Functional Imaging Reveals Respiratory Network Activity During Hypoxic and Opioid Challenge in the Neonate Rat Tilted Sagittal Slab Preparation

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Submitted 3 October 2006; accepted in final form 19 December 2006

Barnes BJ, Tuong C-M, Mellen NM. Functional imaging reveals respiratory network activity during hypoxic and opioid challenge in the neonate rat tilted sagittal slab preparation. J Neurophysiol 97: 2283–2292, 2007. First published January 10, 2007; doi:10.1152/jn.01056.2006. In mammals, respiration-modulated networks are distributed rostrocaudally in the ventrolateral quadrant of the medulla. Recent studies have established that in neonate rodents, two spatially separate networks along this column—the parafacial respiratory group (pFRG) and the pre-Bötzinger complex (preBoC)—are hypothesized to be sufficient for respiratory rhythm generation, but little is known about the connectivity within or between these networks. To be able to observe how these networks interact, we have developed a neonate rat medullary tilted sagittal slab, which exposes one column of respiration-modulated neurons on its surface, permitting functional imaging with cellular resolution. Here we examined how respiratory networks responded to hypoxic challenge and opioid-induced depression. At the systems level, the sagittal slab was congruent with more intact preparations: hypoxic challenge led to a significant increase in respiratory period and inspiratory burst amplitude, consistent with gasping. At opioid concentrations sufficient to slow respiration, we observed periods at integer multiples of control, matching quantal slowing. Consistent with single-unit recordings in more intact preparations, respiratory networks were distributed bimodally along the rostrocaudal axis, with respiratory neurons concentrated at the caudal pole of the facial nucleus, and 350 microns caudally, at the level of the pFRG and the preBoC, respectively. Within these regions neurons active during hypoxia- and/or opioid-induced depression were ubiquitous and interdigitated. In particular, contrary to earlier reports, opiate-insensitive neurons were found at the level of the preBoC.

INTRODUCTION

In mammals, breathing is a continuous rhythmic behavior that constantly adapts itself to maintain blood-gas homeostasis (Khoo 2000). Because essential circuits for respiratory rhythm generation are localized in ventrolateral medulla, this behavior provides a platform for elucidating how cellular and network properties interact to generate a mammalian adaptive behavior. In a landmark series of experiments (Smith et al. 1991), networks capable of generating respiratory-related rhythm were isolated from rodent rostral medulla in a 300-μm slice. In this preparation, a circumscribed region in the ventrolateral quadrant, the pre-Bötzinger complex (preBoC), was identified as necessary and sufficient for respiratory rhythm generation in the slice. The preBoC was found to have a high concentration of propriobulbar respiratory neurons (Dobbins and Feldman 1994), a subset of which had endogenous bursting properties (Johnson et al. 1994). Because an earlier study established that in vitro respiratory rhythm persisted after blockade of Cl−-mediated inhibition, an endogenous burster-driven mechanism for respiratory rhythm generation was proposed (Feldman and Smith 1989) and led to the development of a generic hybrid-pacemaker model (Butera et al. 1999a,b; Smith et al. 1993), with a number of testable predictions, many of which were verified experimentally (Del Negro et al. 2001; Johnson et al. 2001).

Over time, experimental results accumulated that were not captured by this model: biophysically distinct endogenous bursters were identified within the preBoC (Pena et al. 2004; Thoby-Brisson and Ramirez 2001); respiratory neurons in the parafacial respiratory group (pFRG), well rostral to the preBoC at the level of the facial nucleus, were shown to be sufficient for respiratory rhythm generation (Mellen et al. 2003; Onimaru and Homma 2003); and network properties alone were shown to be sufficient for respiratory rhythm generation in the transverse slice (Del Negro et al. 2002). These accumulated findings suggest that respiratory rhythm arises out of the interaction of multiple, mechanistically distinct elements, each capable of qualitatively producing the same behavior.

Here we present a new tilted sagittal slab preparation, isolated from the brain stem spinal cord of the newborn rat. In this preparation, rhythmically active networks, phase-locked to respiratory motor output, are recorded optically at the level of the pFRG and the preBoC. We hypothesized that this preparation is functionally equivalent to the en bloc brain stem–spinal cord preparation. To test this hypothesis, we used opiates, which at subapneic concentrations produce respiratory periods at integer multiples of control in the en bloc preparation (quantal slowing), but give rise to continuously distributed periods in the transverse slice (Mellen et al. 2003). The observation of quantal slowing and/or the persistence of network activity at control frequency would support the hypothesis that our preparation retains two rhythmogenic networks. In addition, we exposed our preparation to hypoxic challenge, to observe how our preparation’s response compares to other in vivo and in vitro preparations. Because of the unparalleled access to both respiratory networks at the cut surface of our slab preparation, in addition to benchmarking our preparation based on system-level responses to opioids and hypoxia, we describe the overlapping distribution of respiratory neurons robust to these challenges. These findings were previously presented in abstract form (Barnes and Mellen 2005).
METHODS

