Genome and Hormones: Gender Differences in Physiology

Selected Contribution: Time-dependent hypoxic respiratory responses in female rats are influenced by age and by the estrus cycle

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Zabka, A. G., M. Behan, and G. S. Mitchell. Selected Contribution: Time-dependent hypoxic respiratory responses in female rats are influenced by age and by the estrus cycle. J Appl Physiol 91: 2831–2838, 2001.—Age affects time-dependent respiratory responses to episodic hypoxia in male rats, particularly long-term facilitation (LTF), a serotonin-dependent respiratory “memory” [Zabka AG, Behan M, and Mitchell GS, J Physiol (Lond) 531: 509, 2001]. Because age and gender influence serotonergic function, we tested the hypotheses that the short-term hypoxic response (STHR), posthypoxia frequency decline (PHFD) and LTF of phrenic and hypoglossal (XII) motor output change with age and stage of the estrus cycle in female rats. Young (3–4 mo) and middle-aged (13 mo) female Sprague-Dawley rats were anesthetized, paralyzed, vagotomized, and ventilated. STHR was measured during and PHFD after the first of three 5-min episodes of isocapnic hypoxia (arterial PO2 35–45 Torr). LTF was assessed 60 min postepisodic hypoxia. Phrenic and XII STHR increased with age (P<0.05). PHFD was unaffected by age or gender. Phrenic LTF increased with age in both estrus and diestrus (P<0.05), whereas XII LTF increased in middle-aged female rats during diestrus only. Age and gender influence time-dependent hypoxic phrenic and XII responses in a complex manner.

respiratory control; plasticity; hypoxia; serotonin; gender

AGE AFFECTS THE CONTROL of breathing. For example, the short-term hypoxic ventilatory response is attenuated with advancing age in male rats (17). O2 consumption, CO2 production, and the ventilatory response to CO2 are also altered by growth and aging (16). The control of breathing is also influenced by gender hormones, particularly at different stages of the estrus cycle, during pregnancy and after menopause (46, 48). However, little is known concerning the specific mechanisms of respiratory control affected by age and/or gender.

In this study, we investigated the effects of age and the estrus cycle on selected time-dependent respiratory responses to episodic hypoxia (45) including 1) the short-term hypoxic response (STHR), 2) posthypoxia frequency decline (PHFD), and 3) long-term facilitation (LTF). These time-dependent hypoxic responses have been studied extensively in anesthetized male rats (18, 40), including the effects of advancing age (56). Although aging had no effect on the phrenic or hypoglossal (XII) STHR in male rats, it greatly diminished LTF (56).

LTF is an example of plasticity in respiratory control that occurs after episodic hypoxia or electrical stimulation of carotid chemosensory neurons in several species (15, 23, 39, 42, 45). LTF after episodic hypoxia is primarily observed as an augmentation of integrated phrenic and XII burst amplitude (ΔDPhr and ΔDXII), with a smaller increase in burst frequency (4, 18, 45). Serotonin (5-hydroxytryptamine; 5-HT) plays an important role in respiratory motor control (8, 11, 37). Serotonin receptor activation is required to elicit phrenic and XII LTF (18, 40). Specifically, 5-HT2 receptor activation is required during, but not after, episodic hypoxia (20), suggesting that 5-HT2 receptor activation is necessary for the induction but not for maintenance of LTF (40).

Serotonergic function is influenced by age and gender. Age-related alterations in the number of serotonergic neurons, 5-HT and 5-hydroxyindoleacetic acid concentrations, 5-HT receptor density, and 5-HT re-
uptake protein have been reported in different regions of the brain (9, 21, 25, 38, 41, 44, 51) and spinal cord (29). In the XII nucleus, a reduction in 5-HT immunoreactivity has been described in middle-aged male rats (6). Gender hormones also affect the serotonergic system, and their influence can lead to changing levels of 5-HT in the central nervous system (7, 34, 43).

