Temperature- and State-Dependence of Dynamic Phrenic Oscillations in the Decerebrate Juvenile Rat

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Abstract

The aim of the present study was to determine the characteristics of the fast oscillations in the juvenile rat phrenic nerve, and to establish their temperature- and state-dependence. Two different age-matched decerebrate, baro- and chemo-denervated rat preparations, the in vivo and the in situ arterially-perfused models, were used to examine three systemic properties: generation and dynamics of fast oscillations in phrenic (Ph) activity (both preparations); responses to anoxia (both preparations); and the effects of temperature on fast oscillations (in situ only). Both juvenile preparations generated power and coherence in two major bands analogous to adult high- (HFO) and medium-frequency oscillations at frequencies that increased with temperature but were lower than in adults. At < 28 °C, however, Ph oscillations were confined primarily to one low-frequency band (20-45 Hz). During sustained anoxia, both preparations produced stereotypical state changes from eupnea to hyperpnea to ‘transition bursting’ (a behavior present only in vivo during incomplete ischemia) to gasping. Thus, the juvenile rat produces a sequential pattern of responses to anoxia that are intermediate forms between those produced by neonates and those produced by adults. Time-frequency analysis determined that fast oscillations demonstrated dynamics over the course of the inspiratory burst, and a state-dependence similar to that of adults in vivo, in which hyperpnea (and transition) bursts are associated with increases in HFO, while gasping contains no HFO. Our results confirm that both the fast oscillations in Ph activity and the coherence between Ph pairs produced by the juvenile rat are profoundly state- and temperature-dependent.

Keywords: respiratory control; zero-interval subtraction; motor synchrony; diaphragm
Introduction

It is well-established that different mammalian species generate fast respiratory rhythms including medium- (MFO) and high-frequency oscillations (HFO) in phrenic nerve (Ph) activity during inspiration. In cats and rabbits, MFO is in the 20–50 Hz range while HFO is in the 60–110 Hz range (1, 4, 18). In the most common contemporary animal model, the rat, both of these oscillations are at slightly greater than 2-fold higher frequencies, with MFO at ~37–110 and HFO at ~156–230 Hz (13, 14). Oscillations in the major bands also appear to be dependent on developmental stage. In rats, one study (9) reported that ‘neonates’ (P14–P36) only produce ‘MFO’ (<50 Hz) and not ‘HFO’ (>50 Hz), although barbiturate anesthesia, known to depress HFO (7, 18), was used in that study. Also, this study assumed that fast oscillations in rat Ph would be in the same frequency bands as for other (larger) species, an assumption that now appears invalid (13, 14). The results of Tarsiuk and Sica (28), obtained using an in vitro brainstem-spinal cord preparation, also suggested that HFO is absent in neonates. However, these preparations were studied at 27 °C, and the effects of low temperatures on fast Ph rhythms in neonates can be significant (29). In juvenile rats, only two papers devoted to spectral analyses of Ph during eupnea and gasping have been published (10, 26), but these studies are difficult to interpret for a variety of technical reasons. Moreover, no studies have examined these features in age-matched controls with identical neuraxes (i.e., decerebrated juvenile rats in vivo) for direct comparison of fast rhythm generation. Because of these developmental and experimental variables, the character and dynamics of the fast oscillations produced by juvenile rats remains unclear.

In the present study, we test the hypotheses that: (1) decerebrate juvenile preparations generate anoxia-induced behaviors qualitatively intermediate between neonatal and adult
responses; (2) the temporal dynamics of fast oscillations mimic those of the adult; (3) the in situ juvenile model produces fast Ph oscillations similar to the in vivo unanesthetized decerebrate rat of the same age; (4) state-dependent fast oscillations produced by juvenile preparations are identical to the state-dependence exhibited by decerebrate adult rats in vivo; and (5) fast oscillations are highly temperature-dependent. Using time-frequency analysis, we recently characterized the dynamics of the Ph and hypoglossal nerve (XII) spectra in the unanesthetized, decerebrate adult rat in vivo during three different functional states: eupnea, hyperpnea and gasping (14). In addition to demonstrating the ability to detect features that may be missed by parametric spectral methods, these time-frequency results provide us with a standard with which we may evaluate responses to anoxia and generation of dynamic fast oscillations in the juvenile.

In addition to production of fast oscillations, bilateral coupling of Ph activity appears to be age-dependent. For example, Zimmer and Goshgarian (30), working in P0–P4 rats in vitro, found that younger preparations demonstrated higher degrees of C4/C5 ventral root phasic activity ipsilateral to C2 hemi-section than preparations made from older neonates, suggesting a higher degree of shared descending input or local functional coupling caudal to C2 in younger animals. A commonly-used measure of phase constancy in coupled oscillations is coherence, which may be caused by common inputs, direct coupling, or both. In an earlier study, we examined the intra-burst dynamics of Ph and XII coherence in the adult unanesthetized decerebrate rat and found significant, dynamic coupling between the left and right Phs, the left and right XII, and between Ph and XII activity during eupnea, hyperpnea and gasping, with the highest coherence observed during the hyperpneic state (15). Applying this metric to pairwise relations between Ph, XII, and vagal efferent activity in the juvenile rat in situ, Lieter & St.-John (10) concluded that significant coherence existed between different nerves only during gasping.
In another study (26), the same authors detected a single peak in Ph-Ph coherence at a specific temperature, but did not report values at other temperatures. In the present study, we use time-frequency coherence analysis to test the hypothesis that (dynamic) coupling between left and right Phs is temperature- and state-dependent in the juvenile rat in situ.
Methods

All procedures were approved by the University of Delaware Institutional Animal Care and Use Committee and consistent with the Animal Welfare Act. Juvenile male Sprague-Dawley rats (90-110 g; 27-32 days old) were used for both in situ and in vivo experiments.