Dissection

In accordance with methods approved by the Institutional Animal Care and Use Committee, we anesthetized neonatal rats (P1–P3; n = 13) with isoflurane and quickly isolated the brain stem and spinal cord (Feldman et al. 1990) under chilled artificial cerebrospinal fluid (aCSF) containing (in mM): 128.0 NaCl, 3.0 KCl, 1.5 CaCl2, 1.0 MgSO4, 21.0 NaHCO3, 0.5 NaH2PO4, and 30.0 glucose, equilibrated with 95%O2-5%CO2. After isolation of the neuraxis, a reticle was used to measure the width of the brain stem and this width was used to determine the mediolateral level of section. To cut the tilted sagittal slab, we used a compound angle chuck consisting of two plates mounted at right angles to each other tilted at 3.5° and 11°, which set the rostrocaudal and ventrodorsal tilt, respectively (Fig. IAii). The chuck was mounted on a vibrating microtome (Vibratome Plus, Vibratome, St. Louis, MO). The brain stem–spinal cord was pinned out on its flat dorsal surface along the midline, with care taken to ensure that the midline was parallel with the blade, ensuring that the angles of the tilting chuck were appropriately cut. After the brain stem was mounted on the chuck and immersed in aerated chilled aCSF, it was gradually raised until the Vibratome blade just touched the lateral edge of the brain stem. Using this position as the origin, the preparation was raised further to cut the lateral edge of the brain stem–spinal cord was mounted on the chuck and immersed in aerated chilled aCSF, it was mounted on a digital translation stage (MT-2000, Sutter Instruments). The sagittal slab was stabilized on a Sylgard (Dow-Corning) block, incubated for 2 h in an aerated solution containing the high-affinity cell-permeant Ca2+/Ca2+ indicator fluo-4 AM (50 μg, TEFLabs), 25 μL of the surfactant pluronic F-127, and 750 μL aCSF for a final concentration of 60 μM fluo-4, and then transferred to the recording chamber (JG 23 W/HP, Warner Instruments, Hamden CT), which was mounted on an upright microscope (Axioskop 2, Zeiss instruments) that in turn was mounted on a digital translation stage (MT-2000, Sutter Instruments).

Data acquisition and signal processing

Motor output was recorded by a suction electrode placed on one of ventral roots C1–C4, and optical signals, elicited using a collimated cyan-emitting LED (peak luminance = 500 nm, luxeon LXHL-NE98, Future Electronics) were recorded using a CCD camera (Orca ER, Hamamatsu, Bridgewater, NJ). Motor output was digitized at 1 kHz and written to disk using an A/D board (PCI-MIO-16XE-10, National Instruments, Austin, TX). Images were high-pass filtered, during the inspiratory burst, both rostral and caudal networks are brighter than background. This figure was generated by averaging images of 3 high-pass filtered images obtained during peak inspiratory activity. ΔF/F scale bar applies to the raw trace only because high-pass filtering sets F = 0. See Supplemental materials for movies of both raw and high-pass filtered versions of these data.
were obtained at 2–4 Hz using a frame grabber (IMAQ PCI-1422, National Instruments) and written to hard drive as a directory of tag image files (TIFs). To eliminate the possibility for variability in frame-to-frame delays, all data were buffered in RAM, and only at the end of acquisition written to disk. Image time stamps were set to the midpoint of the acquisition interval.

The voltage signal was rectified and integrated (τ = 20 ms). The optical signal was high-pass filtered in the time domain by subtracting older images from the current image (1.5-s delay). Regions of interest (ROIs), hewing closely to the soma perimeter, were detected algorithmically and checked manually using custom software (LabVIEW, National Instruments). The stack of high-pass filtered images was then analyzed and mean luminance for each ROI in each frame was obtained. To further eliminate slow fluctuations in baseline, the whole-frame mean intensity was subtracted from each ROI. The fluctuating luminance associated with each ROI was then plotted as a function of time. As a result of high-pass filtering, the resulting traces distorted the slowly decretering ROIs. Because the canvas on which ROIs were plotted matched the surface discernible in the slab, we were able to identify ROI locations such as flou-4 do not reflect [Ca^{2+}]_i, but rather the high K_D of the indicator.

Experimental procedure

Once a viable ventral root signal was established, optical recording using a ×10 water-immersion lens was begun. Typically, Ca^{2+} signals from both the pFRG and preBoTc were both visible at this magnification (Fig. 1C) and recorded at 2–3 Hz. After these regions were localized, a ×20 water-immersion objective was used to record control network activity from each of these regions at 2–4 Hz for 120 s. Each recording location’s coordinates were used for subsequent optical measurements.

