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Graded reductions in oxygenation evoke graded reconfiguration of the isolated respiratory network

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Response of respiratory network to reduced oxygenation

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Abstract (191 words; max = 250 words)

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 preBötzinger complex (preBötC), a neuronal network critical to the central control of breathing. Rhythmogenesis was measured from the preBötC in transverse neonatal mouse brainstem slices which were exposed to graded reductions in O₂ between 0 and 95% O₂, producing tissue oxygenation values ranging from 20 ± 18 (mean ± S.E.M.) 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.

Keywords Oxygen, Hypoxia, PreBötzinger Complex
Introduction (843 words)

While it is generally accepted that blood oxygen (O\textsubscript{2}) 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 O\textsubscript{2} supply to the central nervous system (CNS) (for review of O\textsubscript{2} sensing in the CNS see Neubauer and Sunderram 2004). In the CNS, it is essential that the partial pressure of O\textsubscript{2} (PO\textsubscript{2}) remains tightly regulated over a fine range as hyperoxia can be as detrimental as hypoxia (D’Agostino et al. 2007; Haddad and Jiang 1993). Tissue PO\textsubscript{2} in the CNS is maintained in a narrow range between 10 and 34 Torr (corresponding to a range of 1 to 4% O\textsubscript{2}; Mulkey et al. 2001). Thus, neurons, which are very metabolically active, exist in a microenvironment with an O\textsubscript{2} tension only slightly higher than the threshold for aerobic metabolism (~1% O\textsubscript{2}; Clemens et al. 2001). The challenge of maintaining an optimal O\textsubscript{2} tension is made more difficult by heterogeneous metabolic O\textsubscript{2} demand throughout the CNS and dynamic network activity, which leads to rapid changes in local tissue oxygenation (Ndubuizu 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 O\textsubscript{2} 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 O\textsubscript{2} 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 preBötzinger complex (preBötC) and characterized its response to graded reductions in O\textsubscript{2}. The preBötC is a neuronal network involved in respiratory rhythmogenesis during both well-oxygenated and hypoxic conditions (Solomon et al. 2000; Peña...
Previous studies of the isolated brainstem 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 riluzole (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, inhibit the rhythm, but co-application of these substances eliminates rhythmicity (Peña et al. 2004; Del Negro et al. 2005). 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 (I_{NaP}), which is RIL-sensitive; and the Ca²⁺-activated non-specific cation current (I_{CAN}), which is FFA-sensitive (Peña et al. 2004; Del Negro et al. 2005; Koizumi and Smith 2008). In conjunction with a hypoxia-induced reduction in synaptic inhibition (Ramirez et al. 1997b; Lieske et al. 2000), 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 2007). It has been hypothesized that during hypoxia a reduction in I_{CAN}-dependent mechanisms renders the respiratory network dependent on I_{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 post-inspiratory to inspiratory activity, and a change in burst shape from an augmenting/bell shaped inspiratory burst to a decrementing burst shape (Ramirez et al. 1997b; Lieske et al. 2000). 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 O₂ 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 O₂ levels decrease? We tested the hypothesis that population activity of the preBötC is modulated in a progressive manner over a broad range of O₂ 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 O₂. 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 O₂ sensitivities.
Materials and 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 brainstem slice preparation. Experiments were performed using a transverse brainstem slice preparation of the medulla of mice (Fig. 1) (Smith et al. 1991; Ramirez et al. 1996). 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 to P11) were decapitated, and the brainstem and a short length of spinal cord were dissected in ice cold artificial cerebral spinal fluid (aCSF) containing the following (in mM): 118 NaCl, 25 NaHCO3, 1 NaH2PO4, 1 MgCl2×6H2O, 3 KCl, 30 D-glucose, 1.5 CaCl2. The pH of this aCSF was 7.4 when equilibrated with a gas mixture containing 5% CO2 at room temperature (~21 ºC). The brainstem and spinal cord were mounted with cyanoacrylate glue such that the brainstem was above the spinal cord and the dorsal side of the brainstem faced the sloping face of an agar block (23° from vertical). Serial sections (100 µm) were cut in the rostro-caudal direction using a vibrating blade microtome (Leica VT 1200). Neuroanatomical landmarks such as the compact division of the nucleus ambiguous and the rostral border of the inferior olive were used to identify the appropriate rostro-caudal level to cut the final a 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 ~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 ºC. 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), containing 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 ambiguous 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 (100Hz to 1kHz) 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 preBötC 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: 30s and 100s. 30s time bins were necessary to capture the
dynamics of rapidly changing phenomenon, whereas 100s time bins were appropriate for slowly changing
phenomenon 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 are means of normalized data ± the standard error of the mean (S.E.M.).