In Situ Preparation

A variant of the “working heart-brain stem preparation” in the rat (25) was used for the in situ preparation (see 6 for description). Under deep isoflurane anesthesia, rats (n=10) were bisected just below the diaphragm, decerebrated at the precollicular level, and skinned while immersed in 4°C, carbogen-saturated solution identical to that used to perfuse the preparation (below). Following transfer to a recording chamber, the descending aorta was cannulated (tip facing rostral), and rats were perfused using non-pulsatile flow with a carbogen-saturated (95% O₂ / 5% CO₂; 4 L/min gas in 2L perfusate) solution consisting of 125 mM NaCl, 1.25 mM NaHCO₃, 4 mM KCl, 2.5 mM CaCl₂, 1.25 mM MgSO₄, 1.25 mM KH₂PO₄, 20 mM dextrose, 1.0 mg/L vecuronium bromide, and 2.5% dextran (230 kDa) at ~80 mmHg initially. The diaphragm, heart, lungs, and portions of the rib cage were removed to prevent cardiac-related mechanical and electrical artifacts, and the internal thoracic and branches of the subclavian arteries (e.g., auxiliary) were ligated to prevent leakage of the perfusate from the thorax. After these maneuvers, perfusion at the rate required to produce eupnea resulted in pressures of 100-110 mmHg. The temperature of the perfusate was then gradually raised to 34 °C (see below).

In Vivo Preparation

General Surgical Procedures. Spontaneously breathing rats (n=8) were anesthetized with isoflurane vaporized in O₂ (MDS Matrix; 4–5% induction, 2.0–2.5% maintenance). A tracheotomy was performed via a ventral approach, and animals were intubated with an
atraumatic glass tube, after which they were artificially ventilated (60 cycles/min, 1.5–2.0 ml tidal volume; Columbus Apparatus) with the same gas mixture. A femoral artery and vein were then cannulated for measurement of arterial pressure and infusion of drugs, respectively. Arterial and tracheal cannulae were connected to pressure transducers (CDXII, Argon Medical) for monitoring blood pressure (BP) and inflation pressure (TP) using conventional amplifiers (Gould Statham). Signals from three small subcutaneous electrodes were amplified and filtered (Neurolog; Digitimer, Hertfordshire, England) and used to monitor EKG via an audio amplifier (model AM10; Grass Instruments). The depth of anesthesia was determined sufficient if the withdrawal reflexes and changes in heart rate and blood pressure in response to pinches of the distal hind limbs were absent. End-tidal CO₂ was maintained between 4.5 and 5.0% (Capstar CWE) by adjusting minute volume. During all surgical procedures, rectal temperature was maintained at 37.0 ± 0.3°C via a servo-controlled heating blanket coupled to a rectal thermometer (Harvard Apparatus). The left and right medial branches of the hypoglossal nerves (hereafter referred to as XII) and the phrenic nerves (Phs) were dissected free from the surrounding tissue, transected, and desheathed. Both internal carotid arteries were tied off just below the pterygopalatine artery (22) to prevent bleeding after decerebration.

Decerebration. After this initial surgical preparation, the rats were placed prone in a stereotaxic device. Using a variable-speed surgical drill (Foredom Electric), the parietal bones were removed, the sagittal sinus venosus was ligated using surgical suture, and the neuraxis was gently transected at the rostral border of the superior colliculus using a microspatula. Brain tissue rostral to the transection was removed by suction, and any bleeding was arrested by filling the empty skull space with small pieces of gelfoam (USP, Pharmacia) soaked with cold thrombin solution (USP, 50 units/ml, dissolved in 0.9% saline). Ten to fifteen minutes after the
decerebration, anesthesia was slowly withdrawn, and the animals were paralyzed by intravenous bolus injection of 2 mg/kg of vecuronium bromide (Abbott Labs), followed by continuous infusion (3 mg·kg⁻¹·h⁻¹). Ph recordings were not initiated until at least 1 h after decerebration, with mean blood pressures ≥ 90 mmHg. If necessary, animals were continuously infused with 5.0% dextrose in 0.9% saline (1.0–1.5% body weight or 1.5–2.0 ml/h) to maintain a mean BP of at least 90 mmHg.