Because LTF is 5-HT dependent (4, 40) and preliminary observations suggest that 5-HT immunoreactivity is increased in the XII nucleus of middle-aged female rats (M. Behan and M. S. Brownfield, unpublished observations), we tested the hypothesis that XII and possibly phrenic LTF in female rats increases with age, unlike in male rats. Furthermore, because the stage of the estrus cycle influences both ventilatory control (48, 49) and serotonergic function (22), we predicted that LTF would differ between estrus and diestrus.

**METHODS**

**Experimental groups.** Experiments were performed on intact female Sasco Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA; rat colony K-62, Kingston, NY). Two age groups were studied in both estrus and diestrus (Table 1): young female (3–4 mo; YFE and YFD, respectively) and middle-aged female (13 mo; AFE and AFD, respectively). All experimental procedures were approved by the University of Wisconsin-Madison Animal Care and Use Committee.

**Determination of estrus cycle.** Rats were briefly anesthetized with isoflurane daily to assess the progression of the estrus cycle. Epithelial cells were collected by vaginal swab for at least one complete estrus cycle as assessed under light microscopy (24). Only animals clearly in estrus or diestrus were studied.

**Experimental preparation.** Anesthesia was initiated with isoflurane in an induction chamber and maintained with a nose cone and then through a tracheal cannula (2.5–3.0% in 50% O₂, balance N₂). The tracheal cannula was placed to allow artificial pump ventilation (model 683, Harvard Apparatus, Holliston, MA) and for monitoring tracheal pressure. Rats were slowly converted to urethane anesthesia (1.6 g/kg i.v.) and placed on bipolar silver wire electrodes. Nerve activities were amplified (10,000), band-pass filtered (100 Hz to 10 kHz) (model 1700, A-M Systems, Carlsborg, WA), and integrated (time constant 3 kHz) (model 1265, Novametrix; Wallingford, CT), with sufficient response time to measure expiratory gases in rats.

**Experimental protocol.** Nerve signals were allowed to stabilize for ~60 min after surgical procedures under hyperoxic (P<sub>ₐO₂</sub> > 150 Torr) and normocapnic conditions. The CO₂ apneic threshold was determined by monitoring end-tidal P<sub>CO₂</sub> while increasing the ventilation pump rate to lower P<sub>ₐCO₂</sub> until phrenic nerve activity ceased and then decreasing ventilation until rhythmic phrenic nerve activity resumed. The end-tidal P<sub>CO₂</sub> at the resumption of phrenic activity was designated as the apneic threshold. Baseline nerve activities were established by increasing end-tidal P<sub>CO₂</sub> 2–3 Torr above this CO₂ apneic threshold by increasing inspired CO₂ and/or decreasing respiratory pump rate. After phrenic and XII nerve discharge had become stable, baseline conditions of blood gases, pH, and base excess were assessed by an initial blood sample. All subsequent blood samples were compared with this initial baseline value. Strict isocapnic conditions (~1 Torr from baseline P<sub>ₐCO₂</sub>) were maintained throughout an experiment by monitoring end-tidal P<sub>CO₂</sub> and making adjustments in ventilation rate and/or inspired CO₂ as necessary. To prevent alveolar atelectasis, the lungs were hyperinflated approximately every 60 min.

A schematic presentation of a protocol is shown in Fig. 1. The protocol started with three episodes of isocapnic hypoxia of 5-min duration (inspired O₂ fraction = 0.11–0.12, target P<sub>ₐO₂</sub> = 35–45 Torr), separated by 5 min of hyperoxia. The rats were then monitored for 60 min while isocapnic conditions were maintained in hyperoxia. Arterial blood samples were drawn at 15, 30, and 60 min after the final hypoxic episode to confirm isocapnic conditions. A protocol ended with 5 min of hypoxia to compare nerve activity to that during the first hypoxic episode. Subsequently, 5 min of hypercapnia (end-tidal P<sub>CO₂</sub> 80–90 Torr) was administered to assess maximal (CO₂-stimulated) nerve activity.

