0, 21, 50, 75, 95%). Error bars represent SD.

![Fig. 4](http://jn.physiology.org/) Fictive sighs increase in number during the transition from high O$_2$ to low O$_2$. A: fictive sighs occur at a low frequency in 95% O$_2$ but increase in frequency during the transition to reduced O$_2$. In this example from a postnatal day 9 mouse, 6 sighs occurred within the first 100 s of exposure to 0% O$_2$. As in Fig. 3, dots were placed above sigh bursts. The arrows marked B and C denote windows of time that are expanded in B and C, respectively. B: fictive sighs were identified based on amplitude, shape, and by the presence of a fictive gasp. C: fictive gasps can be identified based on a lower frequency and faster rise time than eupneic bursts (Lieske et al. 2000). D: a comparison of the number of fictive sighs within the first 100 s of the transition to reduced O$_2$ revealed that there was a significantly higher number of sighs in response to 0, 21, and 50% compared with the control experiment in which O$_2$ was maintained at 95%. After an initial increase in the number of sighs during the first 100 s, sighs were very rare during the second 100 s and no sighs were observed in the third 100 s of a 5-min exposure, except in the control experiment in which the level of O$_2$ was maintained at 95%.
compared with the control experiment (95% O2), yet no difference in TTFB was observed between 75 and 95% O2.

During the recovery phase, frequency augmentation occurred following exposure to 0 and 21% O2 (Fig. 9A). Similarly, both burst area (Fig. 9B) and amplitude (Fig. 9C) overshot baseline during the recovery phase following 0 and 21% O2. Half-width increased only following exposure to 21% O2 (Fig. 9D), whereas rise time (Fig. 9E) was unaffected during the recovery phase. Linear regression of the relationship of each metric to surface PO2 revealed that all metrics during the recovery phase have r² values <0.4 and slopes <0.1 (Fig. 9F). Similar to the depression phase, the absolute slopes of frequency and burst area were the largest among the various metrics.

Riluzole-sensitive and FFA-sensitive mechanisms are differentially affected by graded reductions in oxygenation

To test the involvement of riluzole (RIL) and flufenamic (FFA) sensitive mechanisms in shaping the network response to graded reductions of O2, a series of pharmacological experiments were conducted. Similar to the results described previously (Peña et al. 2004), neither 10 μM RIL (Fig. 10A, n = 4) nor 50 μM FFA (Fig. 10B, n = 8) stopped...
rhythmogenesis in 95% $O_2$, but coapplication of both agents eliminated rhythmogenesis (Fig. 10C, n = 6). Furthermore, hypoxic exposure (0% $O_2$) in the presence of RIL alone blocks rhythmogenesis (Fig. 10, A, D, and E), whereas 0% $O_2$ in the presence of FFA alone does not prevent the rhythmic activity (Fig. 10, B, F, and G; Supplemental Fig. S1). No differences were identified for either normalized frequency or burst area during the augmentation phase in the presence of either RIL or FFA compared with untreated controls (data not shown). Note that although the results of these experiments were similar to those reported by Peña et al. (2004), the concentrations of the two drugs used were lower: 50 versus 500 $\mu$M for FFA and 10 versus 20 $\mu$M for RIL. The use of lower concentrations of both drugs was an attempt to limit the effects of these drugs to the blockade of $I_{\text{NaP}}$ by RIL and $I_{\text{CAN}}$ by FFA. The difficulties of ascribing the effects of these two drugs, even at these lower concentrations, to only the blockade of these two currents in a rhythmically active network is addressed in the discussion.

To extend the results of Peña et al. (2004) we tested the effects of blocking RIL- and FFA-sensitive mechanisms under conditions of intermediate $O_2$ levels (21–75%). During the depression phase, reducing $O_2$ from 95 to 21% $O_2$ in the presence of RIL eliminates rhythmogenesis (Fig. 10, D and E; n = 4), but in the presence of FFA neither frequency nor burst area was affected at this $O_2$ tension (Fig. 10, F and G; n = 5). In the presence of RIL, reducing $O_2$ from 95 to 50% completely blocked rhythmic activity in 4 of 11 experiments (Fig. 10, D and E). However, in the remaining 7 preparations where the rhythm continued, reducing $O_2$ to 50% did not significantly influence frequency (Fig. 10D) or burst area (Fig. 10E). In contrast, the reduction of $O_2$ to 50% while exposed to FFA had no effect on the frequency or burst area (Fig. 10, F and G). Thus RIL-sensitive mechanisms are important for basic rhythmogenesis at $\leq$50% $O_2$, whereas FFA-sensitive mechanisms appear to have no impact on rhythmogenesis at $\leq$50%.

