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The long-term impact of ovariectomy on ventilation and expression of phrenic long-term facilitation in female rats

Rebecca Barok[1] | Jessica M. L. Grittner[1] | Brendan J. Dougherty[2]

[1] Rehabilitation Science Graduate Program, Department of Rehabilitation Medicine, University of Minnesota Medical School, Minneapolis, Minnesota, USA

[2] Divisions of Physical Therapy and Rehabilitation Science, Department of Rehabilitation Medicine, University of Minnesota Medical School, Minneapolis, Minnesota, USA

Correspondence
Brendan J. Dougherty, 420 Delaware Street S.E. (MMC 388), Minneapolis, MN 55455, USA.
Email: bdougher@umn.edu

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Abstract

Sex hormones are necessary to enable respiratory neuroplasticity, including phrenic long-term facilitation (pLTF), a form of respiratory motor plasticity elicited by acute, intermittent hypoxia (AIH). Female rats exhibit a progressive increase in phrenic nerve amplitude after AIH characteristic of pLTF only during pro-oestrus, the stage of the oestrous cycle notable for elevated circulating oestradiol levels. Removal of the ovaries [ovariectomy (OVX)], the primary source of circulating oestradiol, also eliminates AIH-induced pLTF after 1 week. Ovariectomy is used routinely as a model to examine the impact of sex hormones on CNS structure and function, but the long-term impact of OVX is rarely examined. Extra-ovarian sites of oestradiol synthesis, including multiple CNS sites, have been identified and might possess the capacity to restore oestradiol levels, in part, over time, impacting respiratory function and the expression of respiratory neuroplasticity. We examined both ventilation in awake, freely behaving female rats, using barometric plethysmography, and the expression of AIH-induced pLTF in anaesthetized, ventilated female rats 2 and 12 weeks after OVX and compared them with age-matched ovarian-intact female rats. Our findings indicate that chronic OVX had little impact on baseline breathing or in the response to respiratory challenge (10% O_2, 5% CO_2, balance N_2) during plethysmography. However, OVX rats at both 2 and 12 weeks demonstrated a persistent loss of AIH-induced pLTF relative to control animals ($P < 0.01$), suggesting that other sources of oestradiol synthesis were insufficient to restore pLTF. These data are consistent with our previous work indicating that oestradiol plays a key role in expression of AIH-induced respiratory neuroplasticity.

KEYWORDS
long-term facilitation, oestrogen, oevarectomy, respiratory neuroplasticity

1 | INTRODUCTION

Sex hormones play a crucial role in the expression of respiratory neuroplasticity (Behan et al., 2003; Dougherty et al., 2017; McIntosh & Dougherty, 2019; Zabka et al., 2001a, 2006). Specifically, recent evidence links 17β-oestradiol (E_2), the most prevalent and neuroactive form of oestrogen, to induction of respiratory neuroplasticity in females (Dougherty et al., 2017a; McIntosh & Dougherty, 2019). Young-adult female rats express phrenic long-term facilitation (pLTF; Bach & Mitchell, 1996; Fuller et al., 2000; Hayashi et al., 1993), a form of respiratory neuroplasticity characterized by a progressive increase in the amplitude of phrenic motor discharge after acute, intermittent hypoxia (AIH). However, the expression of pLTF occurs only during the pro-oestrous phase of the oestrous cycle, which is notable for

high levels of circulating E_2 (Dougherty et al., 2017a). Female rats in the oestrous phase, notable for reduced E_2 levels, do not express AIH-induced pLTF (Dougherty et al., 2017a). At the systems level, AIH also induces plasticity of respiratory function measured with barometric plethysmography. A progressive increase in minute ventilation after AIH is observed in rats (McGuire et al., 2002; Olson et al., 2001) and humans (Babcock & Badr, 1998; Babcock et al., 2003; Harris et al., 2006; Wadhwa et al., 2008), termed ventilatory long-term facilitation. Similar to findings using phrenic nerve recordings, the expression of ventilatory long-term facilitation in rats depends on the availability of oestradiol in females and is present during the pro-oestrous but not the oestrous phase of the normal oestrous cycle (McIntosh & Dougherty, 2019).