**Recording**

All animals were baro- and chemoreceptor denervated via bilateral transection of the vagus (just below the nodose ganglion) and carotid sinus nerves to prevent cardiorespiratory reflex influences on motor nerve outputs. In the in vivo experiments, a bilateral pneumothorax was performed before recording to eliminate lung inflation-related movement artifacts, with a positive end expiratory load of 1.0 cm H₂O applied. With the rat in the supine position, the central ends of the Ph and XII were placed on bipolar silver electrodes and immersed in a mineral oil pool formed by skin flaps. Monophasic recordings (0.5–5,000 Hz; Neurolog, Digitimer) of efferent activity were obtained in both preparations after crushing the peripheral end of each nerve between the silver bipolar electrodes (see 14, 18). The electrical activity of the four nerves, perfusion pressure (in situ), expiratory CO₂ level (in vivo), arterial blood pressure (in vivo), and intratracheal pressure (in vivo) were recorded onto the hard disk of a PC at a sampling rate of 10,000 samples/s for each channel using a 16-bit analog-to-digital converter system (AD Instruments).

**Anoxia and Classification of States**

In order evaluate the transition from eupnea to gasping, anoxic tests were performed in both preparations. In all in situ preparations (n=10), this was achieved by switching to a
perfusate saturated with 95% N₂ / 5% CO₂ and delivering it at the same flow rate, temperature, and pressure as the carbogen-saturated solution. Anoxia was produced in vivo by asphyxia (i.e., cessation of the ventilator). In both preparations, anoxia produced a sequence of respiratory behaviors (Figures 1 and 2). Eupnea, hyperpnea, and gasping were classified as described in the classic literature (8, 12, 16, 17, 20) and in our previous publications (14, 15). According to these studies, respiratory responses to hypoxia in mammals typically progress through four phases: hyperpnea, primary apnea, gasping, and terminal apnea. As in these studies, we define “primary apnea” as the period just following the final hyperpneic burst and ending with the onset of the first gasp. In the present study in juveniles, there was a superposition onto primary apnea of a group of bell-shaped or slowly decrementing Ph discharges in situ and in vivo (see Figures 1 and 2). We refer to these Ph bursts as ‘transition’ bursts. These were not categorized as gasps since gasping is characterized by simultaneous onset of hypoglossal and phrenic discharges (e.g., 10, 14, 21, 27) and in transition bursts the onset of Ph discharge was delayed relative to XII discharge (Figs. 1 and 2). In vivo, transition bursts occurred only if blood pressure did not drop rapidly (i.e., ischemia was delayed; Fig. 2A) and we have never observed them in adults rats in vivo, presumably because rapid development of ischemia was uniformly observed (14). In freely-behaving young rats (5, 10 and 15 days old; 5) respiratory responses to anoxia were reported to progress through prototypical patterns, beginning with initial hyperpnea, followed by primary apnea, ‘early gasps’, and another apnea, and terminal gasps. The responses we describe presently as transition bursts appear to be related to stage I (‘early gasps’), and those we categorize as gasps resemble stage III (terminal gasps) in neonates (5). In the present study, multiple inductions of gasping behaviors were produced in the same in situ preparations, with at least 20 minutes between anoxic challenges, after which the Ph discharges generated eupneic
bursts with spectral characteristics, durations and intervals indistinguishable form those produced prior to the first anoxic challenge.

**Temperature Manipulation.**

In order to determine the effects of temperature on the power and coherence of fast oscillations, data were collected from 6 of the 10 in situ preparations at specific temperature ranges of 21–23, 24–26, 28–30, and 32–34 °C during the normal procedure of gradual warming. Perfusion temperature was changed at a rate 0.33 °C/min, measured via a fine thermocouple wire and thermometer (Digi-Sense; Cole Palmer, Vernon Hills, IL) at the point of aortic cannulation. Only eupneic bursts were compared at these various temperatures.

**Analyses**

**TIME-FREQUENCY POWER AND COHERENCE ESTIMATES.** We refer the reader to our previous studies (14, 15) for a detailed description of the analytical methods. All analyses were performed on inspiratory bursts, which were compared to the mid-expiratory (E-2) phase for statistical significance. Nerve records were digitally filtered between 10 and 1000 Hz. Using the ‘zero-interval subtraction’ method (ZIS, see 14, 15 for details), time-averaged spectra and coherence functions were then computed from inspiratory bursts for each animal under each condition (see below). In addition, time-frequency representations of the spectra and coherence were calculated. For time-frequency analysis, inspiratory bursts were selected within categories according to their mean duration, with tolerances of ± 5% for eupnea, and ± 10% for hyperpnea, transition phase, and gasping. Only those gasps greater than 3 times the amplitude of the average inter-gasp background activity (calculated from the middle third of inter-gasp intervals) were analyzed. Frequency-domain analyses were performed with 2.44 Hz resolution over a 4096-data point window from 0–400 Hz. Using 50% overlap of consecutive zeroed segment positions, the
inspiratory bursts’ time-frequency estimates were computed at each sliding window positions (see 14, 15 for details).

Power spectral estimates were calculated within each condition (temperature or respiratory state). In order to demonstrate the effect of temperature or respiratory state on power distribution, total power was normalized to the 32–34 °C eupnea (in situ) or to eupnea (in vivo) condition, which was set to a total power of 100%. Thus, the percentages in Figures 4 through 6 are expressed relative to eupnea.