Both RIL and FFA also had significant effects on the rhythm at 75 and 95% $O_2$. The burst area in RIL-exposed slices was lower in both 75 and 95% $O_2$ compared with untreated controls (Fig. 10E). This reduction in burst area is to be expected if the RIL-sensitive mechanisms contribute to rhythmogenesis at all $O_2$ tensions tested (0–95%). Additionally, in 75% $O_2$ there was a significant decrease in frequency after exposure to RIL, suggesting that RIL-sensitive mechanisms may help to accelerate the frequency at this $O_2$ tension, but are not essential for the basic expression of rhythmic activity since all preparations continued to burst. Likewise, there were significant decreases in frequency in FFA-treated preparations compared with untreated controls in 75 and 95% $O_2$ (Fig. 10F), suggesting that FFA-sensitive mechanisms may accelerate the rhythm in 75 and 95% $O_2$. Both frequency and area in FFA-treated preparations are unaffected from between 0 and 50% $O_2$. One possible explanation for this phenomenon is that there is an $O_2$-dependent reduction in the expression of FFA-sensitive mechanisms in this range of $O_2$ values. Thus there is no difference between control preparations and FFA-exposed preparations.

**FIG. 6.** Changes in frequency and shape metrics in response to graded reductions in $O_2$ tension during the augmentation phase. A: frequency was significantly higher in 0% ($P < 0.05$), 21% ($P < 0.05$), and 50% ($P < 0.05$) $O_2$ compared with that in 95% $O_2$. B: burst area was significantly lower in 0% ($P < 0.001$), 21% ($P < 0.05$), and 50% ($P < 0.05$) compared with that in 95% $O_2$. C: burst amplitude was significantly lower in 0% compared with that in 95% $O_2$ ($P < 0.05$). D: half-width was significantly lower in 0% ($P < 0.001$) and 21% ($P < 0.01$), and 50% ($P < 0.01$) compared with that in 95% $O_2$. E: rise time was significantly lower in 0% ($P < 0.01$) and 21% ($P < 0.05$) compared with that in 95% $O_2$. * denotes significance in A–E.

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1 The online version of this article contains supplemental data.
DISCUSSION

The ability to respond to changes in arterial $P_{O_2}$ by changing the rate and depth of breathing is important for survival. Previous studies using the transverse brain slice preparation demonstrated that the rhythm generated by the preBötC changes in waveform and frequency during hypoxia, suggesting that there is a reconfiguration of the underlying neural network (Lieske et al. 2000; Peña et al. 2004). The question remained, however, as to how sensitive the preBötC is to smaller changes in $O_2$ and whether network reconfiguration occurs as a discrete switch from one state to another or whether different aspects of the network are differentially affected as tissue oxygenation is progressively reduced. We hypothesized that a broad range of $O_2$ tensions progressively modulates network activity of preBötC, as determined by measurable changes in metrics of rhythmicity. To test this hypothesis, we first established that it was possible to produce unique states of tissue oxygenation by altering the oxygenation level of the media. We then found that these graded reductions in tissue oxygenation differentially affected several metrics of population activity from the preBötC. Some metrics responded gradually over a broad range of oxygenation, whereas others were unaffected by small reductions in oxygenation but then changed abruptly in response to larger reductions in tissue $P_{O_2}$. To better understand the cellular mechanisms that contribute to these transitions in network activity, the response of the network to graded reductions in tissue $P_{O_2}$ was measured in the presence of RIL and FFA, which have been shown to block the persistent Na$^+$ current ($I_{NAP}$) and the Ca$^{2+}$-activated nonspecific cation current ($I_{CAS}$) in preBötC neurons, respectively (Del Negro et al. 2005; Koizumi and Smith 2008; Pace et al. 2007). We found that RIL-sensitive mechanisms are crucial to the expression of a respiratory rhythm in low tissue $P_{O_2}$ but also contribute to the burst area and frequency in high tissue $P_{O_2}$. In contrast, FFA-sensitive mechanisms appear to contribute to the rhythm only at high tissue $P_{O_2}$. The implications of these novel findings will be discussed in further detail in the following text.