Hypoxic challenge was applied by replacing the 95% O_2-5% CO_2 gas mixture with 95% N_2-5% CO_2. Motor output before and during the transition to gasping was recorded. Once the preparation had stabilized, optical recording of network activity accompanying gasping was recorded for 120 s. Normoxic aCSF was then restored and, thereafter to eupneic rhythm, network activity was again optically recorded. In three additional experiments, we recorded exclusively during the transition from normoxia to hypoxia. These recordings lasted 200 s.

Opiate-induced inhibition was obtained by bath-application of the selective µ-opioid receptor agonist [d-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO, Sigma–Aldrich), at concentrations sufficient to slow (75–100 nM) or stop (300–450 nM) respiratory motor output. Motor output before and during drug washin was recorded. After 10 min at steady state, 120 s of optical activity from preBoTc and pFRG was sampled. We then applied the µ-opioid receptor antagonist naloxone (10 µM, Sigma–Aldrich) to restore respiratory rhythm and, after recovery, recorded 120 s of optical activity. The order in which DAMGO or hypoxia was presented was randomized, to control for long-term effects of either protocol.

Data analysis

Optical signals provide information about the spatial distribution of a network and about the activity of network constituents as a function of time. Within each ×20 field of view, we calculated ROI area (based on a pixel size of 0.65 μm^2) as a surrogate for soma size (Fig. 1A) and used the center of mass of each ROI to calculate distances between ROIs. Because the canvas on which ROIs were plotted matched the ×20 field of view in size, we were able to identify ROI locations relative to the boundary of the facial nucleus (VII n) and the ventral surface discernible in the ×10 image obtained at the start of each experiment. By aligning raw ×20 images with appropriately scaled ×10 images and aligning ×10 images from each experiment using the caudal pole of VII n and the ventral surface of the preparation as landmarks, we generated dot diagrams from collated ROIs (Figs. 2 and 5). All these procedures were carried out using Photoshop (Adobe).

FIG. 2. Regions of interest (ROIs) and resulting traces from one sagittal slab. A: rostral (left) and caudal (right) ROIs, outlined in highlighted white dashed lines and numbered according to trace order below. In some cases somata of neurons are visible within the ROI outline, indicating the fit between ROI and cell area. Black dotted line outlines VII n and the ventral surface. As was done for subsequent dot diagrams, the ×10 images that were used to generate the traces below were aligned with the ×10 view used to detect population activity. ROIs were obtained automatically using spatial filtering (see METHODS). B: ventral root (bottom trace) and optical traces from rostral and caudal recording locations; traces were sorted top to bottom, based on peak cross-correlation values obtained with respect to ventral root activity, and the top panel numbering reflects this order. Gray bars aligned with inspiratory burst onsets reveal relative phase and variability of optically recorded units. In the rostral data set 4 skipped inspiratory bursts are marked by arrowheads; most but not all respiratory neurons produced a synchronous Ca^{2+} peak at the midpoint of the cycle in which the skip occurred. Adjacent cell pairs in A are linked by lines labeled with the pair numbering. No obvious relationship between cell proximity and activity pattern was apparent. C: representative burst triggered averages in a 9-s window with a 6-s pretrigger reveal the heterogeneity of respiratory phases displayed by cells in each network. Sampling rate 3 frames/s. Movies associated with these data sets are available in Supplemental materials on-line.
Each ROI’s pattern of activity was displayed as changes in luminance as a function of time (Fig. 1B). Traces were sorted according to their similarity to population motor output, using the peak cross-correlation value as the estimator. We used a quadratic fit algorithm to identify peaks in the ROI and voltage traces. Peaks in the rectified, integrated voltage trace were matched with corresponding peaks in the optical traces to obtain peak-to-peak delay. Neurons were classified based on their firing pattern with respect to ventral root activity. Descriptive statistics and simple tests such as two-sample t-tests assuming equal variances were carried out in Excel (Microsoft), with Bonferroni correction for repeated tests. Two-level ANOVAs and Shapiro–Wilk test for normality were carried out using Origin (OriginLab, Northampton, MA). Data are presented with means and SEs.

RESULTS
Baseline system-level and cellular activity

For the first time, we visualized robust respiratory network activity in both the pFRG and the preBötC with single-cell resolution (Fig. 2). In nine experiments, no significant difference ($P = 0.9$) was found between rostral (25.1 ± 2.7) and caudal (25.5 ± 4.5) cell counts. Over the course of these experiments (234 ± 20 min), respiratory periods did not change significantly (5.3 ± 0.4 vs. 5.4 ± 0.3 s; $P = 0.33$), but optically recorded cell counts did (25.3 ± 2.5 vs. 14.8 ± 1.8 cells; $P = 0.001$); thus rundown effects likely led us to underestimate DAMGO- and/or hypoxia-insensitive neurons. The rate of rundown was uniform in the pFRG and preBötC.