Removal of the ovaries [ovariectomy (OVX)] undermines expression of plasticity (Dougherty et al., 2017a). Given that the ovaries are the primary source of circulating E_2 in females (along with multiple other sex hormones), OVX quickly and significantly reduces circulating E_2 availability. Accordingly, OVX is a commonly used model to determine the impact of sex hormones on CNS structure and function. The time frames used to assess the impact of OVX-induced reductions in circulating hormones typically fall from 24 to 48 h after surgery (Woolley & McEwen, 1993), corresponding to the initial period of reduced E_2 levels, up to 2 weeks post-OVX. Our previous studies demonstrated that a loss of AIH-induced plasticity in OVX females took place 7 days post-surgery (Dougherty et al., 2017a). It is unknown whether later post-OVX time points would demonstrate altered respiratory function or expression of respiratory neuroplasticity or whether compensatory sources of non-ovarian E_2 synthesis would be sufficient to restore the capacity for AIH-induced plasticity over time. The aim of the present study was to explore these topics by: (1) quantifying ventilation in awake, female rats; and (2) measuring the magnitude of AIH-induced pLTF in anaesthetized, mechanically ventilated female rats. Experimental groups were 2 and 12 weeks post-OVX, and they were compared with age-matched rats receiving sham OVX surgery during pro-oestrus. Given that previous studies have indicated that baseline ventilation and ventilatory responses to hypoxia are impacted minimally by circulating hormones (Marques et al., 2015, 2017; McIntosh & Dougherty, 2019; Zabka et al., 2001a, 2003), we hypothesized that ventilatory function in awake female rats would be similar regardless of the time post-OVX. However, given that our previous work established that AIH-induced pLTF requires sufficient circulating E_2 in females (Dougherty et al., 2017a), we hypothesized that non-ovarian sources of E_2 synthesis would be insufficient to restore conditions needed for plasticity and that female rats would experience sustained loss of AIH-induced pLTF after OVX.

New Findings

- **What is the central question of this study?**
  Would ovariectomy cause prolonged changes in ventilation and sustained loss of acute, intermittent hypoxia-induced neuroplasticity or would these outcomes be restored with time?
- **What is the main finding and its importance?**
  Our main findings demonstrate that ovariectomy elicits minimal alteration in overall breathing function but impairs acute, intermittent hypoxia-induced plasticity for ≤ 12 weeks.

2 | METHODS

2.1 | Ethical approval

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota (approval no. 2003-37989A) and conformed to policies detailed in the IS National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Experiments were performed in 6-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 h–12 h light–dark cycles (lights on 06.00–18.00 h). A total of 15 rats were assigned randomly to one of three experimental groups: 2 weeks post-OVX (2wk OVX; $n = 5$); 12 weeks post-OVX (12wk OVX; $n = 5$); or Sham OVX ($n = 5$). All groups were age matched such that our outcome measures were assessed at 6 months of age (Figure 1). Sham-operated rats were studied during the pro-oestrus stage of the oestrous cycle (Dougherty et al., 2017a; McIntosh & Dougherty, 2019). Cycle stage was determined using daily examination of vaginal cell characteristics from vaginal smears under light microscopy (Dougherty et al., 2017a; Marcondes et al., 2002).