In vitro tissue oxygenation

Although only a handful of brain slice studies have shown that neuronal activity is affected throughout a broad range of $O_2$ tensions outside of hypoxia (0% $O_2$) and the conventional control condition (95% $O_2$), these studies have shown that graded changes in $O_2$, and not only hypoxia, can influence biochemical activity (D’Agostino 2007; Fowler 1993) and excitability of neurons within local networks (Fowler 1993; Garcia 3rd et al. 2010; Hoffmann et al. 2006; Mulkey et al. 2001). Similarly, in invertebrate systems, small changes in oxygenation also have been shown to influence network activity (Clemens et al. 2001). Understanding how network behavior changes at multiple $O_2$ tensions is of particular relevance to the respiratory network because mechanisms of rhythmicity in the preBötC are altered during the transition from 95 to 0% $O_2$, thus raising the important question of whether these mechanisms are altered in an all-or-none or graded fashion in response to progressive reductions in tissue oxygenation levels.
A range of physiologically realistic tissue oxygenation states could be achieved by varying the O₂ level of the aCSF. However, the results described in the following text should not be misconstrued as an attempt to define an “optimal” O₂ level that yields a range of physiologically realistic tissue P₂O₂ values within the brain slice preparation.

Neural tissue O₂-depth profiles have been previously reported in vivo (for summary see Garcia 3rd et al. 2010), in situ in the working heart–brain stem preparation (Wilson et al. 2001), as well as in vitro in both isolated brain stem–spinal cord preparations (Brockhaus et al. 1993; Okada et al. 1993) and brain slices (Bingmann and Kolde 1982; Garcia 3rd et al. 2010; Mulkey et al. 2001). Several factors influence the degree of in vitro tissue oxygenation within a brain slice. Some, like the fractional O₂ value (F₂O₂) of the gas mixture used to equilibrate the media are obvious, yet others are often overlooked. These include biological factors such as cell density and metabolic activity in the region of interest. For example, a region of a slice that generates bursting activity such as the preBötC might use more energy and thus require more O₂ than a quiescent region (for differences in O₂-depth profiles between regions of a slice see Bingmann and Kolde 1982). Other factors related to experimental design also affect tissue oxygenation. These include media temperature, which affects metabolic activity; the rate and method of superfusion (i.e., single-sided vs. double-sided); and the ambient barometric pressure (Dean et al. 2003; Fong et al. 2008; Jiang et al. 1991; Mulkey et al. 2001). Moreover, comparisons of our profiles to those made in adult medullary brain stem slices <600 μm thick (Mulkey et al. 2001) demonstrate that slice thickness, and possibly age, affect the O₂-depth profile of the slice (see also Jiang et al. 1991). For all of these reasons, it was impossible to predict tissue oxygenation levels a priori and it was necessary to measure O₂-depth profiles resulting from different media O₂ levels.

We found that two-sided superfusion produced a U-shaped O₂ profile in the slice where maximal tissue P₂O₂ values are found at the surfaces and the nadir was found at the core. Steep O₂ gradients were formed outside the tissue, presumably because the metabolic demand of the slice causes it to act as an O₂ sink (Mulkey et al. 2001). Although media P₂O₂ in the superfusion bath was different when the reservoir was aerated with 21% compared with 0% O₂, neither slice surface nor core P₂O₂ values were significantly different. We interpret these findings to indicate that aCSF with either 0 or 21% O₂ provides insufficient tissue oxygenation. This result differs from the results of a study by D’Agostino et al. (2007), in which 400-μm-thick hippocampal slices appeared to be adequately oxygenated in 20% O₂ based on the ability of individual CA1 neurons to generate evoked action potentials. The discrepancy between these results emphasizes the complexity of tissue oxygenation in vitro. For example, differences in slice thickness (600 μm thick preBötC slices compared with 400 μm thick hippocampal slices) and possibly metabolic activity of the region of interest may markedly affect O₂ profiles within the slice. Despite the similarity of P₂O₂ slice values at 0 and 21% O₂ found here, other O₂ values produced unique O₂-depth profiles, albeit with some convergence of core P₂O₂ values. The observation that unique states of tissue oxygenation could be achieved by varying the media F₂O₂ allowed us to proceed to then test the effects of graded reductions in O₂ on the network activity of the preBötC.