When traces were sorted according to their similarity to population motor output (Fig. 2B), adjacent (i.e., very similar) traces typically arise from somata far apart (Fig. 2A); conversely, adjacent cells (Fig. 2A, linked by lines in Fig. 2B) often have very different patterns of activity (see traces 20 and 22 from the rostral recording location or traces 13 and 15 from the caudal recording location). We carried out cross-correlations between each cell and all others in every data set and plotted cross-correlation peak values against cell-to-cell distances (not shown) and found no structure. Thus based on these crude measures of coupling strength, we failed to find any functionally relevant structure in the spatial organization of the respiratory networks we recorded from.

Expiratory neurons, routinely found in vivo (Ezure 1990), were optically recorded here for the first time (trace 15, Fig. 2B, right). The heterogeneity of phase relations seen in our preparation is readily apparent in burst-triggered averages of selected optical traces (Fig. 2C). In addition to neurons with a fixed phase relation to motor output, we also observed neurons whose phase relations varied (traces 25 rostral, and 25 caudal, Fig. 2B).

During the acquisition of the rostral data set in Fig. 2, four inspiratory bursts were skipped at the level of motor output (arrows). During these skipped cycles, phase-locked peaks in most of the respiratory neurons can be seen. This was a common feature in both rostral and caudal networks, when inspiratory bursts were skipped. Another common feature (apparent in Fig. 5, traces 1, 5, 9, 18, and 26, left column) were neurons that, in addition to generating peaks in phase with motor output, also produced ectopic peaks midway through the respiratory cycle. We did not find neurons rostrally with a biphasic pattern of activity characteristic of pre-I neurons. Because of the low sampling rates imposed on us by the sensitivity of our camera, it is possible that these neurons were present, but could not be resolved because of poor temporal resolution. To test this, we carried out four additional experiments using an electron-multiplier CCD (Hamamatsu 9100–13) that permitted us to record at 20 frames/s. At this temporal resolution, neurons matching the pre-I pattern were detected (Supplemental materials, Fig. 1).1

The dot diagram and histograms (Fig. 3) from control data obtained at the start of each experiment reveal a bimodal distribution along the rostrocaudal axis (top histogram), with one peak at the caudal pole of the VIn and the other about 350 μm caudal. Noninspiratory neurons (i.e., neurons with an expiratory or pre- or postinspiratory phase of firing) are distributed along the neuraxis, but are concentrated at the rostral edge of the caudal distribution. Neurons formed a ventrally skewed unimodal distribution along the ventrodorsal axis.

1 The online version of this article contains supplemental data.
We obtained the mean burst amplitude increased in amplitude (Fig. 4 i). Averaged inspiratory burst under normoxia and hypoxia (black). Hypoxic burst onset and offset, occurred by normoxic burst, is indicated by fine black lines (arrows). Hypoxic burst amplitude is significantly greater than control. Ai: rectified integrated trace of respiratory motor output before and during hypoxic challenge (white bar). After an initial apneic response, respiratory rhythm resumed at a slower period. Ai: averaged inspiratory burst under normoxia and hypoxia (black). Hypoxic burst onset and offset, occurred by normoxic burst, is indicated by fine black lines (arrows). Hypoxic burst amplitude is significantly greater than control. Ai: rectified integrated trace of respiratory motor output before and during hypoxic challenge (white bar). After an initial apneic response, respiratory rhythm resumed at a slower period. Ai: averaged inspiratory burst under normoxia and hypoxia (black). Hypoxic burst onset and offset, occurred by normoxic burst, is indicated by fine black lines (arrows). Hypoxic burst amplitude is significantly greater than control.

Figs. 4 Ai: rectified integrated trace of respiratory motor output before and during hypoxic challenge (white bar). After an initial apneic response, respiratory rhythm resumed at a slower period. Ai: averaged inspiratory burst under normoxia and hypoxia (black). Hypoxic burst onset and offset, occurred by normoxic burst, is indicated by fine black lines (arrows). Hypoxic burst amplitude is significantly greater than control. Ai: rectified integrated trace of respiratory motor output before and during hypoxic challenge (white bar). After an initial apneic response, respiratory rhythm resumed at a slower period. Ai: averaged inspiratory burst under normoxia and hypoxia (black). Hypoxic burst onset and offset, occurred by normoxic burst, is indicated by fine black lines (arrows). Hypoxic burst amplitude is significantly greater than control. Ai: rectified integrated trace of respiratory motor output before and during hypoxic challenge (white bar). After an initial apneic response, respiratory rhythm resumed at a slower period. Ai: averaged inspiratory burst under normoxia and hypoxia (black). Hypoxic burst onset and offset, occurred by normoxic burst, is indicated by fine black lines (arrows). Hypoxic burst amplitude is significantly greater than control.