2.2 | Ovariectomy

Surgical procedures were detailed previously (Dougherty et al., 2017a). Sustained release buprenorphine analgesia (1 mg kg^{-1}; ZooPharm, LLC, Laramie, WY, USA) was injected s.c. 2 h before the start of surgery. Rats were anaesthetized by inhalation of isoflurane (Piramal, Telangana, India) in a closed chamber, and general anaesthesia was maintained via nose cone (2–3% isoflurane in O_2) for the duration of surgery. Adequacy of anaesthesia was confirmed by the lack of response to toe pinch and by eye-blink reflexes. Bilateral incisions were made through the dorsolateral skin and muscle layers to expose the ovarian fat pads. After bilateral removal of the ovaries, the muscle layers were approximated and sutured using absorbable 4–0 suture material and the skin incision closed with wound clips. Rats recovered in their home cages until plethysmographic testing. For Sham OVX rats, surgical procedures were identical with the exception that the ovarian fat pads were exteriorized briefly and then replaced; rats underwent identical recovery procedures.

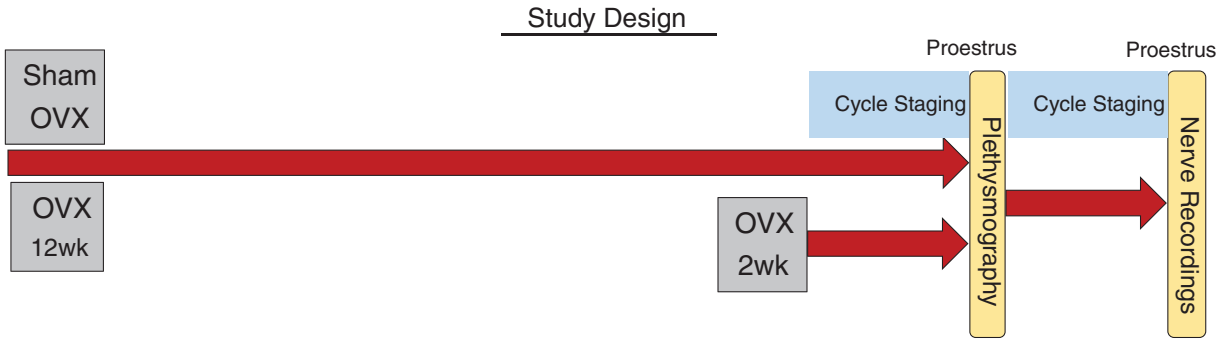

FIGURE 1 Study design. Experiments were designed to ensure that rats were similar in age during plethysmographic and phrenic neurophysiological testing. Ovariectomy (OVX) was completed either 2 weeks (2wks) or 12 weeks (12wks) before plethysmographic testing. Sham OVX surgeries were completed 12 weeks before testing. Sham rats were staged daily leading up to plethysmography and phrenic nerve recordings to ensure that testing was completed during pro-oestrus, the stage of highest circulating oestradiol, in accordance with our previous study (Dougherty et al., 2017a)

2.3 | Barometric plethysmography

Barometric plethysmography (Data Sciences International, St Paul, MN, USA) was used to quantify breathing in awake female rats during the pro-oestrous phase of the oestrous cycle and after OVX. All Sham OVX rats demonstrated a minimum of two complete oestrous cycles before plethysmographic testing and were characterized as being in pro-oestrus immediately before entering the plethysmograph chambers on the day of testing. After an acclimation period of ≥ 45 min, baseline (BL) ventilation was recorded in normoxic breathing conditions (20.9% O_2, balance N_2) for 10 min. Rats were then exposed to a respiratory challenge of hypercapnic hypoxia (Max; 10% O_2, 5% CO_2, balance N_2) for 10 min. All experiments were performed at the same time of day to minimize circadian effects (Seifert & Mortola, 2002).