**Attributes of rhythmogenesis during and following exposure to graded reductions in O₂**

We hypothesized that the preBötC may respond to a wide range of reductions in tissue oxygenation values as determined by changes in metrics such as frequency and shape of the population burst waveform. Additionally, we hypothesized that some metrics may change progressively over a broad range of O₂ values, whereas others may change abruptly. It has been shown previously that there is a stereotypical aggregate frequency response to hypoxia (0% O₂) followed by reoxygenation. First, during the initial drop in tissue oxygenation, burst frequency accelerates during what is known as the augmentation phase, and then during the depression phase it decelerates to a value that is far below the baseline frequency in 95% O₂ (Ramirez et al. 1997a, 1998; Telgkamp and Ramirez 1999). Moreover, on reoxygenation to 95% O₂, there is a further deceleration of frequency followed by a rebound that overshoots the original baseline frequency (Ramirez et al. 1997a, 1998; Telgkamp and Ramirez 1999). Here we demonstrate that graded reductions in oxygenation result in a frequency pattern similar to the stereotypical pattern of augmentation, depression, and overshoot observed in response to severe hypoxia, albeit the changes in frequency were generally smaller in response to more moderate changes in tissue oxygenation. To quantify the effects of different degrees of reduction in O₂ on...
rhythmogenesis we measured frequency and other metrics of population activity at specific time points: the early period of reduced O₂ (i.e., augmentation phase), at the end of the period of reduced O₂ (i.e., depression phase), and following return to 95% O₂ (i.e., recovery phase).

During the augmentation phase, there was an increase in frequency over a broad range of O₂ values from 0 to 50%. Over this same range there was also a transient increase in the number of sighs generated and a decrease in half-width of respiratory bursts. In contrast, other metrics such as rise time and amplitude were influenced only by low levels of O₂ (0 to 21% and 0%, respectively) during augmentation. Burst area, and amplitude were influenced only by low levels of O₂ (i.e., recovery phase).

During the depression phase, frequency was sensitive to a broad range of O₂ values from 0 to 75%. Burst area and half-width continued to be O₂ sensitive over the same broad range as that during the augmentation phase (0–50%). Close examination of these three metrics (frequency, burst area, and half-width) reveals that frequency varies continuously with PO₂, whereas burst area or half-width appears to plateau between 75 and 95% O₂ (Fig. 7, A, B, and D). Media PO₂ was at steady state during the depression phase (Fig. 3A) and thus the tissue PO₂ may also have been at steady state. For this reason it is appropriate to test for possible linear relationships between the change in each metric and issue PO₂. Using linear regression analysis, we found that changes in frequency and burst area had relatively large r² values (0.51 and 0.57, respectively), suggesting that the relationships of these two metrics to tissue PO₂ may be well described by a linear function. In addition, frequency and burst area had relatively large slopes, indicating that these metrics show a high degree of sensitivity to tissue PO₂. Half-width also has a relatively high r² value, suggesting that it may also change in a linear manner with tissue PO₂. Its smaller slope, however, indicates that it is less sensitive than frequency or burst area.

In contrast to these relatively progressive changes, rise time and amplitude change in a more abrupt manner in response to O₂. These metrics do not change significantly except when the level of O₂ is 0% for amplitude and 0 to 21% for rise time. Similar restricted sensitivities to O₂ were observed during both the depression phase and the augmentation phase. During the depression phase, the r² values and slopes of these two metrics are relatively low, further supporting the idea that there may not be a linear relationship between these metrics and the state of tissue oxygenation. Taken together these data suggest that some metrics and their underlying processes have relatively linear and progressive relationships with tissue PO₂, whereas other metrics express sensitivity to O₂ only when O₂ deprivation is severe.

