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Development of ventilatory long-term facilitation is dependent on estrous cycle stage in adult female rats

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ABSTRACT

Ventilatory long-term facilitation (vLTF) is a form of respiratory plasticity characterized by a progressive and sustained increase in minute ventilation over time following acute, intermittent hypoxia (AIH). Though vLTF has been repeatedly demonstrated in adult males (rats and humans), few studies have assessed vLTF in adult females and no studies have explored differential expression of vLTF across the normal female estrous cycle. We recently reported that AIH-induced plasticity of phrenic motor output (phrenic long-term facilitation, pLTF), a phenotypically similar form of respiratory plasticity presenting as a sustained increase in phrenic nerve amplitude, develops in adult female rats only during the proestrus stage of the estrous cycle, notable for high levels of serum estrogen. Here, we tested the hypothesis that AIH-induced vLTF would also be estrous-stage dependent; developing in female rats during proestrus, but not estrus. Barometric plethysmography in adult (4–5 months), normally cycling female rats revealed a progressive increase in minute ventilation for 60 min following AIH (5 × 5 min episodes; 10% O_2) during proestrus indicative of vLTF, while estrus rats showed no changes in minute ventilation over the same time period. The development of vLTF in proestrus rats was driven by changes in tidal volume production versus respiratory frequency consistent with prior studies. These data are the first to investigate differential vLTF expression across the estrous cycle in adult female rats and highlight the importance of female estrous cycle stage as a critical physiological variable to consider in studies of AIH-induced plasticity.

1. Introduction

Brief exposures to modest levels of reduced oxygen (acute intermittent hypoxia; AIH) induce respiratory plasticity in laboratory rodents (reviewed in Dale et al., 2014; Devinney et al., 2013; Gonzalez-Rothi et al., 2015; Navarrete-Opazo and Mitchell, 2014a) and in human clinical studies (Harris et al., 2006; Mateika and Narwani, 2009; Syed et al., 2013; Tester et al., 2011), making AIH an appealing, non-invasive strategy to strengthen respiratory motor output in conditions of respiratory compromise. The specific mechanisms leading to AIH-induced respiratory plasticity are yet to be fully elucidated, however, our extensive knowledge of AIH mechanisms has derived mostly from carefully controlled experiments in anesthetized and ventilated rats. Under these conditions, AIH leads to a progressive and persistent increase in the motor output of respiratory nerves known as long-term facilitation (LTF). LTF has been shown in the motor outputs of phrenic (pLTF; Bach and Mitchell, 1996; Fuller et al., 2000; Hayashi et al., 1993), hypoglossal (Bach and Mitchell, 1996; Baker-Herman and Strey, 2011; Fuller, 2005; Golder and Martinez, 2008; Wilkerson et al., 2018), and intercostal (Fregosi and Mitchell, 1994; Navarrete-Opazo and Mitchell, 2014b) nerves, informing our understanding of region specific mechanisms of AIH-induced plasticity. However, studies in unanesthetized, freely breathing rodents and in humans are also clinically meaningful measures of AIH-induced respiratory plasticity. These experimental conditions inform whether AIH as a general respiratory stimulus can augment respiratory function at a systems level. Indeed, AIH has been shown repeatedly to progressively enhance minute ventilation in rats (McGuire et al., 2002; Olson et al., 2001) and humans (Babcock et al., 2003; Babcock and Badr, 1998; Harris et al., 2006; Wadhwa et al., 2008); termed ventilatory long-term facilitation (vLTF).

Most studies of AIH-induced plasticity have been completed with male rats, or in the case of human studies, with a weighted mix of male over female participants. Thus, our understanding of potential sexually dimorphic mechanisms of AIH-induced plasticity, or even more fundamentally, how females respond to AIH at a systems level, is significantly underdeveloped relative to what we know in males. Recently, our group demonstrated that female rats express AIH-induced pLTF only during the proestrus stage of the estrous cycle, notable for high

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levels of systemic estrogen (Dougherty et al., 2017). Female rats in estrus or with their ovaries removed (ovariectomy), physiological states with significantly reduced serum estrogen levels, did not express pLTF (Dougherty et al., 2017). These data provided evidence that estrogen may be a necessary component for AIH-induced plasticity in females (Dougherty et al., 2017; Zabka et al., 2001). Here we tested whether the estrous cycle dependence of AIH-induced pLTF translates to the unanesthetized, freely breathing model of vLTF. Consistent with our prior studies (Dougherty et al., 2017), we hypothesized that adult female rats would only express vLTF during proestrus, and would not express vLTF in the estrus phase when estradiol levels are reduced. Since previous studies of AIH-induced vLTF were performed using male rats (McGuire et al., 2002, 2008; Nakamura et al., 2010; Olson et al., 2001), these studies represent the first quantification of AIH-induced vLTF expression in female rats.

2. Materials and methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and conformed to policies detailed in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were performed with 4–5 month old female Sprague Dawley rats (Envigo; colony 206). Rats were housed in pairs in a mixed-sex rodent room within an AAALAC-accredited animal facility. They had access to food and water ad libitum and were maintained in 12 hr light-dark cycles (6 am–6 pm).