The BL and Max conditions were established in the respiratory chambers by mixing O_2 and N_2 using a customizable, computer-controlled gas mixer (GSM-3; CWE, Ardmore, PA, USA) to obtain the desired inspired gas concentrations. Gas flowed continuously through each 4.0 litre chamber at 2.5 l min^{-1}, allowing for fast equilibration of gas mixes. 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 s. Customizable plethysmographic data acquisition software (Ponemah; Data Sciences International) recorded respiratory frequency (in breaths per minute) and incorporated chamber temperature, atmospheric and chamber pressure, humidity and rectal temperature to calculate tidal volume (V_T; in millilitres per breath; Drorbaugh & Fenn, 1955) and minute ventilation ($\dot{V}_E$; in millilitres per minute). Chamber temperature, humidity and pressures were constantly recorded; rectal temperatures were taken immediately before each rat entered the plethysmograph chamber and immediately after the conclusion of the experiment. If temperatures changed by > 0.5°C over the course of an experiment, V_T and $\dot{V}_E$ were recalculated using the final temperature (Doperalski et al., 2008; Dougherty et al., 2017b; Fuller et al., 2008, 2009). All rats demonstrated a reduction in temperature over time, consistent with prior studies (McIntosh & Dougherty, 2019; Nakamura et al., 2010); however, no differences in pre- or post-experiment temperatures were noted between groups (Table 1). Respiratory frequency data are expressed in absolute units (e.g., breaths per minute). The values of V_T and $\dot{V}_E$ are reported in absolute units per 100 g body weight (Table 1). The BL data represent mean values during a stable 10 min period before respiratory challenge; Max data are taken from a stable 2 min period in the second half of the 10 min challenge.

2.4 | Neurophysiological preparation

After plethysmographic testing, OVX rats returned to their home cages for a minimum of 7 days before phrenic nerve recording studies. Sham-operated rats were staged daily for 7 days plus at least one complete oestrous cycle before neurophysiology experiments. Nerve recordings in Sham rats were initiated immediately after the morning confirmation of pro-oestrus with vaginal smears. These procedures have been described in detail in our previous publications (Dougherty et al., 2015, 2017a). Briefly, rats were anaesthetized initially by inhalation of isoflurane, tracheotomized and mechanically ventilated (RoVent; Kent Scientific, Torrington, CT, USA; tidal volume, 1.5–2.5 ml; frequency, 75 breaths min^{-1}). Anaesthesia was maintained with isoflurane (SomnoSuite; Kent Scientific; 3–4% in 50% O_2, balance N_2) for the duration of the surgical procedures, and the adequacy of general anaesthesia was confirmed by lack of response to toe pinch and eye-blink reflexes. Rats were slowly converted to urethane anaesthesia (1.8 mg kg^{-1}) via a right femoral vein catheter. Surgery was performed on a temperature-controlled stainless-steel surgical table. Rectal temperature was monitored continuously with a temperature sensor (RightTemp; Kent Scientific) and maintained by adjusting the temperature of the surgical table. The concentration of inspired O_2 was monitored throughout all experiments using a fuel-cell O_2 sensor (AII 3000A; Analytical Industries, Pomona, CA, USA). Rats were vagotomized, and a catheter was inserted into the right femoral artery to monitor blood pressure using a calibrated pressure

TABLE 1 Physiological variables and ventilatory data with barometric plethysmography

Parameter	Sham	2wk OVX	12wk OVX
Age (days)	180 ± 10.0	181.3 ± 0.89	180 ± 10.0
Weight (g)	283 ± 11.15	311 ± 13.08	334 ± 8.23**
Pre-plethysmography temperature (°C)	37.6 ± 0.26	37.7 ± 0.11	37.5 ± 0.27
Post-plethysmography temperature (°C)	37.2 ± 0.18	37.2 ± 0.07	36.8 ± 0.22
Baseline ($F_{I,O_2} = 0.21$)			
Breathing frequency (breaths min^{-1})	59.0 ± 1.65	60.9 ± 5.43	53.8 ± 1.93
V_T [ml (100 g^{-1}) breath^{-1}]	0.48 ± 0.03	0.7 ± 0.10	0.50 ± 0.05
$\dot{V}_E$ [ml min^{-1} (100 g^{-1})]	29.2 ± 2.71	43.6 ± 8.72	30.4 ± 3.48
Max challenge ($F_{I,O_2} = 0.10$; $F_{I,CO_2} = 0.05$)			
Breathing frequency (breaths min^{-1})	107.5 ± 6.61†††	118.8 ± 12.14†††	98.9 ± 62.27†††
V_T [ml (100 g^{-1}) breath^{-1}]	0.61 ± 0.04	0.7 ± 0.11	0.8 ± 0.04†††
$\dot{V}_E$ [ml min^{-1} (100 g^{-1})]	65.2 ± 4.50†††	88.1 ± 17.84†††	82.7 ± 4.92†††