It has been previously reported that frequency augmentation occurs following a 600-s hypoxic exposure (Ramirez et al. 1997a, 1998; Telgkamp and Ramirez 1999). Extending
these observations, we demonstrated that a 300-s exposure to insufficient oxygenation (i.e., 0 and 21% O2) can cause frequency, burst area, and amplitude augmentation. Interestingly, whereas 21% O2 led to a significant augmentation of half-width during the recovery phase, exposure to 0% O2 did not. Although tissue PO2 values between 0 and 21% O2 were not different, we found significant differences found in media PO2. Thus one potential explanation for the difference in response between 0 and 21% O2 is that the production of reactive oxygen species during and following reoxygenation may be different and, as a result, may affect half-width differently.
Network reconfiguration and multiple network states

Hypoxia-mediated reconfiguration of the preBötC appears to involve changes in the relative contribution of different rhythm-generating mechanisms to the population respiratory rhythm (Peña 2008; Peña et al. 2004; Ramirez and Garcia 2007). Specifically, both riluzole (RIL)-sensitive and flufenamic acid (FFA)-sensitive mechanisms contribute to rhythmogenesis in well-oxygenated states, whereas the relative contribution of FFA-sensitive mechanisms wanes under O2-limited conditions, making RIL-sensitive mechanisms crucial for rhythmogenesis in low O2 (Peña et al. 2004). A mechanistic interpretation for the basis of the effects of both RIL and FFA is limited by the fact that even at low concentrations multiple subcellular targets may be affected by RIL and FFA; 10 μM RIL principally affects $I_{Ca}$ but it may also affect other membrane Na$^+$ currents (Del Negro et al. 2005; Ptak et al. 2005) and background (i.e., leak) K$^+$ currents (Lamanauskas and Nistri 2008), resulting in a reduction in neuronal excitability. Similarly, although FFA blocks $I_{Ca}$ (Pace et al. 2007), it may also: 1) inhibit Ca$^{2+}$ currents (Wang et al. 2006), 2) affect a variety of K$^+$ currents (Kochetkov et al. 2000; Takahira et al. 2005), 3) block gap junctions (Harks et al. 2001; Srinivas and Spray 2003); and 4) inhibit voltage-gated Na$^+$ currents (Yau et al. 2010).

RIL alone did not block the basic ability of the network to generate respiratory population bursts in 75 and 95% O2, indicating that RIL-insensitive mechanisms help to sustain the rhythm at these O2 levels. However, in 95% O2, the blockade of RIL-sensitive mechanisms did lead to a reduction in burst area and in 75% O2, both burst area and frequency were reduced. In 50% O2, RIL application led to the cessation of the rhythm in 4 of 11 experiments, whereas in the remaining 7 experiments reductions in frequency and area were not significant. A further reduction in O2 to 0 or 21% led to a complete cessation of rhythmic activity in all preparations tested. These findings indicate that 1) RIL-sensitive mechanisms are essential for bursting activity at 0 and 21% O2, 2) the O2 threshold at which the contribution of RIL-sensitive mechanisms to rhythmogenesis becomes essential is roughly 50% O2, and 3) RIL-sensitive mechanisms contribute to burst frequency at high levels of O2 (75 and 95%).

The relatively limited effect of 10 μM RIL in high O2 suggests that complementary mechanisms aid in the maintenance of rhythmic network activity. Peña et al. (2004) showed that in 95% O2, these complementary mechanisms are sensitive to FFA. In 95% O2, the coapplication of RIL and FFA leads to a cessation of fictive rhythmic activity, whereas the individual application of either drug individually did not lead to a cessation of activity (Peña et al. 2004). This observation was confirmed here using lower concentrations of FFA (50 vs. 500 μM) and RIL (10 vs. 20 μM). Despite the use of lower concentrations, the application of RIL followed by the application of FFA led to a complete cessation of bursting in all preparations tested (Fig. 10C; n = 6), confirming the results of Peña et al. (2004) and also demonstrating that lower concentrations of these drugs are efficacious.