A crossover experimental design was employed to minimize the number of animals used. In total, 36 virgin female rats were studied (Fig. 1). Daily examination of vaginal cell characteristics in vaginal smears using light microscopy (Dougherty et al., 2017; Goldman et al., 2007; Marcondes et al., 2002) was used to track estrous cycles in all 36 rats beginning the morning after arrival to our facility. All rats demonstrated a minimum of two complete estrous cycles prior to initial plethysmography testing. Rats were characterized as being in estrus or proestrus immediately prior to entering the plethysmography chambers on the day of initial testing. Following the first round of plethysmography, all rats were returned to their home cages and staged daily for an additional 7 days, plus at least one complete estrous cycle, before being re-tested with plethysmography during the opposite estrous cycle stage as assessed just prior to entering the plethysmography chamber for the second time.

2.1. Barometric plethysmography

Barometric plethysmography (Data Sciences International, St. Paul, MN, USA) was used to quantify breathing in unanesthetized female rats during both the estrus and proestrus stages of the estrous cycle. We were specifically interested in whether the magnitude of ventilatory long- term facilitation (vLTF) following acute intermittent hypoxia (AIH) was impacted by estrous cycle stage. The protocol used to induce vLTF has been described previously (McGuire et al., 2002, 2008; Nakamura et al., 2010). Rats were acclimated to the plethysmography chambers for a minimum of 2 hours on a day prior to initial testing. Following an additional 60 min acclimation period on the day of testing, baseline ventilation was recorded under normoxic conditions (20.9% O_2, balanced N_2) for 30 minutes. During baseline measures, all rats were observed through the clear walls of the plethysmography chambers to be in a presumed state of quiet sleep with a recumbent posture and closed eyes (Navarrete-Opazo and Mitchell, 2014b). Experimental groups were subsequently exposed to AIH consisting of 5 × 5 min hypoxic exposures (10% O_2, balance N_2) separated with 5 min intervals of normoxia (McGuire et al., 2002, 2008; Nakamura et al., 2010). All experimental rats were aroused during hypoxia and remained awake and alert with eyes open through entire AIH period. After the fifth and final hypoxic exposure, rats breathed normoxic air for an additional 60 min, during which all experimental rats resumed a recumbent, eyes closed position indicative of a presumed state of quiet sleep. A separate group of rats was used as Time Controls to ensure stability of breathing measures (i.e., ensuring that there were no time-dependent changes in ventilation). These rats were in the chambers for equal lengths of time, but *did not receive AIH*. Time control rats had occasional periods of arousal that included positional adjustments, but these were sporadic and not observably different across stages of the estrous cycle; time control rats mostly demonstrated a posture consistent with a presumed state of quiet sleep, similar to experimental rats. No differences in ventilation were noted in estrous ($n = 12$) versus proestrus ($n = 12$) rats during Time Control experiments (2 way ANOVA; cycle stage × time interaction: $p = 0.86$), so data from all Time Control experiments were pooled into one group ($n = 24$) for final analysis. All experiments were performed at the same time of day, beginning 3–5 hours into the light phase of a 12 hr light/dark cycle.

Normoxic and hypoxic conditions were established in the respiratory chambers by mixing O_2 and N_2 using a customizable, computer controlled gas mixer (GSM-3, CWE, Inc.) to obtain desired inspired gas concentrations. Gas flowed continuously through each 4.0 liter chamber at 2.5 L × min^{-1} allowing for equilibrium of hypoxic gas mixes to take place in 76 ± 4 sec. Chamber temperatures remained between 22.5 and 24.0 °C. Daily chamber calibrations were completed using a standard 2.5 L × min^{-1} flow over 2 sec. Customizable plethysmography data acquisition software (Ponemah; Data Sciences International, St. Paul, MN, USA) recorded respiratory frequency (breaths × min^{-1}) and incorporated chamber temperature, atmospheric and chamber pressure, humidity and rectal temperature to calculate tidal volume (V_T; ml × br^{-1}; Drorbaugh and Fenn, 1955) and minute ventilation ($\dot{V}_E$; ml × min^{-1}). Chamber temperature, humidity