Abbreviations: F_{I,CO_2}, fraction of inspired carbon dioxide; F_{I,O_2}, fraction of inspired oxygen; OVX, ovariectomy; $\dot{V}_E$, minute ventilation; V_T, tidal volume; 6wk, 6 weeks; 12wk, 12 weeks. Values are means ± SD for each parameter. No significant differences in age were observed between groups ($P = 0.82$, one-way ANOVA). Ovariectomy had a significant effect on body weight (one-way ANOVA; $P = 0.01$), such that 12wk OVX rats were significantly heavier than Sham rats ($P = 0.009$); 2wk and 12wk OVX rats were similar in weight ($P = 0.16$). All rats showed a reduction in temperature during plethysmographic testing, but no differences were observed between groups pre-test ($P = 0.65$) or post-test ($P = 0.39$). There was a significant condition effect ($P \leq 0.001$; two-way ANOVA), in that all groups showed significantly increased breathing frequency and $\dot{V}_E$ between Baseline and Max. However, only the 12wk OVX group showed an increase in V_T during Max challenge relative to Baseline ($P \leq 0.001$). There were no measured treatment effects between experimental groups. Sham, 2wk OVX and 12wk OVX rats displayed similar respiratory values within Baseline and Max. **$P < 0.01$ relative to Sham; †††$P \leq 0.001$ relative to Baseline.

TABLE 2 Physiological variables with phrenic nerve recordings

Condition	Treatment	Temperature (°C)	pH	P_{aCO_2} (mmHg)	P_{aO_2} (mmHg)	SBE (mequiv l^{-1})	MAP (mmHg)
Baseline	Sham	36.48 ± 0.19	7.38 ± 0.01	43.78 ± 1.87	240.2 ± 10.4	0.05 ± 0.43	117.73 ± 2.34
	2wk OVX	37.08 ± 0.23	7.39 ± 0.02	45.38 ± 2.74	224.8 ± 17.3	1.35 ± 0.60	112.74 ± 32.65
	12wk OVX	36.82 ± 0.25	7.39 ± 0.0	43.12 ± 0.75	197 ± 22.7	0.24 ± 0.56	116.15 ± 6.37
Hypoxia	Sham	36.52± 0.16	7.38 ± 0.01	45.26 ± 1.68	47.2 ± 5.2	1.06 ± 0.29	108.62 ± 3.10
	2wk OVX	37.15 ± 0.26	7.39 ± 0.01	46.3 ± 2.89	38.5 ± 1.3	1.63 ± 1.03	100.49 ± 30.23
	12wk OVX	34.96 ± 1.90	7.39 ± 0.01	43.24 ± 1.11	40.6 ± 2.7	0.64 ± 0.63	101.58 ± 4.53
60 min	Sham	36.53 ± 0.28	7.38 ± 0.01	44.00 ± 1.76	214.4 ± 19.5	0.1 ± 0.21	107.73 ± 3.37
	2wk OVX	37.23 ± 0.43	7.39 ± 0.02	44.93 ± 3.02	225.8 ± 9.8	1.3 ± 0.38	94.02 ± 29.36
	12wk OVX	37.08 ± 0.17	7.40 ± 0.02	42.74 ± 1.18	197 ± 18.9	0.46 ± 0.63	100.68 ± 7.53