Prior to the present study, the influence of different intermediate O2 levels on FFA-sensitive mechanisms was unknown. In 50 μM FFA there was a reduction in frequency in preparations exposed to 75 and 95% O2, suggesting that in relatively high levels of O2 FFA-sensitive mechanisms accelerate the rhythm. At lower O2 levels (0–50%), there was no difference in the frequency of FFA-treated preparations compared with untreated preparations, indicating that FFA-sensitive mechanisms do not contribute to rhythm at ≤50% O2. RIL and FFA have been successfully used to differentiate multiple network states under well-oxygenated conditions and hypoxia within the inspiratory network in vitro (Del Negro et al. 2005; Peña et al. 2004). We have extended these findings by showing that there are specific O2 thresholds for RIL- and FFA-sensitive mechanisms. RIL-sensitive mechanisms are essential for network bursting in low O2 (0 and 21%). In contrast, FFA-sensitive mechanisms accelerate the rhythm in 75 and 95% O2 but do not contribute to the rhythm in 0–50% O2. The waning of FFA-sensitive mechanisms at ≤50% O2 may explain why fictive respiratory activity ceases at 50% O2 in a subset of preparations in the presence of RIL. Additionally, the variability in the ability for RIL-treated preparations to continue bursting at 50% O2 may be related to small differences in the position of the preBötC in relation to the slice surface. In slices that ceased to burst, the preBötC may have been

### Table 2. Response to reduction in Fo2 (nonnormalized values)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fo2 = 0% (n = 9)</th>
<th>P Value</th>
<th>Fo2 = 21% (n = 10)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>0%</td>
<td></td>
<td>21%</td>
</tr>
<tr>
<td>Frequency, Hz</td>
<td>0.36 ± 0.26</td>
<td>0.12 ± 0.13*</td>
<td>0.0034</td>
<td>0.27 ± 0.12</td>
</tr>
<tr>
<td>Half-width, s</td>
<td>0.55 ± 0.15</td>
<td>0.34 ± 0.07*</td>
<td>0.0026</td>
<td>0.43 ± 0.14</td>
</tr>
<tr>
<td>Rise time, s</td>
<td>0.22 ± 0.04</td>
<td>0.16 ± 0.02*</td>
<td>0.0030</td>
<td>0.22 ± 0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Fo2 = 50% (n = 12)</th>
<th>P Value</th>
<th>Fo2 = 75% (n = 9)</th>
<th>P Value</th>
<th>All Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency, Hz</td>
<td>0.30 ± 0.12</td>
<td>0.18 ± 0.12*</td>
<td>0.0029</td>
<td>0.21 ± 0.09</td>
<td>0.16 ± 0.08*</td>
</tr>
<tr>
<td>Half-width, s</td>
<td>0.42 ± 0.07</td>
<td>0.35 ± 0.03*</td>
<td>0.0007</td>
<td>0.55 ± 0.23</td>
<td>0.57 ± 0.23</td>
</tr>
<tr>
<td>Rise time, s</td>
<td>0.18 ± 0.02</td>
<td>0.17 ± 0.03</td>
<td>0.3916</td>
<td>0.20 ± 0.08</td>
<td>0.19 ± 0.07</td>
</tr>
</tbody>
</table>

In all experiments control O2 = 95%, whereas Test O2 tension varied from 0 to 75%. The control data values are means ± SD in the 100-s bin just prior to the test. The test data are the data collected during a 100-s bin during the last third (200–300 s) of a 5-min exposure to reduced O2. Note that this comparison is different for the statistical comparisons presented elsewhere, which were between time-matched responses to “test” levels of O2 (0–75%) and the “control” level of O2 (95%). Control and test values that are significantly different based on a paired, two-tailed t-test are followed by an asterisk (*). The column “All Control” contains the average of all the mean control values for a particular metric.
closer to the slice core, resulting in a lower oxygenation state. Because the synaptic and intrinsic currents that are affected by RIL and FFA were not measured here, it is not possible to conclude that RIL and FFA acted solely to block \( I_{\text{NaP}} \) and \( I_{\text{CAN}} \). However, our findings are consistent with previous cellular electrophysiological studies in the preBöC showing that RIL and FFA block \( I_{\text{NaP}} \) and \( I_{\text{CAN}} \)-mediated bursting, respectively (Del Negro et al. 2005; Koizumi and Smith 2008; Pace et al. 2007; Peña et al. 2004).