**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. Pre-mixed tanks of O₂, CO₂, and N₂ were used with various FO₂ values (0%, 21%, 50%, 75%,
and 95%). In all tanks FCO₂ 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 (FO₂
= 95%), and another saturated with a test gas (FO₂ = 0% to 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 paper we refer to the FO2 of gases used to saturate the aCSF as simply percent 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) employ the mean PO2 at the slice surface as determined empirically (Fig. 2; Table 1) rather than the corresponding FO2 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 by A-M Systems) was used to measure PO2 in the superfusion bath and in the slice. Flush-tip electrodes were made by cutting 30-50 μm diameter Teflon-insulated or Isonel-insulated platinum wire (A-M Systems). The electrode was polarized to a voltage of between -675 to -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, NaSO3, 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 was 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 (up to 400 μm below the lower slice surface). Contact with the upper surface of slice was identified visually using a stereoscope.
Upon 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 sec 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. While 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 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 (Kyence Inc., Tokyo, Japan) or Graphpad InStat (GraphPad Software Inc., 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 FO2 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 while 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 to corresponding measurements made in O2 \leq 75. Core PO2 in 95% O2 is different from core PO2 values in O2 \leq 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 \leq 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 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} (r^2 = 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} (r^2 = 0.99) while the calculated slope of the core PO2 to media relationship is 0.08 Torr FO2^{-1} (r^2 = 0.98).

*Rhythmogenesis responds to progressive reductions in oxygenation.* While 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, therefore, individual slices were only exposed to a single bout of reduced O₂. Exposure to reduced oxygenation levels from 0% to 75% for 300s from a baseline level of 95% produces a stereotypical biphasic frequency response: augmentation followed by depression (Fig. 3B-E). In addition to quantifying the frequency modulation 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 gasp-like 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 either fictive eupneic bursts or fictive gasps (Fig. 4A-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 if the increase in sigh frequency is dependent on the degree of reduction in O₂, we recorded the number of sighs that occurred during the 100s time bin immediately after the transition to reduced O₂. This time bin was chosen because almost all sighs occurred within this time span upon exposure to reduced O₂. We found that there were significantly more sighs in response to 0, 21, and 50% O₂ in comparison to control (95% O₂) (Fig. 4D).

To quantify change in burst frequency, we normalized and averaged data from different preparations. A biphasic response to a reduction in O₂ is evident in the change in mean frequency (Fig. 5A-C) while mean burst area decreased monotonically during exposure to reduced O₂ (Fig. 5D-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 O₂ (i.e. augmentation phase), the second corresponds to the final 100s 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. 5A, 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, burst area decreased when exposed to 50% O$_2$ and below (Fig 7B). However, burst amplitude only decreased in 0% O$_2$ (Fig 7C), which is similar to the response to 0% found during the augmentation phase. Similar to burst area, 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 to the others.

**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$_2$ compared to the control experiment (95% O$_2$); yet no difference in TTFB was observed between 75 and 95% O$_2$.

During the recovery phase, frequency augmentation occurred following exposure to 0 and 21% O$_2$ (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) while 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 $r^2$ values < 0.4 and slopes less than 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% O2 (Supplemental Fig. 1), but co-application of both agents eliminated rhythmogenesis (Fig. 10C, n=6). Furthermore, hypoxic exposure (0% O2) in the presence of RIL alone blocks rhythmogenesis (Fig. 10A, D, E) whereas 0% O2 in the presence of FFA alone does not prevent the rhythmic activity (Fig. 10B, F, G). No differences were identified for either normalized frequency or burst area during the augmentation phase in the presence of either RIL or FFA compared to untreated controls (data not shown). Note that while 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 µM for FFA and 10 versus 20 µM for RIL. The use of lower concentrations of both drugs was an attempt to limit the effects of these drugs to the blockade of the persistent Na$^+$ current ($I_{NaP}$) by RIL and the Ca$^{2+}$-activated non-specific cation current ($I_{CaN}$) 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 upon the results of Peña et al. (2004) we tested the effects of blocking RIL- and FFA-sensitive mechanisms under conditions of intermediate O2 levels (21% to 75%). During the depression phase,
reducing O₂ from 95 to 21% O₂ in the presence of RIL eliminates rhythmogenesis (Fig. 10D, E; n=4) but in
the presence of FFA neither frequency nor burst area were affected at this O₂ tension (Fig. 10F, G; n=5). In the
presence of RIL, reducing O₂ from 95 to 50% completely blocked rhythmic activity in 4 of 11 experiments
(Fig. 10D, E). However, in the remaining 7 preparations where the rhythm continued, reducing O₂ to 50% did
not significantly impact frequency (Fig. 10D) or burst area (Fig. 10E). In contrast, the reduction of O₂ to 50%
while exposed to FFA had no effect on the frequency or burst area (Fig. 10F, G). Thus, RIL-sensitive
mechanisms are important for basic rhythmogenesis at 50% O₂ or below, whereas FFA-sensitive mechanisms
appear to have no impact on rhythmogenesis at 50% or below.