Rats with deviations in P<sub>ₐCO₂</sub> greater than 1 Torr from the baseline value were excluded from analysis. Therefore, changes in P<sub>ₐCO₂</sub> had minimal impact on the results of this study. Also excluded were animals with a blood pressure decrease of more than 20 mmHg from baseline conditions at 60 min posthypoxia.

**Data analysis.** Phrenic and XII nerve activities were recorded throughout the protocol. Peak integrated amplitude (ΔPhr and ΔXII), burst frequency (bursts/min), and mean arterial pressure were measured at the following time points: baseline; last minute of first hypoxic episode (STHR); 15, 30, and 60 min after the final hypoxic episode; and during the final minute of the hypercapnic response. Nerve activities were averaged over ~60 s in each condition. Changes in amplitude from baseline were normalized as a percentage of

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**Table 1. Experimental groups**

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Estrus</th>
<th>Diestrus</th>
<th>Estrus</th>
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</thead>
<tbody>
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<td>YFD</td>
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<td>YFD</td>
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<td>256 ± 8</td>
<td>253 ± 9</td>
<td>340 ± 11</td>
<td>345 ± 15</td>
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</table>

Values are means ± SE. XII, hypoglossal.
baseline nerve activity (%baseline) and as a percentage of the hypercapnic response (%maximum). Changes in burst frequency were expressed as a difference from baseline in bursts per minute.

Depending on the variable, either a one-way or a two-way ANOVA with a repeated measures design (SigmaStat Version 2.0, Jandel, San Rafael, CA) was performed, followed by a least significant difference post hoc test for individual comparisons. Differences were considered significant if \( P < 0.05 \). All data reported are means ± SE.

RESULTS

Apneic threshold, baseline, and \( \mathrm{CO}_2 \) regulation. The \( \mathrm{CO}_2 \) apneic threshold was not significantly different among the four groups of rats, and thus baseline \( \mathrm{PaCO}_2 \) values were similar (Table 2). Within each group, mean \( \mathrm{PaCO}_2 \) remained within 1 Torr of this baseline value throughout the protocol.

Baseline respiratory activity. The ratio of baseline to maximal \( \mathrm{CO}_2 \) response for phrenic nerve activity did not differ between young and middle-aged rats in estrus or diestrus (YFE = 0.50 ± 0.07, YFD = 0.45 ± 0.03, AFE = 0.31 ± 0.04, AFD = 0.36 ± 0.07; \( P > 0.05 \)), and thus differences in the range from baseline to maximal phrenic activity should not have influenced our results. However, there was a difference in this ratio for XII activity (YFE = 0.46 ± 0.05; YFD = 0.35 ± 0.04; AFE = 0.30 ± 0.03; AFD = 0.26 ± 0.04; YFE was significantly greater than AFE and AFD, \( P < 0.05 \)), suggesting that the dynamic range was greater in middle-aged female rats. The impact of this difference in baseline activity relative to maximal activity is unclear.

STHR. \( \mathrm{PaO}_2 \) during hypoxic episodes was comparable in the four groups of rats (YFE = 38 ± 1, YFD = 38 ± 1, AFE = 35 ± 2, and AFD = 39 ± 2 Torr; \( P > 0.05 \)). The phrenic STHR, expressed as %baseline, was significantly greater in middle-aged vs. young rats (\( P = 0.02 \)) but did not differ between estrus and diestrus within an age group (Fig. 2A). When expressed as percentage of maximal \( \mathrm{CO}_2 \) response (%\( \mathrm{CO}_2 \) max), the phrenic STHR was greater in the AFE group vs. YFE, YFD, and AFD (Fig. 2B; \( P = 0.01 \)). The XII STHR did not differ among groups when expressed as %baseline or as %\( \mathrm{CO}_2 \) max (Fig. 2, C and D). Frequency responses during hypoxia did not differ among groups (Fig. 2E).