Changes in respiratory rhythm and pattern in response to hypoxic challenge and opioid-induced depression were previously described both in vitro and in vivo (Lieske et al. 2001; Paton et al. 2006; Solomon et al. 2000; St-John and Paton 2000). Thus these challenges are useful benchmarks to measure system-level function of the tilted sagittal slab. Hypoxic challenge was applied by perfusing the preparation with ACSF aerated with 95% N₂-5% CO₂. Shortly after onset of hypoxic challenge, a prolonged apnea was seen in five of nine preparations. Irrespective of whether prolonged apnea occurred, the subsequent respiratory period was slower and inspiratory bursts increased in amplitude (Fig. 4 Ai). When respiratory rhythm resumed, the period was slower and inspiratory bursts increased in amplitude (Fig. 4 A i). We obtained the mean burst envelope from 10–20 inspiratory bursts under normoxia and hypoxia for each experiment and averaged these means to obtain the averaged traces shown in Fig. 4 Ai. In addition to a significantly increased inspiratory burst amplitude under hypoxia (paired t-test, P = 0.008), burst onset was more abrupt and the burst duration was shorter (arrows, Fig. 4 Ai). In eight of nine experiments, the mean respiratory period under hypoxia was significantly longer than control (P < 0.01, with Bonferroni correction, Fig. 4 Ai). A comparison of pooled mean periods under hypoxia was significantly longer (5.8 ± 1.0 vs. 9.2 ± 1.6 s; P = 0.0003) than control (Fig. 4 Ai).

Administration of μ-opiate receptor agonists, at concentrations sufficient to slow but not stop respiration (n = 4; Fig. 4 B, left), gave rise to respiratory periods at integer multiples of control period (quantal slowing; Fig. 4 B, right). In addition, inspiratory burst amplitudes increased during quantal slowing, but because of the low number of inspiratory bursts during opioid-induced quantal slowing, the significance of this observation could not be tested. At concentrations sufficient to induce apnea (n = 5), cycles immediately after drug washin were likewise at integer multiples of control period.

At the cellular level, during hypoxic challenge and DAMGO-induced apnea, we encountered substantial numbers of interdigitated DAMGO- and/or hypoxia-insensitive neurons in both rostral and caudal regions. In Fig. 5, examples of DAMGO- and/or hypoxia-insensitive neurons from one caudal recording are shown and provide examples of some of the cellular responses that we observed. As in Fig. 2, traces are numbered from top to bottom and this numbering is carried over to the cropped ×20 image, in which ROIs are shown (Fig. 5 B i). Only traces active during DAMGO application and/or hypoxic challenge are shown. Traces are not sorted in relation to motor output, but rather are grouped according to their responses to hypoxia and opioid-induced depression.

In comparing fictive eupnea to fictive gasping, the most obvious change in neuronal activity was the loss of respiratory modulation or its weakening (Fig. 5). Other neurons, weakly modulated under eupneic conditions, showed stable and robust respiratory modulation during hypoxic challenge (Fig. 5, traces 14 and 16). We also observed ectopic bursting among neurons active during hypoxic challenge; thus in traces 14, 22, 25, and 26, peaks are apparent between inspiratory bursts. Although in this data set, expiratory neurons retained their expiratory phase of activity during hypoxic challenge (Fig. 5, traces 4 and 18), in other data sets, we found expiratory neurons that shifted to an inspiratory pattern during hypoxic challenge.

A consistent finding of this study was the presence of respiratory neurons in or near the preBoTC that remained rhythmically active at or near the frequency of the control respiratory rhythm during opioid-induced depression. The concentration used in this case (300 nM) is twice the concentration necessary to induce apnea in the sagittal slab and above the apneic threshold in the en bloc preparation (Mellen et al. 2003). Although respiratory rhythm was eliminated, periodic firing persisted. Importantly, gray bars aligned with peaks in trace 1 reveal that phase relations between neurons seen in the intact network persisted after DAMGO-induced depression (Fig. 5 A i, middle column); in particular, expiratory neurons remained in antiphase to inspiratory neurons (traces 4 and 11). Burst-triggered averages (triggered off trace 1 rather than the ventral root) of traces 1–4 (Fig. 5 A ii) show that DAMGO activity...
matches activity under control (black line) and hypoxic conditions (blue line). This particular optical recording was made from a region extending from 150 to 500 μm caudal to the caudal pole of VII (Fig. 5Bi). Within this network, neurons insensitive to DAMGO and/or hypoxia were interdigitated without any obvious topographic organization (Fig. 5Bii); this was a consistent feature within all networks sampled. In particular, cells close together may have differing patterns of activity under control conditions, but the same insensitivity to hypoxia and DAMGO (traces 1 and 3), or differing patterns of control activity and different responses to hypoxia and DAMGO (pairs 12 and 17; 12 and 2).