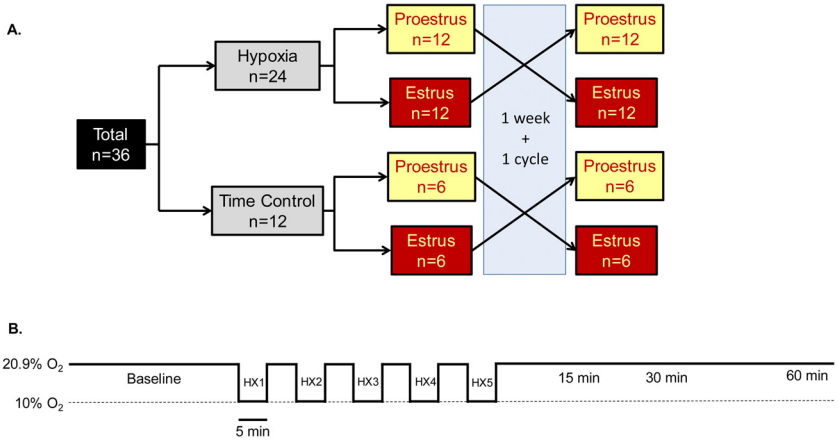

Fig. 1. Experimental design and plethysmography protocol. Ventilation was assessed with barometric plethysmography in 36 adult female rats using a cross-over design (A). All 36 rats experienced two full estrous cycles and were assigned to an initial group based on their estrous cycle stage just prior to entering the plethysmography chamber. Following the initial respiratory assessment, all 36 rats were returned to their home cages and staged daily for a period of 7 days plus one full estrous cycle before being re-tested in the opposite estrous cycle stage as assessed just prior to entering the plethysmography chambers. The experimental protocol consisted of a 30 min period of room air breathing (i.e., baseline) followed by 5, 5-min exposures to hypoxia (Hx; 10% O_2) with 5 min room air intervals (B). Rats returned to baseline, room-air breathing for 60 minutes following the final hypoxic episode. Rats designated as Time Controls were in the chambers for an equivalent period of time, but were not exposed to intermittent hypoxia.

Table 1
Physiological variables and ventilatory data. No significant differences in age ($p = 0.83$) or body weight ($p = 0.35$) were observed across experimental and time control groups. All rats showed a reduction in rectal temperature during plethysmography ($p < 0.001$ for all groups), however, no differences were noted in pre- or post-plethysmography temperatures between groups. During baseline, all respiratory parameters were equivalent. Proestrus and estrus groups received intermittent hypoxia and elevated all respiratory parameters when compared to the Time Control group which did not receive hypoxia. $\dot{V}_E$ was significantly higher in proestrus rats ($p < 0.01$) at 60 min compared to Time Controls.

Group	Proestrus	Estrus	Time Control
Age (days)	138 ± 3	134 ± 4	136 ± 5
Weight (g)	256 ± 4	257 ± 2	256 ± 4
Pre-Temp (°C)	37.7 ± 0.1	37.7 ± 0.1	37.7 ± 0.1
Post-Temp (°C)	36.8 ± 0.1^{+++}	36.9 ± 0.1^{+++}	36.8 ± 0.1^{+++}
Baseline			
$\dot{V}_E$ (ml min^{-1} 100 g^{-1})	41.4 ± 1.6	43.6 ± 1.3	41.4 ± 1.1
V_T (ml 100 g^{-1} breath^{-1})	0.69 ± 0.02	0.73 ± 0.01	0.74 ± 0.02
Freq. (breaths min^{-1})	57.5 ± 1.3	57.5 ± 1.4	55.0 ± 0.7
Hypoxia			
$\dot{V}_E$ (ml min^{-1} 100 g^{-1})	92.5 ± 2.6***	96.9 ± 2.6***	42.6 ± 1.1
V_T (ml 100 g^{-1} breath^{-1})	0.84 ± 0.03*	0.86 ± 0.02**	0.76 ± 0.03
Freq. (breaths min^{-1})	111 ± 3.9***	116 ± 5.0***	56.4 ± 1.3
15 min post-hypoxia			
$\dot{V}_E$ (ml min^{-1} 100 g^{-1})	42.5 ± 1.8	44.7 ± 1.2*	39.6 ± 1.0
V_T (ml 100 g^{-1} breath^{-1})	0.70 ± 0.02	0.74 ± 0.02	0.72 ± 0.02
Freq. (breaths min^{-1})	60.0 ± 1.4*	60.0 ± 1.0*	54.3 ± 1.1
30 min post-hypoxia			
$\dot{V}_E$ (ml min^{-1} 100 g^{-1})	43.2 ± 1.4	44.3 ± 1.4*	40.0 ± 0.9
V_T (ml 100 g^{-1} breath^{-1})	0.70 ± 0.02	0.73 ± 0.01	0.70 ± 0.02
Freq. (breaths min^{-1})	61.0 ± 1.9*	60.0 ± 1.7	55.2 ± 1.4
60 min post-hypoxia			
$\dot{V}_E$ (ml min^{-1} 100 g^{-1})	47.8 ± 1.9**	46.2 ± 1.4	41.2 ± 1.2
V_T (ml 100 g^{-1} breath^{-1})	0.77 ± 0.02	0.77 ± 0.02	0.73 ± 0.02
Freq. (breaths min^{-1})	61.0 ± 2.0	59.4 ± 2.0	56.2 ± 1.5

* $p < 0.05$ from Time Controls.
** $p < 0.01$ from Time Controls.
*** $p < 0.001$ from Time Controls.
$^{+++}$ $p < 0.001$ from Pre-Temp.

and pressures were constantly recorded; rectal temperatures were taken immediately prior to entering the plethysmograph chambers and immediately following the conclusion of the experiment. If temperatures changed by more than 0.5 °C over the course of an experiment, V_T and $\dot{V}_E$ were re-calculated at 60 min post-hypoxia (or equivalent time point in time control rats) using the final temperature (Doperalski et al., 2008; Dougherty et al., 2018; Fuller et al., 2006, 2008, 2009). All rats demonstrated a reduction in temperature over time consistent with prior studies (Nakamura et al., 2010), however, no differences in pre- or post-temperatures were noted between groups (Table 1).