Phrenic LTF. When all four groups are included in the ANOVA, phrenic amplitude (%baseline) progressively increased with time postepisodic hypoxia (\( P < 0.001 \)), indicating the development of LTF in female rats. However, individually, young female rats in estrus and diestrus did not show significant LTF at any time postepisodic hypoxia (Fig. 3A). In the AFD group, phrenic amplitude was significantly higher relative to baseline at 15, 30, and 60 min postepisodic hypoxia and significantly higher at 30 and 60 min compared with both groups of young rats (YFE, YFD) (Fig. 3A).

When expressed as %\( \mathrm{CO}_2 \) max, phrenic amplitude showed a significant (\( P < 0.001 \)) overall increase postepisodic hypoxia, confirming the development of LTF. Phrenic LTF (%\( \mathrm{CO}_2 \) max) showed the greatest age-associated increase in AFD. YFD and AFE rats had significant LTF at 60 min and the AFD group at 15, 30, and 60 min postepisodic hypoxia (\( P < 0.05 \)). LTF was significantly greater in the AFD group vs. YFE at 30 and 60 min postepisodic hypoxia (\( P < 0.05 \)) (Fig. 3B).

XII LTF. Typical examples of a XII neurogram of two young (estrus and diestrus) and two middle-aged (estrus and diestrus) female rats are shown in Fig. 4. There was a significant (\( P < 0.001 \)) overall time effect on XII burst amplitude after episodic hypoxia (%baseline), indicating the development of XII LTF in female rats. However, individual groups did not show significant XII LTF vs. baseline, with the exception of AFD (Fig. 3C). At 30 and 60 min, \( \Delta \)XII in the AFD group showed significantly greater LTF compared with AFE, YFE, and YFD (Fig. 3C).

When expressed as %\( \mathrm{CO}_2 \) max, XII amplitude showed a significant (\( P < 0.001 \)) overall time effect,
confirming the existence of XII LTF in female rats. Significant LTF vs. baseline occurred in the AFE group at 60 min and in the AFD group at 15, 30, and 60 min postepisodic hypoxia (all \( P < 0.05 \)). LTF in AFD was significantly greater vs. YFE at 15, 30, and 60 min (\( P < 0.05 \)) (Fig. 3D).

Burst frequency. Although all four groups of rats showed an increase in burst frequency after episodic hypoxia, there were no significant differences among individual groups at any time point (Fig. 3E), and only the YFD had a significant increase vs. baseline at 60 min postepisodic hypoxia (\( P < 0.05 \)).

Mean arterial blood pressure. Mean arterial blood pressure was similar in all four groups of rats (Fig. 5). During all three hypoxic episodes, mean arterial pressure decreased significantly, as is commonly seen in anesthetized rats (4, 27), but was not different from baseline at any time postepisodic hypoxia.

DISCUSSION

The major findings in the present study on female rats are 1) the magnitude of the phrenic (but not XII) STHR increases with age but is not estrus cycle dependent; 2) PHFD is not affected by age or the estrus cycle; 3) LTF of phrenic and XII motor output increases with age, with greater increases in phrenic vs. XII LTF; and 4) LTF differs with the stage of the estrus cycle, being more prominent during diestrus. These results provide a striking contrast with male rats, in whom age does not affect phrenic STHR and actually decreases phrenic and XII LTF (56).