Although within networks, hypoxia- and/or DAMGO-insensitive neurons were interdigitated, when data were pooled, a degree of segregation was apparent along the rostrocaudal axis of the sagittal slab. Using the same methods as for Fig. 3, we generated a dot diagram of hypoxia-insensitive neurons (white), DAMGO-insensitive neurons (black), and DAMGO- and hypoxia-insensitive neurons (gray) (Fig. 6). As the Venn diagram indicates (Fig. 6, top right), hypoxia-insensitive neurons outnumber DAMGO-insensitive neurons and a substantial number of neurons are insensitive to both. The histograms show exclusively DAMGO- and hypoxia-insensitive histograms (black and white bars, respectively) stacked on top of the DAMGO- and hypoxia-insensitive histogram (gray bars). Although all three classes of neurons are found along the sagittal slab, their distributions differ. Rostrally, all three classes of neurons approximate the same relative distribution as inspiratory neurons under control conditions (bars with diagonal lines). Caudally, the largest concentration of DAMGO-insensitive neurons and neurons insensitive to both DAMGO and hypoxia were found in the bin 400 μm caudal to the caudal pole of VII, whereas neurons that were exclusively hypoxia-insensitive reached their maximum 100 μm more caudally. Thus neurons insensitive to DAMGO and hypoxia as well as neurons insensitive to DAMGO were most concentrated 100 μm caudal to the control histogram peak, whereas the largest number of neurons insensitive to hypoxia only were 100 μm more caudal still. Along the ventrodorsal axis, distributions of DAMGO- and/or hypoxia-insensitive neurons matched the control histograms in their relative numbers.

Because hypoxia transforms motor pattern, the cellular and synaptic activity of respiratory networks is transformed as well. This transformation can be observed in parallel using optical recordings. To gain a qualitative sense of how intrinsic
activity and network properties change during hypoxic challenge, we looked at respiratory networks in the transition from eupnea to gasping ($n = 3$), recording 200 s of activity 1 min after anoxic aCSF washin (Fig. 7). Whereas in a large subset of neurons activity and phase relations to motor output remained constant (group 1, group 5), others showed an increase (group 2) or decrease (group 3) in respiratory drive. Another subgroup, initially weakly modulated, displayed strong rhythmic activity at greater than respiratory frequency (group 4). Because motor output remained phase-locked with this activity, these neurons likely receive respiratory drive and may also contribute to inspiratory burst timing during hypoxia. Because ectopic activity was phase-locked across groups 4 and 5 (dotted lines, Fig. 7), it is likely that these neurons were synaptically coupled.

**DISCUSSION**

Converging evidence suggests that in the ventrolateral medulla, two spatially separate networks are each sufficient for respiratory rhythm generation (Mellen et al. 2003; Onimaru and Homma 2003; Onimaru et al. 2006). We present here a novel in vitro preparation, the tilted sagittal slab, offering easy and reproducible optical recording from both respiratory networks with single-neuron resolution.

Unlike an earlier attempt to expose these networks at the surface of a slice (Paton et al. 1994), this preparation was not developed to isolate a minimal circuit, but rather to provide an easily reproducible preparation permitting optical and intracellular recording at the surface of the slab. Because preparations cut slightly too lateral or medial showed no respiration-modulated optical activity or were silent, these superficial networks are likely necessary for respiratory rhythm generation. Because of the circuits retained in this preparation or because its thickness may have mitigated the perturbations attendant with the isolation of any slice preparation (Steriadi 2001), the tilted sagittal slab generated stable rhythmic activity in physiological $[K^+]_o$ over the 4 h that these experiments lasted.

Mappings between the neurons we recorded from and respiratory neuron taxonomies developed using intracellular methods (Onimaru and Homma 1992; Rekling et al. 1996) cannot be made because intracellular taxonomies are based on onset time differences in the 100-ms range, whereas we sampled at 2–3 fps. Our temporal resolution was sufficient to differentiate between inspiratory, early, and late expiratory neurons, all of which were found in our preparation, consistent with studies in other, less-reduced preparations (Dutschmann and Paton 2003; Ezure et al. 1988). In addition, in the absence of information about projection pattern or phenotype, we were unable to distinguish between propriobulbar rhythm-generating interneurons and (pre-) motoneurons. These include VIIIn motoneurons innervating nasal airways and laryngeal and pharyngeal motoneurons within the nucleus ambiguus, which include subgroups active during both expiration and inspiration (Grelot et al. 1989; Hwang et al. 1988; reviewed in Bianchi et al. 1995). Thus units recorded in these regions likely include upper airway (pre-) motoneurons. In addition, respiration-modulated cardiovascular neurons have been recorded from in this region and these too are active at a range of phases (Miyawaki et al. 1995). Because the regions from which respiratory activity was
bursts suggests coupling (dotted lines). Phase-locking between ectopic respiration-modulated neurons (group 4) or the emergence of robust ectopic bursting (i.e., an
response to hypoxia is attributed to the absence of pontine and afferent inputs, which together contribute to eupneic rhythm in vivo and which are depressed during hypoxia (Rybak et al. 2004). On this view, because these inputs are absent to begin with in vitro, the hypoxic response is correspondingly smaller.