Respiratory frequency data are expressed in absolute units (e.g., breaths × min^{-1}). V_T and $\dot{V}_E$ are reported in both absolute units per 100 g body weight (Table 1) and as a % change from baseline for calculation of vLTF. Baseline (BL) data represent mean values during a stable 10-min period prior to the start of hypoxia. Subsequently, 30 sec of stable breathing was averaged and analyzed during the first hypoxic exposure for assessment of hypoxic ventilatory response (HVR) in experimental groups, and 2-mins of stable breathing was averaged and analyzed at 15, 30 and 60 min post-hypoxia. Data at equivalent time points were analyzed in Time control rats. Analyses were completed using commercially available statistical software (GraphPad Prism, Version 7, La Jolla, CA, USA) and are presented as means ± 1 SE with graphical representations of individual data points to demonstrate spread of data about the mean. We employed one-way ANOVAs to compare ages and body weights (Table 1). One-way ANOVAs were also used to compare absolute values of respiratory parameters during BL, during the first hypoxic challenge (or equivalent time point in Time Control rats), and at 15 min, 30 min and 60 min (Table 1). An additional one-way ANOVA was used to compare HVR as a % change from BL across groups (Fig. 3). Two-way ANOVA was used to evaluate changes in body temperature within and across groups pre- and post-plethysmography (Table 1). To determine time-dependent changes in ventilation within and across groups during the post-hypoxic interval, or equivalent period in Time Control rats (% change from BL), we used a 2-way ANOVA with repeated measures. Tukey post hoc tests were used to identify statistically significant individual comparisons when significant main effects or interactions were present. With all statistical measures, differences were considered significant if $p < 0.05$.

3. Results

There were no differences in age ($p = 0.83$) or body weight ($p = 0.35$) across experimental and time control groups as shown in Table 1. Initial rectal temperature (immediately prior to entering the plethysmograph; pre-temp) was similar across groups and not affected by estrous cycle stage ($p \geq 0.93$; Table 1). A significant reduction in core body temperature was measured in all groups during plethysmography (2 way ANOVA, main effect of time, $p < 0.001$ $F(1, 69) = 316.4$) reflected in significantly reduced rectal temperatures immediately following plethysmography (post-temp; $p < 0.001$ for each group; Table 1). The temperature drop was experienced equally regardless of estrous cycle stage or treatment; all groups had similar post-plethysmography temperatures ($p \geq 0.71$; Table 1).

3.1. Baseline ventilation was similar in estrus and proestrus rats

Absolute ventilatory data are presented in Table 1 and representative traces from our barometric plethysmograph are presented in Fig. 2. Overall, BL $\dot{V}_E$ was similar across experimental groups ($p \geq 0.50$ for all BL comparisons) and was not impacted by estrous cycle stage. BL V_T ($p \geq 0.18$) and respiratory frequency ($p \geq 0.45$) were also similar across experimental groups; no statistical differences were noted.

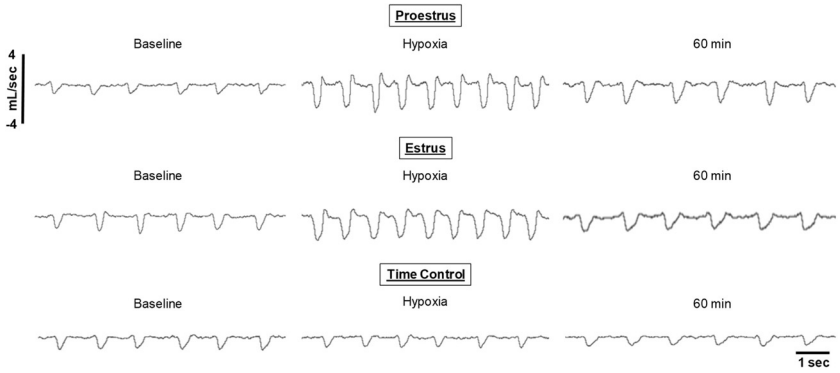

Fig. 2. Representative barometric plethysmography traces. All traces are identically scaled. No differences were observed with baseline ventilation or with ventilatory responses to hypoxia between experimental groups (estrus vs. proestrus). However, rats in the proestrus phase of the estrous cycle (Top trace) demonstrated an increase in the amplitude of the plethysmography trace over 60 min following acute intermittent hypoxia (AIH). This reflects an increase in tidal volume production and increased minute ventilation consistent with ventilatory long-term facilitation (vLTF). Time Control groups (bottom trace) were not exposed to AIH and were included to ensure stability of our respiratory measures (i.e., ensuring that there were no time-dependent changes in ventilation with plethysmography).