Abbreviations: MAP, mean arterial pressure; OVX, ovariectomy; P_{aCO_2}, partial pressure of CO_2; P_{aO_2}, partial pressure of O_2; SBE, standard base excess; 6wk, 6 weeks; 12wk, 12 weeks. Values are means ± SD for each parameter. No significant differences were observed in temperature, pH, P_{aCO_2}, P_{aO_2}, SBE or MAP between any of the treatment groups in any condition ($P > 0.05$; one-way ANOVA for each comparison).

transducer (SP844; MEMScap, Isere, France). Blood samples obtained from the femoral artery were analysed for partial pressures of O_2 (P_{aO_2}) and CO_2 (P_{aCO_2}) and pH with a blood gas analyser (CCA-TS2; OPTI Medical, Roswell, GA, USA); standard base excess, calculated by the analyser, was also used as an indicator of metabolic acid–base disturbances. A slow, continuous infusion of a 5:1 mix of veterinary lactated Ringer solution and sodium bicarbonate was maintained via the femoral vein catheter after conversion to urethane anaesthesia to maintain blood pressure and acid–base balance throughout the experiment. The left phrenic nerve was dissected, exposed via a dorsal approach, cut distally and desheathed. The nerve was submerged in mineral oil and placed on bipolar silver recording electrodes to record spontaneous neural activity. The adequacy of anaesthesia was tested before protocols commenced and immediately after the protocol was completed. The adequacy of anaesthetic depth was assessed as the lack of pressor or respiratory neural response to a toe pinch. We did not observe increased blood pressure or respiratory nerve activity in any of the rats after toe pinch. Neuromuscular block with pancuronium bromide (~1.2 ml, i.v., 1 mg ml^{-1}) was initiated after confirmation of adequate anaesthesia, in order to remove excessive movement artefacts associated with respiratory muscle activity. Instantaneous end-tidal CO_2 was monitored from the expired line (CapnoScan; Kent Scientific) and maintained at ~45 mmHg throughout the surgery to allow stabilization of the preparation and initial nerve signals. Nerve

activity was amplified (gain, 10,000×; A-M systems 1800, Sequim, WA, USA), bandpass filtered (300 Hz to 10 kHz), rectified and integrated (time constant, 50 ms). Resulting signals were digitized, recorded and analysed with PowerLab (LabChart v.8 software, AD Instruments, Colorado Springs, CO, USA).

2.5 | Neurophysiology protocol

Stable nerve activity was established while the rat was ventilated with a hyperoxic inspired gas mixture [fraction of inspired oxygen (F_{I,O_2}), 0.5–0.6; P_{aO_2}, > 150 mmHg], with sufficient levels of inspired CO_2 to maintain constant arterial P_{aCO_2}, preventing the rat from becoming apnoeic (typically between 40 and 45 mmHg). After the preparation was stabilized, the apnoeic threshold for rhythmic phrenic activity was determined by progressively lowering the inspired (and arterial) CO_2 until rhythmic phrenic activity ceased. From apnoea, the end-tidal CO_2 was increased progressively every ~60 s until nerve activity resumed (i.e., the recruitment threshold). Baseline nerve activity was established with end-tidal CO_2 set 2 mmHg above the CO_2 recruitment threshold. This procedure allows a standardized level of respiratory drive during baseline conditions in different rats. A baseline blood sample was taken, and the rats were exposed to AIH, consisting of three 5 min hypoxic episodes (F_{I,O_2}, 0.10–0.12; P_{aO_2}, 35–45 mmHg) interspersed with 5 min intervals of baseline conditions (i.e., F_{I,O_2}, 0.5–0.6). Data were collected from the first hypoxic episode when possible. However, if P_{aO_2} or P_{aCO_2} fell out of a priori ranges during hypoxia, the data were gathered from subsequent hypoxic episodes. Nerve activity was monitored for 60 min after the last hypoxic episode while maintaining baseline levels of arterial blood gases. Blood samples (0.2 ml in a heparinized syringe) were obtained and analysed before hypoxic challenge (baseline), during hypoxic challenge and at 15, 30 and 60 min post-hypoxia. At the conclusion of each experiment, rats were killed by urethane overdose administered via the femoral vein, followed by discontinuation of pump ventilation.