Both RIL and FFA also had significant effects on the rhythm at 75% and 95% O₂. The burst area in RIL
exposed slices was lower in both 75 and 95% O₂ compared to untreated controls (Fig. 10E). This reduction in
burst area is to be expected if the RIL sensitive mechanisms contribute to rhythmogenesis at all O₂ tensions
tested (0 to 95%). Additionally, in 75% O₂ there was a significant decrease in frequency after exposure to RIL,
suggesting that RIL sensitive mechanisms may help to accelerate the frequency at this O₂ 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 to untreated controls in 75 and
95% O₂ (Fig. 10F), suggesting that FFA sensitive mechanisms may accelerate the rhythm in 75 and 95% O₂.
Both frequency and area in FFA treated preparations are unaffected from between 0 and 50% O₂. One
possible explanation for this phenomenon is that there is an O₂-dependent reduction in the expression of FFA-
sensitive mechanisms in this range of O₂ values. Thus, there is no difference between control preparations and
FFA-exposed preparations.
Discussion

The ability to respond to changes in arterial PO$_2$ 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 preB$\text{"o}t$C changes in waveform and frequency during hypoxia, suggesting that there is a reconfiguration of the underlying neural network (Peña et al. 2004; Lieske et al. 2000). The question remained, however, as to how sensitive the preB$\text{"o}t$C 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 preB$\text{"o}t$C as determined by measureable 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 preB$\text{"o}t$C. 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 PO$_2$. To better understand the cellular mechanisms that contribute to these transitions in network activity, the response of the network to graded reductions in tissue PO$_2$ was measured in the presence of RIL and FFA, which have been shown to block the persistent Na$^+$ current (I$_{\text{NaP}}$) and the Ca$^{2+}$-activated non-specific cation current (I$_{\text{CAN}}$) in preB$\text{"o}t$C neurons, respectively (Del Negro et al. 2005; Pace et al. 2007; Koizumi and Smith 2008). We found that RIL-sensitive mechanisms are crucial to the expression of a respiratory rhythm in low tissue PO$_2$ but also contribute to the burst area and frequency in high tissue PO$_2$. In contrast, FFA-sensitive mechanisms appear to contribute to the rhythm only at high tissue PO$_2$. The implications of these novel findings will be discussed in further detail below.
In vitro tissue oxygenation. While only a handful of brain slice studies have shown that neuronal activity is affected throughout a broad range of O₂ tensions outside of hypoxia (0% O₂) and the conventional control condition (95% O₂), these studies have shown that graded changes in O₂, and not only hypoxia, can influence biochemical activity (Fowler 1993; D’Agostino 2007) and excitability of neurons within a local networks (Fowler 1993; Hoffmann et al. 2006; Mulkey et al. 2001, Garcia et al. 2010). 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 O₂ 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% O₂. 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. For this reason, it was critical to measure O₂-depth profiles in order to determine whether unique tissue oxygenation states could be achieved by varying the O₂ level of the aCSF. However, the results described below should not be misconstrued as an attempt to define an "optimal" O₂ level that yields a range of physiologically realistic tissue PO₂ values within the brain slice preparation.