3.2. Hypoxic ventilatory responses were unaffected by estrous cycle stage

Regardless of estrous cycle stage, adult female rats in the proestrus and estrus groups significantly increased minute ventilation in response to hypoxia (Figs. 2 and 3A). This was reflected in significantly larger absolute $\dot{V}_E$, V_T, and frequency in these groups compared to the Time Control group which did not receive hypoxia (Table 1). The magnitude of the HVR (i.e., %change in $\dot{V}_E$ relative to BL) was similar in proestrus and estrus groups ($p = 0.83$; Fig. 3A). Components of $\dot{V}_E$ showed a similar pattern in response to hypoxia. Female rats in estrus and proestrus significantly increased V_T in response to hypoxia ($p < 0.001$) compared to Time Control rats, but to a similar extent ($p = 0.83$; Fig. 3B). Breathing frequency during hypoxia in estrus and proestrus groups was also significantly elevated compared to Time Controls ($p < 0.001$), with a similar magnitude of increased frequency in estrus and proestrus rats ($p = 0.64$; Fig. 3C).

3.3. vLTF was expressed only during proestrus

$\dot{V}_E$ was assessed over 60 min post-hypoxia to determine if AIH would lead to the development of vLTF, defined as a significant increase in $\dot{V}_E$ relative to BL (i.e., % Δ BL). Two-way ANOVA with repeated measures revealed a significant estrous stage × time interaction in $\dot{V}_E$ ($F(6, 207) = 2.217$, $p = 0.04$). With post hoc analysis, proestrus rats showed an increase in $\dot{V}_E$ that was significantly elevated above BL values 60 min post-hypoxia (i.e., vLTF; $p < 0.0001$, Fig. 4A). Neither Time Control ($p = 0.95$), nor estrus groups ($p = 0.18$) showed similar changes in ventilation over the same 60 min period (Fig. 4A). At 60 min, mean $\dot{V}_E$ in proestrus rats was also significantly higher than both estrus ($p = 0.03$) and time control rats ($p < 0.001$; Fig. 4B).

A significant estrous stage × time interaction ($F(6, 207) = 2.525$, $p = 0.02$) was also measured in post-hypoxia V_T. Post hoc analysis revealed that proestrus rats showed a ramping of V_T over 60 min post-hypoxia culminating in significantly enhanced V_T relative to BL at 60 min ($p < 0.0001$, Fig. 4C). No changes in V_T relative to BL were noted in estrus rats following hypoxia or in time control rats (Fig. 4C). V_T at 60 min was also significantly elevated in proestrus rats compared to time controls ($p < 0.0001$) and estrus rats ($p < 0.05$, Fig. 4D). These data indicate that the vLTF exhibited by female rats in proestrus was primarily driven by changes in V_T following AIH. No time dependent changes in respiratory frequency were measured in either experimental group. However, respiratory frequency was significantly lower in the time control group compared to proestrus and estrus groups ($p = 0.02$ for each) at 15 min and remained lower than proestrus rats at 30 min ($p = 0.02$). By 60 min, no differences in frequency were identified across groups.

4. Discussion

We present here the first studies to explore AIH-induced vLTF expression in adult female rats and demonstrate a link between estrous cycle stage and expression of AIH-induced ventilatory plasticity in rodents. Our novel data indicate that adult female rats develop vLTF following AIH only during the proestrus phase of the estrous cycle, while rats in the estrus phase do not develop AIH-induced vLTF. As proestrus is notable for increased serum estrogen levels in adult rats, these results support the hypothesis that expression of AIH-induced respiratory plasticity may require estrogen signaling in the adult female rat as was recently established for AIH-induced phrenic LTF (pLTF; Dougherty et al., 2017). These data reinforce female estrous cycle stage as a critical biological factor to consider in studies of respiratory plasticity.

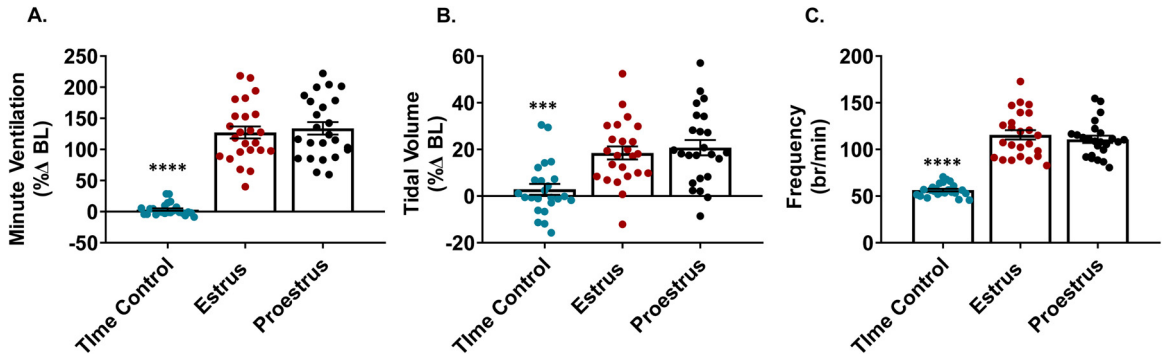

Fig. 3. Hypoxic ventilatory response (HVR) was similar in estrus and proestrus. The HVR was quantified during the first AIH exposure and expressed as a % change from baseline (%ΔBL). Rats in estrus and proestrus robustly responded to hypoxia with an increase in minute ventilation (A) that consisted of increases in tidal volume (B) and respiratory frequency (C). Time control groups did not receive hypoxic exposures and are included to rule out time-dependent changes in ventilation during plethysmography. One way ANOVA with Tukey post hoc analysis. $^{****}p < 0.0001$ from all other groups; $^{***}p < 0.001$ from all other groups. All data points overlaid on the means ± 1SE to demonstrate spread of the data about the mean.