**Statistical Analyses.** During eupneic breathing, the inspiratory (Ti) and expiratory (Te) durations, as well as the Ti/Te ratios, were calculated at each temperature range (in situ) and in vivo. Within each animal, average (gated) power spectral and coherence functions were calculated separately for eupneic (n=20–30), hyperpneic (n=10–15), transition (n=6–9), and gasping (n=10–15) inspiratory bursts, as well as for expiratory control periods. In addition, within a given preparation type (in vivo vs. in situ), data were grouped across animals to determine the relative consistency of the various features of these measures. Specifically, either parametric (t-test) or nonparametric (Wilcoxon and Mann-Whitney U) tests were applied to compare two groups of data, as required by their conformation to a normal distribution. Parametric (one-way and repeated-measure ANOVA) and non-parametric (Friedman & Kruskal-Wallis) tests were used to compare results obtained from different conditions (e.g., temperatures, preparations), states (e.g., eupnea vs. gasping). Data are presented as means ± SE, and P values < 0.05 obtained from any statistical tests were interpreted as significant, as were 95 % confidence level. The borders of frequency bands and their maximal peaks were identified using a custom-written optimization algorithm running in Matlab (versions 6.5.1 and 7.0.4; see 17, 18
for details), and only bands which were statistically-significantly higher than expiratory background levels are listed in the tables.
Results

Responses to Anoxia

All ten rats studied in situ showed stereotypical patterns of change in Ph activity in response to anoxic stress, as illustrated in Figure 1A. Following the start of perfusion with anoxic perfusate, a eupneic pattern (‘E’, slow incrementing ramp) was replaced by a hyperpneic one (‘H’, rapidly incrementing ramp), which was also accompanied by an increase in tonic activity. Hyperpnea was characterized first by an increase in respiratory rate, then by increases in Ph amplitude (Fig. 1A). This hyperpneic phase was followed (during the first anoxic challenge) by a primary apnea during which 6–9 transition bursts appeared in all in situ preparations. As illustrated in Figure 1B, transition burst patterns were either bell-shaped or slowly decrementing. Transition bursts were distinct from gasps, which are characterized by simultaneous onset of Ph and XII, even if the pre-phrenic portion of XII is ignored, (Fig. 1B vs. 1C). The final transition bursts occurred well before the end of primary apnea, which was then followed by gasping. After the 3rd or 4th anoxic challenge in all in situ preparations, silent primary apnea was abolished in responses to anoxia, and respiratory states progressed smoothly from eupnea to hyperpnea to transition to gasping without interruption (Fig. 1D).

Figure 2 provides an example demonstrating that the in vivo preparation responds similarly to the in situ preparation of the same age, where gasps are produced following primary apnea. Only 2 of 8 in vivo preparations produced transition bursts during primary apnea, and these were limited in number to 2 and 3, respectively. Transition bursts occurred only in preparations where blood pressure did not drop rapidly (i.e., ischemia was delayed and anoxia incomplete; Fig. 2A). As with in situ preparations (see above), XII onset preceded Ph onset during transition bursts and coincided with Ph onset during gasping. Finally, in situ and in vivo
burst shapes were well-matched in shape, as exemplified by the bursts in Figure 2D recorded from one in vivo and one in situ preparation.

**Temperature-Dependence of Respiratory Parameters and Fast Oscillations In Situ**

Table 1 provides a summary of the basic respiratory rhythm characteristics of the in situ preparations at different temperatures. As the values indicate, respiratory rate becomes significantly more rapid, and inspiratory duration shorter, as temperature increases (p<0.05 between adjacent temperature conditions). However, duty cycle (Ti/Te) was relatively constant over most temperature ranges, and only significantly smaller (p<0.05) at the lowest temperatures (21–23 °C). The in vivo respiratory parameters suggest an extrapolated continuation of the temperature-dependent trends for all parameters save Ti/Te, which was significantly smaller (p<0.05) than during all in situ temperatures save 21–23 °C.

Figure 3 illustrates the effects of temperature on oscillations in the Ph and XII nerves. At all temperatures during eupnea, hypoglossal activity began before phrenic. At 21–23 °C, XII oscillations were characterized by a mixture of slow and fast frequencies, while the Ph was dominated by a single frequency band, particularly later in the burst (arrows). At the onset of Ph activation, a high-amplitude, slow-wave “start-up” component was observed in Ph activity at all temperatures, akin to that observed in adults in vivo (14). At 24–26 °C, typical XII activity contained a mixture of low and high frequency oscillations, while those in the Ph were still dominated by a single frequency. Beginning at 28 and continuing through 34 °C, XII activity demonstrated a high-amplitude slow wave of near-synchronous of activity simultaneous with Ph onset (dotted line) in all in situ preparations. Meanwhile, at 28–30 °C, low-amplitude, high-frequency Ph oscillations appeared mixed with low-frequency oscillations, and the former became more pronounced at 32–34 °C. In vivo (37 °C), both nerves demonstrated both fast and
slow oscillations, as well as start-up (Fig. 3, bottom). The pre-Ph/Ph ratio of XII activity was consistently reduced with increasing temperature (Table 1).