Neural tissue O₂-depth profiles have been previously reported in vivo (for summary see Garcia et al. 2010), in situ in the working heart brainstem preparation (Wilson et al. 2001), as well as in vitro in both isolated brainstem spinal cord preparations (Brockhaus et al. 1993; Okada et al. 1993) and brain slices (Bingmann and Kolde 1982; Mulkey et al. 2001; Garcia et al. 2010). Several factors influence the degree of in vitro tissue oxygenation within a brain slice. Some, like the fractional O₂ value (FO₂) 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, therefore, 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 versus
double sided), and the ambient barometric pressure (Mulkey et al. 2001, Dean et al. 2003, Jiang et al. 1991;
Fong et al. 2008). Moreover, comparisons of our profiles to those made in adult medullary brainstem 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 \textit{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
PO₂ 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 PO₂ in the superfusion bath was different when the reservoir was aerated with
21% compared to 0% O₂, neither slice surface nor core PO₂ 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 400µm 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 \textit{in vitro}. For example, differences in slice thickness (600µm thick preBötC slices compared to
400µm thick hippocampal slices) and possibly metabolic activity of the region of interest may markedly affect
O₂ profiles within the slice. Despite the similarity of PO₂ slice values at 0 and 21% O₂ found in here, other O₂
values produced unique O₂-depth profiles, albeit with some convergence of core PO₂ values. The observation
that unique states of tissue oxygenation could be achieved by varying the media FO₂ 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, upon 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 only influenced by low levels of O₂ (0 to 21%, and 0%, respectively) during augmentation. Burst area which combines elements of burst amplitude and half-width showed a relatively broad sensitivity to O₂ (0 to 50%), most likely due to changes in half-width rather than amplitude. The significant decrease in amplitude in 0
compared to 21% O\textsubscript{2} is somewhat surprising as no significant difference in tissue oxygenation was found between these two O\textsubscript{2} levels either at the slice surface or at the core. During the depression phase, frequency was sensitive to a broad range of O\textsubscript{2} levels from 0 to 75%. Burst area and half-width continued to be O\textsubscript{2} sensitive over the same broad range as during the augmentation phase (0 to 50%). Close examination of these three metrics (frequency, burst area, and half-width) reveals that frequency varies continuously with PO\textsubscript{2}, whereas burst area or half-width appear to plateau between 75 and 95% O\textsubscript{2} (Fig. 7A, B, D). Media PO\textsubscript{2} was at steady state during the depression phase (Fig. 3A), and, therefore, the tissue PO\textsubscript{2} 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 PO\textsubscript{2}. Using linear regression analysis, we found that changes in frequency and burst area had relatively large \(r^2\) values (0.51 and 0.57, respectively), suggesting that the relationships of these two metrics to tissue PO\textsubscript{2} 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 PO\textsubscript{2}. Half-width also has a relatively high \(r^2\) value, suggesting that it may also change in a linear manner with tissue PO\textsubscript{2}. 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 O\textsubscript{2}. These metrics do not change significantly except in when the level of O\textsubscript{2} is 0% for amplitude and 0 to 21% for rise time. Similar restricted sensitivities to O\textsubscript{2} were observed during both the depression phase and the augmentation phase. During the depression phase, the \(r^2\) 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 PO\textsubscript{2}, whereas other metrics express sensitivity to O\textsubscript{2} only when O\textsubscript{2} deprivation is severe.
It has been previously reported that frequency augmentation occurs following a 600s hypoxic exposure (Ramirez et al. 1997a, 1998; Telgkamp and Ramirez 1999). Extending these observations, we demonstrated that 300s exposure to insufficient oxygenation (*i.e.* 0% and 21% O2) can cause frequency, burst area, and amplitude augmentation. Interestingly, while 21% O2 led to a significant augmentation of half-width during the recovery phase, exposure to 0% O2 did not. Although tissue PO2 between 0 and 21% O2 were not different, we found significant differences found in media PO2. Hence, 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 preBötzC appears to involve changes in the relative contribution of different rhythm-generating mechanisms to the population respiratory rhythm (Peña et al. 2004; Ramirez and Garcia 2007; Peña 2008). Specifically, both riluzole-sensitive (RIL-sensitive) and flufenamic acid-sensitive (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_NaP but it may also affect on 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, while FFA blocks I_CAN (Pace et al. 2007), it may also: (1) inhibit Ca²⁺ currents (Wang et al. 2006), (2) affect a variety of K⁺ currents (Kochetkov et al. 2000; Takahira et al. 2005), and (3) block gap junctions (Harks et al. 2001; Srinivas and Spray 2003).

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 while 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 approximately 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 co-application 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 versus 500 µM) and RIL (10 versus 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 to 50%), there was no difference in the frequency of FFA treated preparations compared to untreated preparations, indicating that FFA-sensitive mechanisms do not contribute to rhythm at 50% O₂ and below. The use of RIL and FFA has been successfully employed to differentiate multiple network states under well-
oxygenated conditions and hypoxia within the inspiratory network *in vitro* (Peña et al. 2004; Del Negro et al. 2005). We have extended these findings by showing that there are specific O$_2$ thresholds for RIL- and FFA-sensitive mechanisms. RIL-sensitive mechanisms are essential for network bursting in low O$_2$ (0, 21%). In contrast, FFA-sensitive mechanisms accelerate the rhythm in 75 and 95% O$_2$ but do not contribute to the rhythm in 0 to 50% O$_2$. The waning of FFA-sensitive mechanisms at 50% O$_2$ and below may explain why fictive respiratory activity ceases at 50% O$_2$ 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% O$_2$ 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 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 preBötC showing that RIL and FFA block $I_{\text{NaP}}$ and $I_{\text{CAN}}$ mediated bursting, respectively (Peña et al 2004; Del Negro et al. 2005; Pace et al. 2007; Koizumi and Smith 2008).