Apneic threshold, baseline, and \( CO_2 \) regulation. Several studies have demonstrated that high circulating levels of progesterone (during diestrus or pregnancy) are associated with an increase in resting ventilation and decreased \( Paco_2 \) (1, 5, 35, 46, 48, 55). Although there were no significant differences in apneic thresholds (and thus baseline values) among rat groups, a trend toward lower baselines in young and middle-aged rats during diestrus was observed (Table 2). A lower baseline \( Paco_2 \) in diestrus would be consistent with increased drive to breathe under baseline conditions, consistent with the predicted actions of progesterone. To avoid any potential complications associated with shifts in the \( CO_2 \) apneic threshold, individual determinations of the \( CO_2 \) apneic threshold were
made. Baseline PaCO₂ was established 2–3 Torr above this level, thus standardizing baseline respiratory drive for all animals. Strict isocapnic conditions with respect to this baseline value were maintained throughout each protocol in all groups of rats. Therefore, uncontrolled changes in PaCO₂ are not likely to have influenced the outcome of our experiments.

Baseline PaCO₂ in female rats (Table 2) was higher than is normally seen in male rats under similar experimental conditions (18, 40, 56). The same result was found in a study on anesthetized female and male rats (50), revealing significantly higher PaCO₂ in normoxia and hyperoxia. Although these authors suggested that this increase may be due to higher compliance and a more efficient O₂ transport system in female rats, this is unlikely in the present study because blood gases were rigorously controlled and pulmonary mechanics were bypassed by recording phrenic motor output.

STHRs. The severity of hypoxia was similar for all groups of rats and, therefore, did not directly influence the experimental outcome. Our findings that the short-term hypoxic phrenic response increased significantly with age (but not the estrus cycle) contrasts with studies on female humans and cats, in which the hypoxic ventilatory response is greater during the luteal phase of the menstrual cycle (i.e., diestrus) (49, 55). Although there was a difference in the baseline-CO₂ maximal response ratio in XII motor output, suggesting the potential for greater XII response in middle-aged female rats relative to young female rats in diestrus and in estrus. In middle-aged rats, XII LTF was significantly greater during diestrus compared with estrus. During diestrus, XII LTF was significantly greater in middle-aged rats than in young rats. There were no age- or estrus cycle-related differences in frequency at 60 min after hypoxic episodes. Values are means ± SE. *Significantly different from baseline; #Significantly different among groups.

PHFD. Posthypoxia frequency decline did not differ among groups of female rats nor was it different from young or middle-aged male rats (Zabka, Behan, and Mitchell, unpublished observations), suggesting that...
PHFD is unaffected by age, gender, and/or the estrus cycle.

LTF. The magnitude of phrenic and XII LTF varies among substrains of Sprague-Dawley rats (18, 19), revealing a genetic influence on the magnitude of LTF. However, repeated studies on young male Sasco Sprague-Dawley rats indicate an LTF of 60–100% in both phrenic and XII nerves 60 min postepisodic hypoxia (4, 18, 19, 27, 56). On the other hand, phrenic LTF decreases with age in male Sasco rats, and XII LTF is abolished (56). An increase in LTF can be elicited through preconditioning treatments such as cervical dorsal rhizotomy (28) or chronic intermittent hypoxia (33). Thus LTF, a form of respiratory plasticity, is in itself plastic (40). However, to date, there have been no other reports concerning variations of LTF with gender or the stage of the estrus cycle.

Regardless of the normalization used, LTF of phrenic and XII motor output in female rats increased with age but in a manner dependent on the stage of the estrus cycle. The most significant increase occurred in middle-aged rats during diestrus when 5-HT levels are high (22, 36).

Neither group of young female rats developed significant LTF at any time postepisodic hypoxia, contrasting with male rats of similar age (18, 40, 56). This finding may indicate a relative lack of serotonergic modulation of respiratory motor output in female rats of this age group. Although the reasons for this gender difference are not understood, gender hormones may play a pivotal role. Young female rats have a slower follicular development (12) and a greater number of smaller follicles with a lower concentration of 17β-estradiol than middle-aged cycling rats (32). Thus lower estrogen levels and/or a different estrogen-progesterone ratio might have been responsible for the failure to develop significant LTF in young female rats. In male rats, 5-HT decreases with age in the dorsal and ventral horns of cervical spinal regions associated with the phrenic motor nucleus (29). However, little is known concerning 5-HT spinal levels in female rats.