Figure 4 (top) provides the power in the right Ph (left), and coherence between the Phs (right), using time-averaged ZIS analysis, averaged over all in situ preparations at four different temperature ranges (black, blue, gold, red traces), as well as for age-matched in vivo rats (green traces). The dominant low-frequency band (20–55 Hz) changed significantly in relative power, but not in position (peaks range: 29–37 Hz), as temperature was increased from 21–30 °C. At temperatures ≥ 32 °C, the low-frequency power band shifted to higher frequencies (peak at ~51 Hz; Table 2). The same general pattern held true for the 55–90 Hz band (peaks at 68–73 Hz), in that an increase in relative power, but not in frequency, was noted for temperatures between 21-30 °C. At 32–34 °C, a significant shift in the peak frequency was apparent, to ~107 Hz (Table 2). However, the peaks in power at the highest temperatures in situ were located at lower frequencies than corresponding peaks in vivo (Tables 2 and 3). All temperature ranges showed significant coherence (Fig. 4, top right) in the frequency bands that dominated their power spectra.

An example of the temperature-dependence of the dynamics in the power and coherence functions in a typical in situ preparation is provided in the time-frequency representations in Figure 4, left and right columns, respectively. At 21–23 °C, power in the 20–45 Hz band was dynamic, exhibiting distinct early (first fifth) and late (last third) components. As the temperature of the preparation was increased further to 24–26 °C, a higher-frequency band emerged (55–90 Hz); and this band gained in both relative power and duration as temperature was increased to 28–30 °C. Finally, at 32–34 °C, this band shifted towards higher frequencies (90–125 Hz) and was concentrated in the first half of the eupneic burst (Fig. 4, bottom left).
The in situ preparation was characterized by strong coherence in the fast oscillations generated in the two Phs. The highest coherence (>0.8) occurred in the lowest temperatures (21–23 °C) over the same frequencies and with the same time course as the dominant power band at that temperature (Fig. 4). As preparation temperature was elevated, the correlation in frequency and time course between coherence and power remained consistent with one exception: At 32–34 °C, there is significant early coherence in the 50–75 Hz range (Fig. 4, bottom right), where only modest power is expressed (bottom left).

**State-Dependent Changes in Power and Coherence In Situ**

Figure 5 illustrates the changes in Ph power (top left) and coherence (top right) associated with state changes in all in situ preparations at 32–34 °C. Progressive anoxia resulted in an increase in power at high frequencies during hyperpnea and transition phases, followed by a selective loss of power at those frequencies during gasping. During eupnea, power at high frequencies was typically contained within a narrow band (90–125 Hz) and restricted to the first third of the burst (Fig. 4, bottom left), with relatively low coherence (Fig. 4, bottom right). Power is also distributed to lower frequencies (30–90 Hz) in eupnea during early and late inspiration with strong coherence (Fig. 4, bottom row; Table 2). Over all animals, power is increased during hyperpnea at all frequencies relative to eupnea (Fig. 5, top left). Also, coherence associated with high frequencies (100–130 Hz) was dramatically increased over the eupneic condition (Fig. 5, top right) particularly during the first half of inspiration (Fig. 5, hyperpnea time-frequency coherence). During transition bursts, over all animals, power was increased compared to eupnea in a manner similar to hyperpnea, with a notable addition of the highest frequencies (130–190 Hz; Fig. 5, top row). In this condition, power was distributed more heavily to the 50-100 Hz band than the other conditions, particularly during the final third of
inspiration (Fig. 5, time-frequency rel. power). During gasping, power was concentrated in two low-frequency bands during the first half of inspiration (Fig. 5, bottom left). During transition and gasping bursts, significant coherence was well-matched to both the frequency and time course associated with significant power.

**State-Dependent Changes in Power and Coherence In Vivo**

In addition to the shift in power peaks to higher frequencies, the other major difference between in vivo and 32–34 °C in situ preparations was that the former had significant power and coherence in the 145–195 Hz band (Fig. 4), while the coherence between left and right Ph activity was relatively low at these frequencies in vivo (Fig. 6). A large fraction of power was contained in the 50–100 Hz band during the latter half of inspiration and in the 100-130 Hz band throughout the entire burst (Figure 6, top row; Table 3). Coherence in these two bands was strongest during the last third and first half of the burst, respectively. During hyperpnea, relative power shifted to the 100–150 Hz domain and appeared ‘fractured’ into many sub-bands (Fig. 6, top left, blue trace), although significant power was retained during late inspiration in the 50–100 Hz band (Fig. 6 time-frequency rel. power). Finally, gasping produced oscillations in two major bands (peaks at 63 and 88 Hz, Table 3), restricted to early inspiration (Fig. 6, bottom left). Significant coherence was noted in all major power bands (Fig. 6, right column), with highest values during hyperpnea.
Discussion

With specific regard to our hypotheses, our results suggest that:

1. juvenile preparations generate responses to anoxia qualitatively intermediate between neonates and adults, as evidenced by the presence of transition bursts, which were not observed in adults in our previous study (14).