In addition, our preparation differs from other in vitro preparations: unlike the en bloc preparation (Duffin 2003), but congruent with in vivo studies, inspiratory burst pattern shifts from augmenting to purely decrementing. This may be because in the en bloc preparation, respiratory networks are relatively hypoxic and acidic even under control conditions (Voipio and Ballanyi 1997) as a result of the long diffusion distances from the preparation’s surface to respiratory networks. Thus in the en bloc preparation, control conditions may be sufficient to transform the inspiratory pattern.

Our preparation also differs from the transverse slice preparation: at the system level in the transverse slice, hypoxia-induced slowing is preceded by an increase in respiratory frequency (Lieske et al. 2000), whereas in our preparation this was not observed. At the level of individual neurons, however, during the transition to hypoxia, we observed subsets of neurons rhythmically active at frequencies higher than motor output; thus the increase in respiratory frequency seen in the transverse slice may be explained by the relatively larger number of these upregulated neurons. It was also reported that hypoxia transforms expiratory respiratory neurons to tonically active neurons (Thoby-Brisson and Ramirez 2000); because of our high-pass filtering, this transformation would appear as the silencing of the expiratory neuron because the tonic signal would be filtered out. Hypoxic challenge recruited neurons quiescent under control conditions. These likely include sympathetic neurons, which have been shown to be entrained by central respiratory rhythm-generating neurons during hypoxic challenge (Dick et al. 2004).

The observation of respiratory periods distributed at integer multiples of control period (quantal slowing) during opiate-induced depression in preparations retaining the pFRG was interpreted as evidence for the rhythmogenic capacity of this network (Mellen et al. 2003). Thus we used opioid-induced depression to test whether putative opiate-insensitive pFRG networks were functional in our preparation. At the system level, we observed quantal slowing, consistent with the hypothesis that our preparation retained an opiate-insensitive rhythmogenic network. In addition, networks of respiration-modulated neurons at the level of the VIIIn were observed, consistent with the rostrocaudal location of the pFRG. Our networks were concentrated at the caudal pole and dorsal edge of the VIIIn, whereas endogenously bursting pre-I neurons, which are hypothesized to drive the pFRG rhythm, were primarily recorded from along the ventral surface of the VIIIn. Because the existing boundaries of the pFRG were drawn to delineate a region in which pre-I neurons were plentiful, and readily accessible to intracellular recordings (Onimaru et al. 1989, 1995), the more dorsal networks described here cannot be excluded a priori as constituents of the same functional network because they have heretofore not been systematically studied. Evidence that neurons surrounding the VIIIn form a functional group can be found in optical recordings in the transverse plane at the rostral pole of the brain stem–spinal cord, which revealed respiratory neurons along the lateral and dorsal edge of the VIIIn active synchronous with ventral pFRG neurons (Onimaru and Homma 2003). The low number of respiratory neurons found near the ventral surface may be a result of the compression-related damage ensuing from the cutting of the slab, or because at the ventral surface, the superficial cells we record from are lateral to the pre-I neurons.

FIG. 7. Respiratory network activity pattern changes in the transition from fictive eupnea to fictive gasping. Recording began after the bath had been perfused with anoxic artificial cerebrospinal fluid for 1 min. Activity level in many neurons remained constant (group 1). Other previously quiescent neurons became respiration-modulated (group 2); conversely, activity in some respiration modulated neurons was attenuated (group 3). Finally, we observed the emergence of robust ectopic bursting (i.e., an n-to-1 relationship between cellular bursts and motor output) in previously quiescent neurons (group 4) or respiration-modulated neurons (group 5). Phase-locking between ectopic bursts suggests coupling (dotted lines).

recorded corresponded with regions that have been proposed as sufficient for rhythm generation, we surmise that propriobulbar rhythmogenic neurons were included in our sample. This is supported by our observation of ectopic “inspiratory” activity during skipped cycles.

An important test for a new preparation is that it match existing preparations in its response to challenges. Because responses to hypoxia and opiates were previously well described in other in vivo and in vitro preparations, we used these here to test our preparation. At the system level, our preparation qualitatively matches other preparations, but differences exist and, in some cases, may be interpretable. Congruent with both in vivo and in vitro studies, hypoxia initially gave rise to apnea, which was followed by a significantly slower respiratory rhythm, with augmented inspiratory amplitude. As with other in vitro preparations, although the respiratory period was significantly longer, the dramatic lengthening accompanying hypoxia-induced gasping in vivo was not observed here. A recent modeling study suggests that this blunted in vitro response to hypoxia is attributed to the absence of pontine and afferent inputs, which together contribute to eupneic rhythm in vivo and which are depressed during hypoxia (Rybak et al. 2004). On this view, because these inputs are absent to begin with in vitro, the hypoxic response is correspondingly smaller.
recorded from en bloc. In addition, the sampling rates used in the bulk of the experiments here may have compromised detection of the biphasic firing pattern characteristic of pre-I neurons. When higher sampling rate recordings were made from neurons dorsal and caudal to the Vthn, transient inhibition during inspiration could be detected in neurons that at 3 fps appeared as inspiratory, or active before or after inspiration. This suggests that pre-I neurons were present in our sample, but identified as inspiratory, or active before and/or after inspiration.