In both young and middle-aged rats, LTF was higher in diestrus relative to estrus (Fig. 3, A–D). Circulating levels of estrogen and progesterone vary with the stage of the estrus cycle, with high progesterone and progressively increasing estrogen levels during diestrus and high estrogen and progesterone levels in early estrus (14). The estrus cycle may influence the development of LTF via serotonin-dependent mechanisms. 5-HT in the central nervous system also varies with the estrus cycle, with higher levels during diestrus and lower levels detected in some brain regions during estrus (22, 36). Estrogen can influence the serotonergic system at a number of different levels. For example, estrogen displaces tryptophan, the 5-HT precursor, from plasma albumin binding sites (3, 34). Estrogen reduces monoamine oxidase, a 5-HT degrading enzyme (43); decreases serotonin reuptake transporter mRNA; and downregulates 5-HT1A autoreceptor activity (7). Each of these effects would enhance serotonergic function at target sites. Furthermore, the dorsal raphe nuclei of rats contain estrogen and progesterone receptors in cells adjacent to serotonergic neurons, raising the possibility that estrogen could influence raphe neurons and in turn modulate respiratory motor output (2, 7). Alternatively, higher circulating progesterone levels during diestrus might augment serotonin-dependent LTF. A study of female and male cats demonstrated that progesterone acts as a respiratory stimulant via progesterone receptors, whereas other steroids such as...
testosterone, estrogen, or cortisol could not elicit this stimulatory effect (5). Given that estrogen is required to induce progesterone-receptor expression (5, 7), it may be that the ratio of estrogen to progesterone is the critical factor in altering serotonergic modulation of the respiratory control system and thus LTF.

Limitations of methods. In this study, rats were anesthetized, vagotomized, paralyzed, and pump ventilated. However, the magnitude of ventilatory LTF in awake male rats is similar to the magnitude of phrenic LTF in anesthetized, vagotomized, paralyzed rats of similar age when isocapnic conditions are maintained (42). To our knowledge, no studies have been performed that compare LTF in awake and anesthetized female rats. We have preliminary, suggestive evidence that general anesthesia influences gender hormone levels in male and female rats. It appears that serum estrogen levels in female rats and testosterone levels in male rats (both young adult) decline with time in anesthesia whereas progesterone levels in both sexes male rats (both young adult) decline with time in anesthesia whereas progesterone levels in both sexes increase. Thus far, no studies have been performed on middle-aged or aged rats. Therefore, we cannot exclude the possibility that a differential anesthesia effect on gender hormones may account for differences in hypoxic ventilatory responses young and middle-aged female rats.

Possible significance. The results of our study demonstrate an increased ability in aging female rats to develop LTF. Serotonergic input to XII motor neurons may contribute to the maintenance of patent upper airways and prevent breathing disorders such as obstructive sleep apnea (OSA) or snoring (30, 52). The occurrence of OSA is minimal in young and middle-aged female rats. Postmenopausal women receiving hormone replacement therapy have a lower incidence of this breathing disorder than women without hormone replacement (10). Because menopause is caused by a decrease of ovarian hormone production (estrogen and progesterone), one could speculate that, before menopause, women might be protected from development of upper airway-associated breathing disorders by a neuroprotective role of ovarian hormones on the serotonergic modulation of upper airway muscles. Female rats do not undergo menopause but instead experience prolonged estrus cycles, finally leading to periods of persistent estrus (53). This might explain the maintained or even increased capacity for serotonergic modulation of respiratory motor output. In contrast to females, middle-aged male rats reveal an age-related decrease (phrenic) or loss (XII) of serotonin-dependent LTF (56), a striking coincidence with the peak occurrence of OSA in middle-aged men (26, 54). Although the physiological role of LTF remains unclear, we suspect that the investigation of its underlying mechanisms will suggest possible causes for age-related and gender-influenced breathing disorders.

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REFERENCES

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