2. responses in situ are history-dependent in that silent primary apneusis in anoxic responses is eliminated by multiple exposures to anoxia in situ, even allowing for complete recovery between them.

3. the two juvenile preparations are similar but not identical, presumably because higher operating temperatures in vivo promote higher frequency oscillations and increased spectral entropy. Fast oscillations demonstrated state-dependent behaviors qualitatively similar to adults, including loss of higher frequencies during gasping.

4. at temperatures above 32 °C, fast oscillations are organized in the juvenile in a manner analogous to adults, with power concentrated in two major bands, the major caveat being that they are located at significantly lower frequencies than adults. Neither preparation produced strong oscillations at frequencies equivalent to those reported as HFO in adults (14).

5. functional coupling between the two phrenic nerves is not significantly temperature-dependent, as significant coherence was observed at all temperatures in frequency bands containing significant oscillations.

In addition, we also found that:

1. while respiratory rate increases with increasing temperature, auto-regulation of the overall respiratory rhythm in the juvenile is evident in the consistency of the Ti/Te ratio, and
2. both Ph and XII were characterized by a high-amplitude synchronous discharge at the onset of Ph activation at temperatures $\geq 28 \, ^\circ C$.

Taken together, these results suggest that the juvenile in situ rat preparation is a reasonable model for certain respiratory behaviors in age-matched in vivo decerebrate preparations. These include general rhythm generation, and the responses to anoxia (with the exception of the presence of transition bursts during primary apnea). The response patterns to anoxia produced during continuous perfusion in situ differ slightly from those produced by asphyxia in vivo, presumably because the latter typically causes ischemia (and when it does, no transition bursts are produced), while anoxic perfusion was continued in situ. Thus, respiratory responses are dependent on the cardiovascular response, and this may or may not be age-dependent. Interestingly, the sequence of systemic responses to repeated anoxic challenges is history-dependent in situ, resulting in the elimination of silent primary apnea. This implies a change in responsiveness that results in an earlier initiation of auto-resuscitation, a system dynamic that may serve to resuscitate the animal sooner in the face of recurring hypoxia. Due mainly to our current inability to cleanly delineate between different states following the 3rd or 4th challenge, we could not define the state-specific changes in fast oscillations caused by multiple anoxic challenges. Advances in analytical tools will be required to do so in future studies of plasticity.

Significant temperature-dependent effects were evident in situ. The most important is that of production of fast oscillations in the Ph activity. Only one other study (26) examined the relationship between power spectral components and temperature in this preparation, and our eupnea results are in general agreement with theirs, with certain exceptions. For example, we report two distinct peaks at 34 $^\circ C$ (Fig. 4, top) while they report just one (Fig. 4 of ref. 26). This
discrepancy may have resulted from different analytical methods, as our ZIS-based method is demonstrated to have better resolving power than standard parametric FFT analysis of the same data (see 14). Alternatively, this difference may reflect a much finer frequency resolution employed in this study, although the authors did not specify this parameter. This methodological issue may also explain why our phrenic power spectra are shaped so differently than those reported by Leiter and St.-John (10). Another difference between our results and the study by St.-John and Leiter (26) is their failure to observe significant coherence at low frequencies (<80 Hz) at 31 °C, while we did at both the 28–30 and 32–34 temperature ranges. The authors’ analytical method may have missed these because of the transient nature of Ph coherence (Fig. 4), for which our time-frequency methods are particularly well-suited. However, significant coherence was clearly discernable in our ZIS time-averaged estimates, even though they were averaged across all animals (Fig. 4, top right), which requires that the results are consistent across animals. Nonetheless, regarding eupnea, our overall observation of an increase in power at higher frequencies as temperature increases supports their findings.

However, our results are in complete discord with those of St.-John and Leiter (26) regarding gasping. This disagreement appears to be due in part to differences in classification criteria. Although the authors did not show the transition from eupnea to gasping in continuous data records (Figs. 1, 2 of the present study), their description that “within ~1 min, the incrementing pattern of eupnea was replaced by the decrementing pattern of gasping” places this behavior during our transition bursts (Fig. 1A). We note that bursts they classify as gasps (Figure 2 of ref. 26) resemble our bell-shaped or slowly-decrementing bursts during hyperpnea or transition (Fig. 1B). Accordingly, the spectral components they attributed to gasping correspond roughly to those we attribute to the hyperpnea or transition bursts at similar
temperatures. Since only the Ph was recorded, the authors could not verify that the Ph bursts they classified as gasps began simultaneously with XII activation, a commonly-recognized criterion (e.g., 14, 21, 27). Furthermore, the authors did not attempt to correlate their observations with age-matched in vivo preparations. We made this direct comparison, and our classification scheme is perfectly consistent with the behavior of pre-collicularly decerebrated in vivo animals of the same age (Fig. 2D). If these bell-shaped or slowly decrementing bursts are the only types of anoxic responses the authors observed, this may be due to the differences in methods used to alter respiratory state. Specifically, we perfused the preparation with an anoxic/normocapnic solution, while they used a hypoxic/hypercapnic solution (26).