Central O$_2$ sensitivity and implications for *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), suggests that the *in vivo* respiratory response to hypoxia may be in part mediated by the O$_2$ sensitivity of the preBötC. In the context of *in vivo* studies, there is a divergence in the data regarding the role of central 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 neonate and adult, carotid body denervation eliminates the augmentation phase (Bureau et al. 1985; Wang et al. 1996; Izumizaki et al. 2004). 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 (Moyer and Beecher 1942; Miller and Tenney 1975; Richter et al. 1991; reviewed in Neubauer et al. 1990). This discrepancy may be explained by the fact that denervation of the peripheral O₂ sensory system results in loss of the tonic input from the carotid bodies. Such loss in tonic input would be similar to the hyperoxic silencing of peripheral chemosensitive input when an animal is breathing a hyperoxic 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 central nervous system stimulates breathing during non-rapid eye movement sleep. Hence, 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 hyperoxic 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 O₂ 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 peripheral chemosensitive input as well as pulmonary afferent input by nerve transaction does not eliminate hypoxic
ventilatory depression (Richter et al. 1991; Melton et al. 1996).

During the recovery phase, the short cessation of fictive respiration observed in vitro is similar to the phenomenon of post-hypoxic inhibition in vivo. Upon reoxygenation, in animals lacking peripheral chemoreceptor input the duration of transient post-hypoxic 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 O2. However, our study does not support the necessity of pontine input for post-hypoxic inhibition (Baekey et al. 2008).

Conclusions. While 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 supply of O2. In summary, the results of this study has expanded our understanding of the nature of O2 sensitivity within the preBötC beyond the range of hypoxia and control O2. We have demonstrated that graded reductions in O2 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 O2 tested which suggests that multiple degenerate mechanisms (for review see Mellen 2010) with various O2 sensitivities shape the assembly of the rhythm originating from the preBötC. 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 O2.

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Disclosures
none
References


Figure Legends

Fig. 1 Rhythmic bursts of activity were recorded extracellularly from the surface of a slice in an area of Ventral Respiratory Group (VRG) called the preBötzinger Complex (preBötC). The upper trace is a raw extracellular recording. The lower trace is a rectified-and-integrated version of the same voltage signal. The light grey line was integrated with analog circuitry with a time constant of 50 ms. The superimposed black line is a digitally filtered version of the same signal created with a 2nd order Butterworth low pass filter with a cut off of 1.5 Hz. This twice-filtered signal was used for subsequent data analysis.

Fig. 2 Reductions in the media O₂ resulted in corresponding reductions in tissue oxygenation. A:

Average O₂-depth profiles showing differences in tissue oxygenation based on superfusing a brain slice with aCSF bubbled with different levels of O₂ (red = FO₂ 95%, orange = FO₂ 75%, yellow = FO₂ 50%, light blue = FO₂ 21%, dark blue = FO₂ 0%). Inset shows mean tissue PO₂ values at the core of the slice (300 μM) with an expanded y-axis. Note that the mean values in 0 and 21% FO₂ are similar. Depth was measured relative to the top surface of the brain slice. Although slices were cut to a nominal thickness of 600 μm, the average measured slice thickness was 575 ± 10 μm (n=7). Beneath the bottom of the slice the oxygen tension rose to values similar to those found above the slice, indicating that the flow rate of media below the slice was similar to that above the slice (data not shown). B: Plot of media PO₂ (400 μm above the tissue) as a function of FO₂ in the equilibrating gas mixture. Although theoretically media saturated with 0% O₂ should have a PO₂ of 0 Torr, the actual ambient PO₂ was 38 Torr in the media above the slice, likely due to incomplete saturation of the aCSF in the reservoir that feeds the superfusion bath as well as gas diffusion at the media-ambient atmosphere interface in the superfusion bath. C: Plots of tissue PO₂ at the upper surface of the slice (depth = 0 μm) and at the core of the slice (depth = 300 μm) as a function of media PO₂.
The response of the \textit{in vitro} respiratory rhythm is dependent on the degree of reduction in oxygen. Representative preparations were each subjected to a single reduction in O$_2$. \textit{A}: PO$_2$ values measured within the superfusion chamber resulting from a switch between a control reservoir saturated with 95\% O$_2$ and a second reservoir saturated with 0\% to 75\% O$_2$. During the 300s test period there was a rapid decrease in the PO$_2$ within the superfusion chamber, which eventually stabilized at a new O$_2$ level. \textit{B-E}, Responses of the \textit{in vitro} respiratory rhythm to 5 min long reductions in O$_2$. The dots placed above certain bursts denote sigh-like bursts, which were distinguished by their large amplitude compared to normal respiratory bursts (Lieske et al. 2000). \textit{F}: An averaged burst waveform calculated from the bursts within a 100s time bin in 95\% O$_2$. 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.