**Central \( \text{O}_2 \) sensitivity and implications for \( \text{in vivo} \) hypoxic ventilatory response**

The biphasic frequency response to graded reductions in tissue oxygenation shown here, taken together with previous in vitro slice studies (Ramirez et al. 1997a, 1998; Telgkamp and Ramirez 1999), would suggest that the in vivo respiratory response to hypoxia may be mediated, in part, by the \( \text{O}_2 \) sensitivity of the preBöC. In the context of in vivo studies, there is a divergence in the data regarding the role of central \( \text{O}_2 \) sensing in the initial increase in frequency during the augmentation phase of hypoxic exposure. Many in vivo studies have suggested that peripheral sensory input is necessary for the expression of the augmentation phase of the hypoxic ventilatory response. In both neonatal and adult, carotid body denervation eliminates the augmentation phase (Bureau et al. 1985; Izumizaki et al. 2004; Wang et al. 1996). Furthermore, in vagotomized neonatal sheep frequency augmentation is diminished due to an inability to shorten expiration (Delacourt et al. 1995; for review see Bissonnette 2000). Conversely, however, in some studies frequency augmentation during initial hypoxic exposure has been observed in peripherally chemoreceptor-denervated animals (Miller and Tenney 1975; Moyer and Beecher 1942; Richter et al. 1991; reviewed in Neubauer et al. 1990). This discrepancy may be explained by the fact that denervation of the peripheral \( \text{O}_2 \) sensory system results in loss of the tonic input from the carotid bodies. Such loss in tonic input would be similar to the hypoxic silencing of peripheral chemosensitive input when an animal is breathing a hypoxic gas mixture. Curran et al. (2000) demonstrated that in the presence of intact carotid body input (maintained under a normoxic state) hypoxia specifically localized in the CNS stimulates breathing during nonrapid eye movement sleep. Thus it is possible that in most cases, the loss of tonic input, in peripherally denervated animals, acts as a signal that the animal is in a hypoxic state, overriding the central drive to increase frequency of ventilation during hypoxia. This phenomenon could explain the lack of early acceleration of frequency in response to hypoxia in some studies in which peripheral sensory input is removed through denervation. The observation that an acceleration of frequency does occur in other studies in which peripheral sensory input is removed could possibly be explained by differences in the degree of reduction in oxygenation. As pointed out by Bissonnette (2000) in vivo studies typically vary in the value of inspired fractional \( \text{O}_2 \) from 8 to 15%. Perhaps a larger reduction in oxygenation leads to acceleration of frequency during the early augmentation phase of the hypoxic response.

In contrast to the augmentation phase, the later frequency depression of rhythmogenesis during reduced oxygenation is in agreement with in vivo data indicating that ventilatory depression during hypoxia is of central origin. For example, in vivo the depressed ventilation during hypoxia is unaccompanied by a parallel reduction in carotid sinus nerve activity (Vizek et al. 1987). Furthermore, the removal of both peripheral chemosensitive input and pulmonary afferent input by nerve transaction does not eliminate hypoxic ventilatory depression (Melton et al. 1996; Richter et al. 1991).

During the recovery phase, the short cessation of fictive respiration observed in vitro is similar to the phenomenon of posthypoxic inhibition in vivo. On reoxygenation, in animals lacking peripheral chemoreceptor input the duration of transient posthypoxic inhibition of phrenic nerve discharge correlates positively with the severity of hypoxic insult (Melton et al. 1996). Similarly, here we found that TTFB positively correlated with the magnitude in the drop of \( \text{O}_2 \). However, our study does not support the necessity of pontine input for posthypoxic inhibition (Baekey et al. 2008).

**Conclusions**

Although in most cases the degree of oxygenation in vivo is fairly constant, there are periods such as apneas during sleep-disordered breathing where the demand of oxygen by neural networks can transiently outweigh the supply of \( \text{O}_2 \). In summary, the results of this study have expanded our understanding of the nature of \( \text{O}_2 \) sensitivity within the preBöC beyond the range of hypoxia and control \( \text{O}_2 \). We have demonstrated that graded reductions in \( \text{O}_2 \) lead to progressive changes in some metrics and abrupt changes in other metrics of respiratory rhythm generation. Moreover, rhythmic network activity was stably expressed at each level of \( \text{O}_2 \) tested, which suggests that multiple degenerate mechanisms (for review see Mellen 2010) with various \( \text{O}_2 \) sensitivities shape the assembly of the rhythm originating from the preBöC. Such degeneracy would be beneficial to network function by creating an avenue for robust function in multiple states of oxygenation while retaining the dynamic responsiveness to changes in \( \text{O}_2 \).

**Acknowledgments**

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**Disclosures**

No conflicts of interest, financial or otherwise, are declared by the authors.

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RESPONSE OF RESPIRATORY NETWORK TO REDUCED OXYGENATION