In addition to differences in behavioral classification, the St.-John and Leiter (26) report inexplicably low respiratory rates for eupnea at 31 °C (11.2 ± 0.7 breaths/min; Table 2 of ref. 26), which is not only many SE units from the rates we presently report at 32–34 (26.4 breath/min) or 28–30 °C (21.7 breaths/min), but also from their own recently published results at 31 °C (25 breaths/min; Table 1 of ref. 11), though this reported value is discordant with the raw data shown in the same paper (~12–14 breaths/min; Fig. 2, ‘Eupnea’ in ref. 11). The combination of differences in gasp classification and inconsistency between basic respiratory rhythmic behavior ultimately precludes us from making meaningful comparisons to our present results.

The in situ preparation is used to study a variety of respiratory behaviors, and researchers have used this preparation at lower temperatures for a host of reasons (e.g., maintenance of the preparation for longer periods). Depending on the goal of these studies, such as functional connectivity within the respiratory network, this may have little consequence. However, our results suggest that temperatures below 32 °C are not appropriate for studying the mechanisms of
fast oscillations at frequencies even remotely similar to those produced in vivo. We did not examine temperatures above 34 °C in situ because perfusion rates required to meet metabolic demands at these temperatures produce significant edema. Thus, direct comparisons between the two preparations should be made with caution. Nonetheless, two dominant frequency bands were observed in both juvenile preparations, suggesting that the developmental precursors to adult MFO and HFO are present and, with the caveat of lower-frequency output, we conclude that this preparation may be used to study mechanisms of fast oscillations in respiratory motor activity. The observation that during gasping power is split between two low-frequency bands also suggests a direct behavioral analogy to the adult rat in vivo (14).

Our results also suggest that the juvenile in situ preparation’s complexity (i.e., spectral entropy; 19) dramatically increases with temperature, implying that the system operates in fundamentally different states at low vs. high temperatures. This increase in complexity is characterized by the progression from a “single band” low-frequency output at low temperatures to a multi-band output at higher temperatures. Likewise, at 32–34 °C in situ eupnea and gasping appear to be relatively low-complexity states, while hyperpnea or transition states produce higher levels of complexity. In vivo, hyperpnea also demonstrates higher complexity than eupnea or gasping, similar to changes described in the adult cat during fictive gasping (2).

Although we found that neither juvenile preparation managed to produce significant power during eupnea at frequencies corresponding to HFO in adults (14), the system is capable of generating oscillations at these frequencies under conditions that produce hyperpneic or transition bursts (Figs. 5 and 6). We conclude that the underlying cause for lack of adult HFO during eupnea is the relative immaturity of the system, as the in vivo juvenile oscillations (Fig. 6) differed significantly in frequency from those reported for adult rats in vivo (14). When
considering our present results with those previously characterized in adult rats in vivo (14, 15), one reasonably concludes that the major fast oscillation bands increase in frequency during development. Although it is not clear if entirely new circuit and cellular mechanisms develop to produce HFO in the adult or if the same mechanisms operate in a different regime, the generation of significant oscillations in this band associated with maturation has been described in pigs (3, 24), cats (9, 23), and rats (9). This conclusion is also supported by another study of young adult rats (13) that generated oscillations at frequencies between those displayed here and those we reported in older animals in vivo (14). Qualitatively speaking, the power spectral dynamics of either juvenile preparation contain the same features as the adult in eupnea (low frequency “start-up”, early HFO, late MFO), hyperpnea (highly fractured frequency bands including at high frequencies), and gasping (split between two early low-frequency bands). Thus, this developmental stage appears to generate similar dynamics in motor neuron synchrony as the adult, if at lower absolute frequencies, implying that efficient diaphragmatic activation requires correlated drive at lower frequencies than in, but similar patterns to, the adult.

Interestingly, significant low-frequency coherence between right and left Ph activity is present even at the lowest temperatures studied in situ (Fig. 4), and as temperatures rise and power is expressed in higher frequencies, coherence is decreased (but still significant) in the representative low frequencies. This observation suggests that increases in temperature cause network instabilities with regard to bilateral phase constancy, resulting in a partial decoupling of left and right Ph activity. Nonetheless, the relative strength of coherence in both preparations implies that these motor neuron pools are driven by common sources and/or are locally coupled. Functional coupling between the left and right Ph output has been noted in neonates (30), and our results suggest that it is quite robust in juveniles. More specifically, HFO tend to have more
consistently high coherence than the MFO in adult rats (15). In the present study, we note that MFO analog in the juvenile rat displays coherence values equal to those of HFO (Figs. 4 and 6), suggesting that the common input associated with MFO is more highly expressed in juveniles than adults.