\textbf{Fig. 4} Fictive sighs increase in number during the transition from high O$_2$ to low O$_2$. \textit{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 100s of exposure to 0\% O$_2$. As in figure 3, dots were placed above sigh bursts. The arrows marked B and C denote windows of time that are
expanded in panels B and C, respectively. B: Fictive sighs were identified based on amplitude, shape, and
by the presence of a longer burst interval immediately after a sigh compared to immediately prior (Lieske et al.
2000). The burst area of sighs was on average 136.6 ± 50.3% greater than that of eupneic bursts (n=6; paired
t-test, P=0.006). 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 1st 100s of the
transition to reduced O2 revealed that there was a significantly higher number of sighs in response to 0%, 21%,
and 50% compared to the control experiment in which O2 was maintained at 95%. After an initial increase in
the number of sighs during the 1st 100s, sighs were very rare during the 2nd 100s and no sighs were observed
in the 3rd 100s of a 5 min exposure except in the control experiment in which the level of O2 was maintained at
95%. Note that empirically derived mean slice surface PO2 values (20, 73, 177, 341, 440 Torr; see Table 1) are
reported on the x-axis rather than the corresponding media FO2 values (0, 21, 50, 75, 95%). Error bars
represent standard deviation.

Fig. 5 Changes in burst frequency and burst area of the in vitro respiratory rhythm in response to
graded changes in O2. A: Changes in mean normalized burst frequency in response to 3 different test O2
values: 0% (n=9), 21% (n=10) and 95% (n=11). In response to 0 and 21% O2, the frequency increased during
the augmentation phase and decreased during the depression phase. During the recovery phase the frequency
overshot the baseline frequency prior to the reduction in O2. B: In response to 50% O2 (n=12) the frequency
increased during the augmentation phase and decreased during the depression phase; however, unlike the
responses to 0 and 21% O2, it did not overshoot during the recovery phase. C: In response to 75% O2 (n=9)
the frequency declined during the depression phase. D: Unlike the biphasic response of frequency, the mean
normalized burst area decreased monotonically during exposure to 0 and 21% O$_2$. During reoxygenation, burst area overshot the baseline pre-hypoxia value. $E$: In response to 50% O$_2$, burst area decreased. Upon reoxygenation, there was a weak overshoot, followed by a return to the pre-hypoxia baseline value. $F$: In response to 75% O$_2$, there was little difference in the burst area compared to the control experiment (O$_2$ = 95%).

**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 to 95% O$_2$. $B$: Burst area was significantly lower in 0% (P<0.001), 21% (P<0.05), and 50% (P<0.05) compared to 95% O$_2$. $C$: Burst amplitude was significantly lower in 0% compared to 95% O$_2$. $D$: Half-width was significantly lower in 0% (P<0.01), 21% (P<0.01), 50% (P<0.01) compared to 95% O$_2$. $E$: Rise time was significantly lower in 0% (P<0.01) and 21% (P<0.05) compared to 95% O$_2$. * denotes significance in $A$-$E$.

**Fig. 7 Changes in metrics in response to graded reductions in O$_2$ tension during the depression phase.**

$A$: Frequency was significantly lower at 0% (P<0.001), 21% (P<0.001), 50% (P<0.001), and 75% (P<0.05) compared to 95% O$_2$. $B$: Burst area was significantly lower in 0% (P<0.001), 21% (P<0.01), and 50% (P<0.01) compared to 95% O$_2$. $C$: Burst amplitude was significantly lower in 0% (P<0.01) compared to 95% O$_2$. $D$: Half-width was significantly lower in 0% (P<0.001), 21% (P<0.001), and 50% (P<0.01) compared to 95% O$_2$. $E$: Rise time was significantly lower in 0% (P<0.01) and 21% (P<0.01) compared to 95%. * denotes significance in $A$-$E$. $F$: Differences in both the absolute values of the slopes of linear regressions and $r^2$ values of the metrics indicate that they are influenced differentially by O$_2$. 