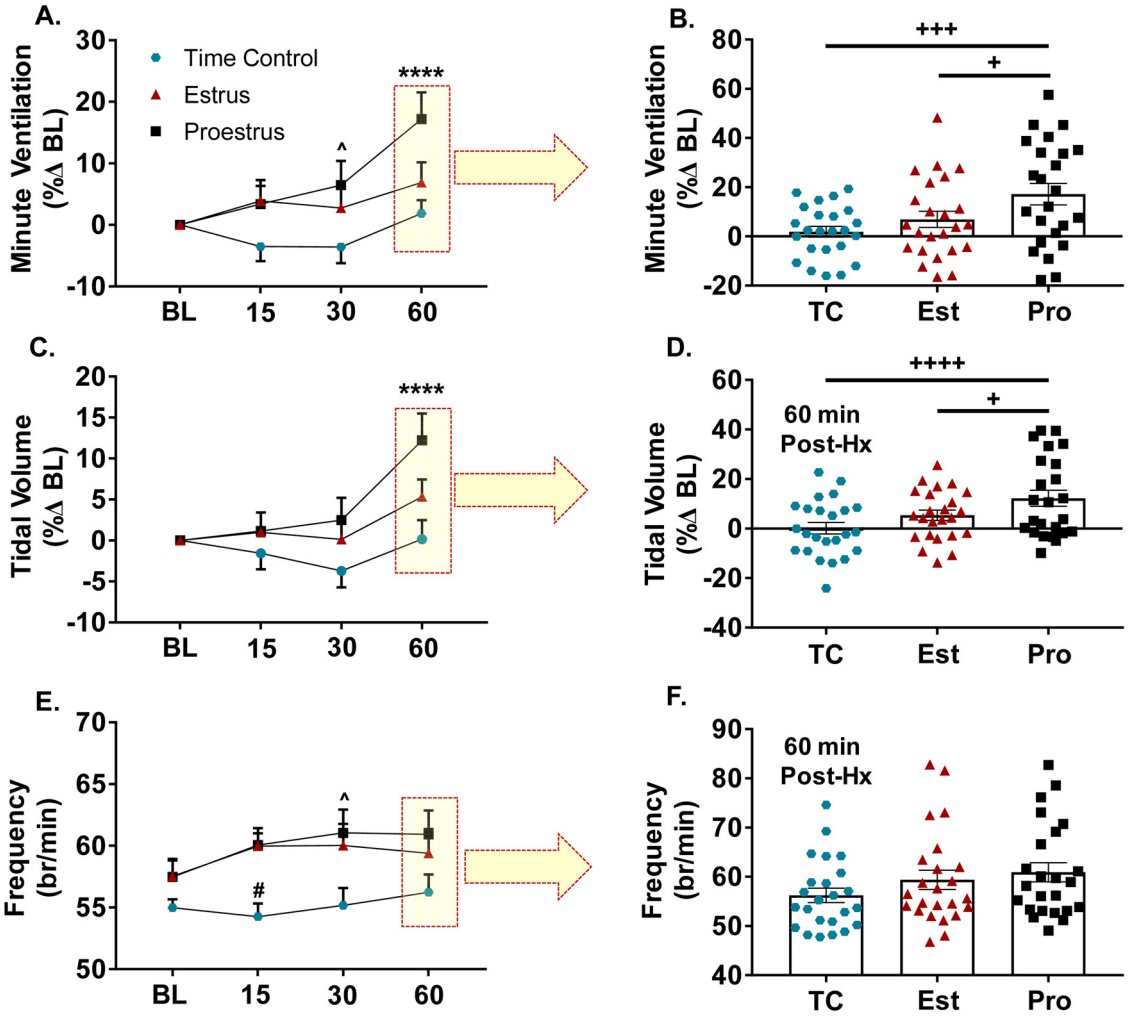

Fig. 4. Only rats in proestrus developed AIH-induced vLTF. We analyzed the development of vLTF by assessing ventilation for 60 min following the final hypoxic episode (or the equivalent time period in the Time Control group) expressed as a % change from baseline (%ΔBL). Rats in proestrus showed an increase in minute ventilation over 60 min following AIH indicative of vLTF (A). Within the 60 min time point, proestrus rats demonstrated elevated minute ventilation compared to both estrus ($^{+}p < 0.05$) and time control ($^{+++}p < 0.001$) rats (B). Changes in tidal volume production appeared to be the primary driver of vLTF in proestrus rats. Proestrus rats showed a progressive increase in tidal volume production in response to AIH (C) and by 60 min, proestrus rats demonstrated higher tidal volume production than estrus ($p < 0.05$) or time control ($^{++++}P < 0.0001$) rats (D). No time dependent changes in respiratory frequency were measured in any treatment group (E). Respiratory frequency was significantly lower in the Time Control group compared to proestrus and estrus groups ($^{\#}p < 0.05$ for each) at 15 min and remained lower than proestrus rats at 30 min ($\hat{p} < 0.05$). However, by 60 min, no differences in frequency were identified between groups (F). $^{****}p < 0.0001$ from BL. Two-way ANOVA with repeated measures and Tukey post hoc analysis. Figures B, D and F include all data points overlaid on the means ± 1SE to demonstrate spread of the data about the mean.