**Perspectives and Significance**

Although not definitively proven, the observation of fast oscillations in respiratory motor neuron pools implies synchronous activation among (a subset of) the motor neurons. This mechanism (along with brief high-frequency stimulation at onset) has been shown to increase the efficiency of muscle activation. Although the juvenile in situ model has been widely used to study cardiovascular and respiratory control function, it has not been carefully characterized in terms of its production, dynamics, and coherence in fast oscillations. Moreover, these preparations are commonly used under a variety of conditions, including different temperatures, without explicit knowledge of how these may affect fast oscillations, if present. This study quantitatively characterizes the fast phrenic oscillations present in equivalent in situ and in vivo preparations, and the temperature- and state-dependence in situ, thereby allowing for interpretation of results from single-cell to systemic function in this model, with particular emphasis on their relationship to the adult. As the in situ preparation affords more accessibility of the brainstem and spinal circuitry, characterization of the fast oscillations in this preparation will provide context for future studies that elucidate the mechanisms underlying production of synchronized oscillations.

**Grants**

This work was supported by a grant (RFR) from the National Institutes of Health, R01 HL68143.
REFERENCES


Figure Legends

Figure 1. Production and classification of anoxia-induced inspiratory behaviors in the juvenile rat in situ at 34 °C. A: the first anoxic stimulus (beginning at arrow and maintained throughout the remainder of the record; see Methods for details) results in progression from eupnea (E) to hyperpnea (H) to a primary apnea that includes transition (T) bursts, followed by gasping (G). Traces: raw hypoglossal (XII, top) and phrenic (Ph, bottom) nerve activity. B: expansion of all bursts during the transition phase in A, demonstrating bell-shaped (b) and decrementing (d) burst patterns, each of which begin after onset of XII activity (lines). \[ \text{XII} \text{ and } \text{Ph} \text{ are integrated activity (} \tau = 50 \text{ ms).} \] C: expansion of traces during gasping (period indicated by gray bar in A), demonstrating simultaneous onset of Ph and XII. D: the fifth anoxic challenge produces a response pattern that is devoid of silent apneusis periods, as phases proceed without interruption from eupnea to gasping.

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Figure 3. Temperature effects on oscillations. Top four pairs of traces: simultaneously recorded individual bursts of XII and Ph activity from one in situ preparation at temperatures indicated above each pair. Ph oscillations (arrows) are evident at all temperatures, but increase significantly in frequency at 32–34 °C. Note large, synchronous Ph activation (‘start-up’) at Ph burst onset, and in XII at temperatures from 28–34 °C. Bottom pair of traces: example of in vivo XII and Ph bursts, showing both fast and slow oscillatory behavior. Time bar applies to all traces.

Figure 4. Temperature-dependence of dynamic power and coherence in situ during eupnea. Top row: right phrenic power spectra (left) and left-right phrenic coherence functions (right) determined by time-averaged ZIS method, averaged over all animals (n=6) during eupnea, at different temperatures (indicated by colors). For comparison, power and coherence of expiratory activity in situ (noise, 32–34 °C) and in vivo eupneic values (green) are shown. Bottom Four Rows: time-frequency power (left) and coherence (right) functions for an individual in situ preparation at temperatures ranges indicated. Note the increase in power at 80–110 Hz, particularly early in the inspiratory burst, as temperature increases. \( x \)-axes: normalized inspiratory time from beginning (0) to end (1) of burst. \( y \)-axes: frequency (Hz). Note different relative power and coherence scales associated with different temperatures.

Figure 5. State-dependent changes in dynamic power and coherence in situ at 32–34 °C. Top: right phrenic power spectra (left) and left-right phrenic coherence functions (right) determined by time-averaged ZIS method, averaged over all animals (n=10) grouped according to state (colored traces). Bottom Three Rows: time-frequency power (left) and coherence (right)
functions for an individual in situ preparation during hyperpnea, transition and gasping. Axes as in Fig. 4. Note different relative power and coherence scales associated with different states.

**Figure 6.** State-dependent changes in dynamic power and coherence in the juvenile rat in vivo at 37 °C. **Top Row:** right phrenic power spectra (left) and left-right phrenic coherence functions (right) determined by time-averaged ZIS method, averaged over all animals (n=8) grouped according to state (indicated by color). **Bottom Three Rows:** time-frequency power (left) and coherence (right) functions for an individual in situ preparation during eupnea, hyperpnea, and gasping. Axes as in Figs. 4 and 5. Note different relative power and coherence scales associated with different states.

**Tables**

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**Table 1.** General eupnic respiratory parameters in situ and in vivo. **Abbreviations:** temp, temperature; T, respiratory period; Rate, respiratory rate; Ti, inspiratory duration; Te, expiratory duration; XII prePh/Ph-r, ratio of pre-phrenic to phrenic-related hypoglossal activity. *P<0.05 between successive temperatures.
### Table 2.

Temperature-dependent Ph power and coherence features in situ. All band range and peak location values are in Hz, and relative amplitude (RelAmpl) of power spectral peak values are in %. *State abbreviations:* E, eupnea; H, hyperpnea; T, transition; G, gasping. Dashed lines separate peaks and bands within the same state-temperature combination.

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Table 3. Spectral and coherence features for in vivo preparations under three states. Power spectral peaks, coherence peaks, and band ranges all in Hz. Power spectral peak amplitudes (RelAmp) in %. Dashed lines separate different bands/peaks present during the same state.
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