* denotes significance in $A$-$E$.
Fig. 8 To quantify the ability of the in vitro respiratory network to recover from hypoxia the time to first burst (TTFB) was measured. A: TTFB was measured from the onset of reoxygenation to the first population burst. B: Significant differences in TTFB were found at 0% (P<0.01), 21% (P<0.01), and 50% (P<0.01) compared to 95% O₂. * denotes significance. Error bars represent standard deviation.

Fig. 9 Changes in metrics in response to graded reductions in O₂ tension during the recovery phase. A: Frequency was significantly higher after exposure to 0% (P<0.01) and 21% (P<0.01) compared to the control experiment (i.e. 95% O₂). B: Burst area was significantly greater after exposure to 0% (P<0.01) and 21% (P<0.01) compared to 95% O₂. C: Burst amplitude was significantly greater after 0% (P<0.001) and 21% (P<0.01) compared to 95% O₂. D: Half-width was significantly greater after 21% (P<0.05) compared to 95% O₂. E: Rise time did not change significantly compared to 95%. F: Differences in both the absolute values of the slopes of linear regressions and r² values of the metrics indicate that they are influenced differentially by O₂.

Fig. 10 The response of the respiratory network to graded changes in O₂ is different under conditions of blockade of the persistent Na⁺ current (I_{NaP}) and the blockade of the Ca²⁺-activated non-specific cation current (I_{CAN}). A: Representative experiment showing that bath application of 10 µM riluzole (RIL) followed by a reduction of the media O₂ to 0% results in a cessation of bursting (n=4). Upon reoxygenation the rhythm recovered (data not shown). B: Representative experiment showing that bath application of 50 µM flufenamic acid (FFA) does not lead to a cessation of bursting in 0% O₂ (n=6). Upon reoxygenation the rhythm recovered its original amplitude and frequency (data not shown). C: Representative experiment showing that bath application of FFA followed by RIL in 95% O₂ leads to a cessation of bursting (n=6). Note
that O₂ was maintained at 95% throughout the experiment. Unpaired \( t \)-tests were conducted comparing

either frequency (\( D \)) or burst area (\( E \)) in RIL to the respective metrics of untreated rhythms at the same O₂ level. \( D \): In RIL treated preparations cessation of the rhythm occurred in 0% (\( n=4 \)), 21% (\( n=5 \)), and 50% (\( n=4/11 \)) while at 75% O₂ (\( n=6 \)) RIL significantly reduced frequency. RIL did not affect frequency at 95% O₂ (\( n=8 \)). In the 7 RIL experiments where the rhythm continued to burst at 50% O₂, there were no significant differences in frequency between this group and the untreated group. Frequency during the last 100s of a 5 min exposure to reduced O₂ was normalized to the mean value in the 100s time bin immediately preceding the test exposure. \( E \): In RIL treated preparations cessation of the rhythm occurred in 0% (\( n=4 \)), 21% (\( n=5 \)), 50% (\( n=4/11 \)) leading to significant differences in burst area. At 75% (\( n=6 \)) and 95% (\( n=8 \)) O₂ RIL significantly reduced burst area. In the 7 RIL experiments where the rhythm continued to burst at 50% O₂ there were no significant differences between this group and the untreated group. Burst area during the last 100s of a 5 min exposure to reduced O₂ was normalized to the mean value in the 100s time bin immediately preceding the test exposure. \( F \): Frequency in preparations treated with FFA was significantly reduced at 75% (\( n=8 \)) and 95% (\( n=11 \)) O₂. FFA did not affect changes in frequency when exposed to 50% (\( n=8 \)), 21% (\( n=5 \)), and 0% (\( n=5 \)) O₂. \( G \): FFA did not affect changes in burst area at 95% (\( n=11 \)), 75% (\( n=8 \)), 50% (\( n=8 \)), 21% (\( n=5 \)), or 0% (\( n=5 \)) O₂. Unpaired \( t \)-tests were conducted comparing either frequency (\( F \)) or burst area (\( G \)) in FFA to the respective metrics of untreated rhythms at the same O₂. n.s. = not significant; \(*=P<0.05; **=P<0.01; ***=P<0.001. \)
A. Relative Distance from upper surface (µm)

B. Media

C. Core (300 µm)

Upper Surface (0 µm)

Media PO₂ (Torr)

FO₂ (%)
Hill et al. Fig 3
Hill et al. Fig 4

A

95% O₂

300 s

0% O₂

B

fictive eupneic burst

C

fictive gasp

D

Number of sighs

Slice surface PO₂ (Torr)