4.1. Baseline ventilation and hypoxic ventilatory responses

Adult female rats in this study showed minimal variation in baseline ventilation across the estrous cycle. Indeed, baseline $\dot{V}_E$ was nearly identical in proestrus and estrus groups with little change in either frequency or V_T. These data are consistent with previous plethysmography reports (Marques et al., 2017) and studies using anesthetized and ventilated experimental rat preparations (Dougherty et al., 2017; Zabka et al., 2001, 2003). Though our study focused on rats in estrus and proestrus, a recent study by Marquez et al. demonstrated that resting ventilation was similar across all four stages of the rat estrous cycle (Marques et al., 2017). In pre-menopausal human females, resting ventilation has been shown to increase during the luteal phase of the menstrual cycle, a ~14 day stage notable for gradually rising levels of serum progesterone (Behan and Kinkead, 2011; England and Farhi, 1976; Schoene et al., 1981; Takano, 1984). Elevated progesterone levels are likely involved in these ventilatory changes since serum progesterone and resting ventilation have correlated in normally cycling female rats (Behan and Kinkead, 2011; Machida, 1981; Popovic and White, 1998; White et al., 1983) and during pregnancy (Jensen et al., 2007). Also, reductions in resting ventilation are seen following menopause and in amenorrheic women (Behan and Kinkead, 2011; Schoene et al., 1981) when serum progesterone levels are significantly reduced. However, several studies have failed to yield clear correlations between serum progesterone and cyclic ventilatory changes in females (Behan and Kinkead, 2011; Beidleman et al., 1999) and serum progesterone levels failed to correlate with expression of AIH-induced pLTF (Dougherty et al., 2017). These contradictory findings obscure a possible direct mechanistic relationship. Further, progesterone sensitivity is highly dependent on prior estrogen signaling (MacLusky and McEwen, 1980; Parsons et al., 1982), complicating the interpretation of human studies that lack accurate serum hormone measures. If rising progesterone levels have an impact on resting ventilation in rats, the timing and duration of our experimental procedure may preclude us from capturing its effects. The truncated duration of estrous cycles in rats (~4 days) compared with the human menstrual cycle (~28 days)

reduces the period of elevated progesterone to only a few hours immediately preceding ovulation. This may explain the frequently observed steady state of baseline ventilation across the rat estrous cycle.

To assess hypoxic ventilatory responses (HVR) we quantified changes in ventilation during the first hypoxic challenge. Rats in estrus and proestrus readily increased $\dot{V}_E$ in response to hypoxia with elevations in both frequency and V_T. The magnitude of these responses was similar regardless of cycle stage in accordance with prior studies (Dougherty et al., 2017; Marques et al., 2017; Zabka et al., 2001, 2003). For example, our previous work demonstrated that phrenic nerve amplitude in response to AIH was similar in rats during estrus and proestrus consistent with our current results (Dougherty et al., 2017). These data are in-line with the idea that rats are naturally able to compensate for normal hormonal fluctuations to maintain hypoxic sensitivity. Body temperature is a key factor in the calculation of V_T and $\dot{V}_E$, and we were unable to capture potential hypoxia-induced temperature changes in this study. Reductions in body temperature during hypoxia are well established, even during short time domains like the 5 min hypoxic challenges used in this study (Marques et al., 2017; Morgan et al., 2014; Nakamura et al., 2010), and are likely to have impacted our quantification of HVR. However, a recent study indicated that the magnitude of hypoxia-induced reductions in core body temperature was similar regardless of estrous cycle stage (Marques et al., 2017) and thus, unlikely to have significantly influenced our interpretation, especially since pre-test body temperatures were similar across groups (Table 1).

4.2. Ventilatory long-term facilitation in estrus and proestrus

Our results indicate that development of AIH-induced vLTF in adult females occurs only in proestrus and not in estrus. All previous reports of AIH-induced vLTF in adult rats used only males (McGuire et al., 2002, 2008; Nakamura et al., 2010; Olson et al., 2001); this is the first report to characterize vLTF in adult females and the first to suggest a relationship between vLTF and estrous cycle stage. These data support and expand our recent results in an anesthetized and ventilated experimental preparation, demonstrating that AIH-induced pLTF also develops only during proestrus (Dougherty et al., 2017). Indeed, those studies directly equated serum estrogen levels (not progesterone) to the development of pLTF and showed that estrogen supplementation was sufficient to restore pLTF following ovariectomy, a procedure that eliminates the development of respiratory motor plasticity (Dougherty et al., 2017). As rats in proestrus display elevated serum estrogen levels and low serum progesterone (Dougherty et al., 2017), the expression of vLTF during proestrus is consistent with a role for estrogen signaling. This may also differentiate the cellular processes underlying resting state ventilation and HVR (progesterone signaling) from the unique cellular mechanisms that promote the development of LTF (estrogen signaling).