A striking difference between this study and earlier reports (Mellen et al. 2003; Takeda et al. 2001) was the observation of opiate-insensitive respiratory neurons at the level of the preBötC, which maintained control frequency and phase relationships after DAMGO-induced apnea. This novel observation may be ascribed to the better oxygenation of respiratory networks in our preparation, as compared with en bloc. In addition, distance from the ventral surface, and perhaps also size (Towe and Harding 1970), may have led single-unit recording methods to miss the DAMGO-insensitive neurons we recorded from here.

Accumulating evidence suggests that the pFRG and preBötC have distinct functional roles, can mediate qualitatively different respiratory patterns, originate from different rhombomeres (Chatonnet et al. 2003), and, under appropriate conditions, are individually sufficient for rhythm generation (Onimaru et al. 2006; Smith et al. 1991). Thus pre-I neurons, concentrated in the pFRG, are opiate insensitive, derive from rhombomeres r3–r4 (Chatonnet et al. 2006), and generate expiratory drive (Janczewski and Feldman 2006). By contrast preBötC neurons act as hypoxia sensors (Solomon et al. 2000), regulate gasp frequency (Solomon 2002; Solomon et al. 2000), and, when NK1R-positive neurons in preBötC are selectively ablated, ataxic breathing but not gasping is observed and hypoxic challenge is lethal (Gray et al. 2001). Thus we were expecting to find some spatial segregation in DAMGO- or hypoxia-insensitive networks, with little spatial or functional overlap. Instead, we found anatomically overlapping networks and a subset of neurons that were active both during hypoxic challenge and opioid-induced depression. This indicates that if hypoxia- or DAMGO-insensitive neurons are spatially segregated, the networks they project to are interdigitated. Coupling between both networks has been inferred from the inspiratory inhibition of pFRG pre-I neurons and from the presence of caudally distributed neurons with pre-I firing patterns (Arata et al. 1990; Kashiwagi et al. 1993), although little is known about how these networks interact. Our observation of the persistence of control phase relations between inspiratory and expiratory neurons in or near the preBötC during DAMGO-induced apnea indicates that drive from opiate-insensitive neurons maintains coordinated network behavior and not just the activity of relay neurons.

Although everywhere we recorded we found neuronal heterogeneity, this heterogeneity was not uniformly distributed. Neurons with phases other than inspiratory were concentrated at the rostral pole of the caudal cluster. Based on functional anatomical descriptions of medullary respiratory networks (Smith et al. 1991), the more rostral region rich in expiratory neurons may correspond to the Bötzinger complex. On this construal, the more caudal region with the highest concentration of DAMGO- and/or hypoxia-insensitive neurons, would correspond to the preBötC. Within such heterogeneous networks, neuronal intrinsic properties are typically yoked by the network within which they are embedded (Seriade 2001, 2006), so that the contribution of individual neurons to the network is apparent only as conditions change. This interplay between intrinsic properties and network constraints is apparent in the coordinated transition from quiescence to activity and activity to quiescence during anoxic acSF washin.

We have presented a novel preparation that 1) retains both the pFRG and the preBötC and 2) permits optical recording from both networks, with single-neuron resolution, and without requiring averaging. This preparation offers a useful platform for characterizing connectivity between respiratory neurons and networks, about which little is known, either by incorporating single-unit recording or, at sufficiently high acquisition rates, by time-series analysis from optical records. Multiple extracellular electrode recordings have already provided a window on the bewilderingly rich phenomenology of respiratory network dynamics and the complexity of the neural interactions that give rise to them (Lindsey et al. 1994). Here, similarly rich data sets are generated, but in addition, the spatial layout of the network can be studied, which may provide new organizing principles to the understanding of respiratory networks. Because this preparation’s responses to hypoxic challenge and opioid-induced depression were qualitatively similar to other in vitro and in vivo preparations, we infer that similar mechanisms underpin these behaviors across preparations. Here, the networks involved can be monitored in parallel at the cut surface of the sagittal slab.

ACKNOWLEDGMENTS

Thanks to Dr. Jack Feldman, in whose laboratory these techniques were first developed. Thanks to Graftek Imaging (Austin, TX) for the loan of the EM-CCD camera and for technical support in getting it to work.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant HL-068007.

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