P<0.01  P<0.01  P<0.01

0 100 200 300 400 500

3.5

3.0

2.5

2.0

1.5

1.0

0.5

0.0
Hill et al. Fig 10

A. 95% O₂
Riluzole (10μM)
0% O₂

B. 95% O₂
FFA (50μM)
0% O₂

C. 95% O₂
FFA (50μM)
Riluzole (10μM)

D. Frequency (% of Control at 95% O₂)
Surface PO₂ (Torr)

E. Burst Area (% of Control at 95% O₂)
Surface PO₂ (Torr)

F. Frequency (% of Control at 95% O₂)
Surface PO₂ (Torr)

G. Burst Area (% of Control at 95% O₂)
Surface PO₂ (Torr)
Table 1 Oxygen-depth profile measurements

Oxygen-depth profile measurements made in media (circulating aCSF) as well as at the upper
surface and core of 600µm thick rhythmic medullary brainstem slices containing the preBötC.

<table>
<thead>
<tr>
<th></th>
<th>95%</th>
<th>75%</th>
<th>50%</th>
<th>21%</th>
<th>0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>6</td>
<td>11</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Media PO₂ (^i)</td>
<td>679 ± 30(^{A})</td>
<td>532 ± 18(^{B})</td>
<td>347 ± 48(^{C})</td>
<td>154 ± 21(^{D})</td>
<td>38 ± 28</td>
</tr>
<tr>
<td>0 µm (surface)</td>
<td>440 ± 56(^{A})</td>
<td>341 ± 51(^{B})</td>
<td>177 ± 46(^{C})</td>
<td>73 ± 8</td>
<td>20 ± 18</td>
</tr>
<tr>
<td>300µm (core)</td>
<td>58 ±16(^{E,F})</td>
<td>43 ± 11(^{G})</td>
<td>34 ± 16(^{H})</td>
<td>9 ± 6</td>
<td>5 ± 6</td>
</tr>
</tbody>
</table>

\(^i\)Measurements taken 400 to 500µm above the upper surface of the slice.  \(^A\)Significantly
different (P ≤ 0.001) from PO₂ values measured at the same distance relative to the slice
surface in aCSF equilibrated at 75, 50, 21 and 0% O₂;  \(^B\)Significantly different (P≤0.001)
from PO₂ values measured at the same relative distance in 50, 21 and 0% O₂;  \(^C\)Significantly
different (P≤0.001) from PO₂ values measured at the same relative distance in 21 and 0% O₂;
\(^D\)Significantly different (P≤0.001) from PO₂ values measured at the same relative distance in
0% O₂;  \(^E\)Significantly different (P≤0.05) from PO₂ values measured at the same relative
distance in 50% O₂;  \(^F\)Significantly different (P≤0.001) from PO₂ values measured at the same
relative distance in 21 and 0% O₂;  \(^G\)Significantly different (P≤0.01) from the PO₂ values
measured the same relative distance in 21 and 0% O₂;  \(^H\)Significantly different (P≤0.05) from
the PO₂ values measured the same relative distance in 21 and 0% O₂.
Table 2: Response to reduction in FO\textsubscript{2} (non-normalized values)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FO\textsubscript{2} = 0% (n = 9)</th>
<th>FO\textsubscript{2} = 21% (n = 10)</th>
<th>FO\textsubscript{2} = 50% (n = 12)</th>
<th>FO\textsubscript{2} = 75% (n = 9)</th>
<th>All control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency (Hz)</td>
<td>control: 0.36±0.26</td>
<td>P Value: 0.12±0.15*</td>
<td>control: 0.14±0.09*</td>
<td>control: 0.001</td>
<td>control: 0.21±0.08*</td>
</tr>
<tr>
<td>Half-width (s)</td>
<td>control: 0.55±0.15</td>
<td>P Value: 0.34±0.07*</td>
<td>control: 0.31±0.09*</td>
<td>control: 0.0007</td>
<td>control: 0.55±0.23</td>
</tr>
<tr>
<td>Rise time (s)</td>
<td>control: 0.22±0.05</td>
<td>P Value: 0.16±0.02*</td>
<td>control: 0.17±0.03</td>
<td>control: 0.3916</td>
<td>control: 0.20±0.08</td>
</tr>
</tbody>
</table>

In all experiments control O\textsubscript{2} = 95% whereas Test O\textsubscript{2} tension varied from 0% to 75%. The control data is mean ± std in the 100s bin just prior to the test. The test data are the data collected during a 100 s bin during the last third (200-300s) of a 5 min exposure to reduced O\textsubscript{2}. Note that this comparison is different for the statistical comparisons presented elsewhere, which were between time matched responses to ‘test’ levels of O\textsubscript{2} (0-75%) and the ‘control’ level of O\textsubscript{2} (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.