Healthy human subjects develop vLTF following AIH when end-tidal partial pressure of carbon dioxide (P_{ETCO_2}) is maintained at a constant level slightly above normocapnic baseline values during and following AIH (Harris et al., 2006). Indeed, Wadhwa and colleagues showed that the magnitude of vLTF under these experimental conditions is similar in male and pre-menopausal females (Wadhwa et al., 2008). Consistent with our results, the female subjects in that study, and in other vLTF studies (Syed et al., 2013), were all in the follicular phase of the menstrual cycle, notable for gradually increasing serum estrogen levels, similar to what might be seen in the proestrus phase in rats. In the one study that tested females during the luteal phase of the menstrual cycle, AIH did not lead to the development of vLTF (Jordan et al., 2002), though $PETCO_2$ was also not maintained above normocapnic levels. Collectively, the minimal number of human vLTF studies that have included women support the notion that menstrual cycle stage may have an impact on development of AIH-induced vLTF. An investigation that rigorously controls for serum sex hormone levels (specifically estrogen and progesterone) is warranted to confirm this hypothesis.

4.3. Methodological considerations

Our study relied on careful and consistent analysis of vaginal epithelial cell characteristics to group rats into the appropriate estrous cycle stage. Our prior studies combined this customary staging with measures of serum estrogen to demonstrate consistency of estrous stage characterization (Dougherty et al., 2017). Therefore, we are confident that our staging procedures accurately reflect the physiological state of our experimental rats. We previously reported a strong correlation between the magnitude of AIH-induced pLTF and serum estrogen (Dougherty et al., 2017), however, there are no studies demonstrating that this relationship translates to studies of vLTF. Further, there is speculation that high progesterone levels may significantly impact vLTF expression in rats since progesterone appears to have such a direct influence on resting ventilation and HVR in humans (Syed et al., 2013). More specific correlations between vLTF and serum estrogen and progesterone in future studies will provide clarity to these topics.

Two other points of consideration: our data does not factor in the metabolic profile or sleep state of our experimental rats. We were unable to capture and assess expired gas concentrations, nor track internal body temperature changes in real time during these experiments. Therefore, the calibration of ventilatory data to each rat's individual metabolic rate was not included. Though these reporting methods are consistent with prior plethysmography studies (Dougherty et al., 2016, 2018; Fuller et al., 2006, 2008, 2009; Lane et al., 2012; Navarrete-Opazo et al., 2015), inclusion of expired V_{O_2} and V_{CO_2} measures would enhance the precision of hypoxic chemosensitivity and vLTF measures (Morgan et al., 2014). However, metabolic standardization in this study may not have significantly impacted our results, as changing estrous cycle stages do not appear to impact $\dot{V}_E/V_{O_2}$ or core body temperature during hypoxia in adult female rats (Marques et al., 2017).

In addition, prior studies clearly demonstrate that the magnitude of AIH-induced vLTF in male rats is dependent on sleep-state, with the highest magnitude of vLTF developing during deep, slow-wave, non-REM sleep (Nakamura et al., 2010). Though our rats were all tested during their predicted period of sleep and visually appeared to be in comparable states of quiet sleep, we are unable to account for variability in sleep state in our interpretations. Female rats spend similar periods of their light cycle in REM and non-REM sleep regardless of estrous cycle stage (Zhang et al., 1995). Though, notable differences in sleep state architecture have been reported during different stages of the estrous cycle, these are revealed mostly during the “dark” (i.e., night) phase of a controlled light/dark cycle (Colvin et al., 1968; Kleinlogel, 1983; Zhang et al., 1995) versus the “light” phase used here. In addition, rats spend considerably more time in deeper sleep states during the day following estrus (Kleinlogel, 1983), and this may impact the development of vLTF. However, our rats were tested on the morning of proestrus and estrus and were likely outside the window of this “rebound phenomenon” (Kleinlogel, 1983). The sleep-state dependent fluctuations in vLTF magnitude measured in male rats are likely to persist in females; but studies of sleep state-dependent differences in vLTF magnitude across the female estrous cycle have yet to be conducted.

5. Conclusion

The present findings indicate that unanesthetized, freely breathing adult female rats develop AIH-induced vLTF only during the proestrus phase of the estrous cycle. These results are consistent with recent studies demonstrating an estrous stage dependence for the expression of AIH-induced pLTF (Dougherty et al., 2017) and may reflect an important role for estrogen signaling in the development of respiratory plasticity. Detailed characterization of how sex steroids, and estrogen in particular, modulate respiratory plasticity is critical for translation of

novel therapeutic interventions to ameliorate respiratory insufficiency resulting from injury or neurodegenerative diseases. Our data suggest that estrous or menstrual cycle stage is a critical consideration in studies of respiratory plasticity and detailed studies investigating vLTF in healthy adult females across the normal menstrual cycle are warranted.

Conflict of interest

The authors declare no competing financial interests.

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