2.6 | Quantification of serum oestradiol

Although visualization of vaginal cell characteristics provide strong indication of circulating sex hormones (in particular, E_2), additional quantification of serum E_2 was performed using enzyme-linked immunosorbent assay (ELISA; Dougherty et al., 2017a; Zabka et al., 2005, 2006). Briefly, blood samples were collected at the conclusion of phrenic neurophysiology experiments, before urethane overdose. Samples were centrifuged at 845 x *g* for 10 min, and serum was collected for storage at −80°C. The concentration of E_2 was determined using ELISAs (Calbiotech, Spring Valley, CA, USA) according to the manufacturer's instructions; all serum samples were run in triplicate. The absorbance was read at 450 nm, and serum concentrations were interpolated from standard curves run in each assay.

2.7 | Statistical analyses

All statistical analyses were completed using commercially available statistical software (GraphPad Prism, v.8; GraphPad, La Jolla, CA, USA). One-way ANOVA was used to compare age, weight and temperature across experimental groups (Table 1). Ventilation data from barometric plethysmography are presented as the means ± SD, with graphical representations of individual data points to demonstrate spread of data about the mean. Two-way ANOVA with repeated measures [factors: treatment (OVX vs. Sham) and condition (BL vs. Max)] was used to compare respiratory parameters between treatments within and across conditions (Table 1). Bonferroni post hoc tests were used to identify statistically significant individual comparisons when significant main effects or interactions were present.

For phrenic nerve recordings, the peak amplitude and burst frequency of phrenic nerve activity were averaged in 1 min bins at each recorded data point (baseline, during hypoxic exposure and 15, 30 and 60 min after the final hypoxic episode). Baseline measurements were taken immediately before the start of the first hypoxic challenge. Burst frequency was analysed and reported in bursts per minute. Statistical comparisons in phrenic burst amplitude and frequency were made for time (baseline and 15, 30 and 60 min after hypoxia) and treatment effects using two-way ANOVA with repeated measures. Given that post hoc analyses of phrenic burst amplitude and frequency measures showed statistical significance only at 60 min post-hypoxia, we limited our figures to this time point for ease of visual comparison. As with our plethysmography data, phrenic nerve data are presented as means ± SD with graphical representations of individual data points to demonstrate spread of data about the mean. Physiological variables during phrenic recording experiments were compared across groups in the same manner, and data are presented for measurements taken at baseline, during hypoxic challenge and 60 min post-AIH (60 min). Separate analyses of the BL phrenic amplitudes and phrenic nerve burst responses to hypoxia were made using a one-way ANOVA. Serum oestradiol concentrations after ELISA were also compared using a one-way ANOVA with Bonferroni post hoc test, and linear regression analysis was used to compare serum E_2 concentrations with phrenic amplitudes 60 min post-AIH (i.e., pLTF). For all statistical comparisons, differences were considered significant at $P < 0.05$.

3 | RESULTS

To evaluate the impact of OVX on ventilation, whole-body barometric plethysmography was completed in three experimental groups of female Sprague–Dawley rats: 2wk OVX, 12wk OVX and a group of rats receiving Sham OVX surgery. Consistent with previous studies, we assessed the Sham rats during the pro-oestrous phase of the oestrous cycle (Dougherty, et al., 2017). All experimental groups were equivalent in age ($P = 0.82$; Table 1). One-way ANOVA indicated that OVX had a significant effect on body weight ($P = 0.01$) such that 12wk OVX rats

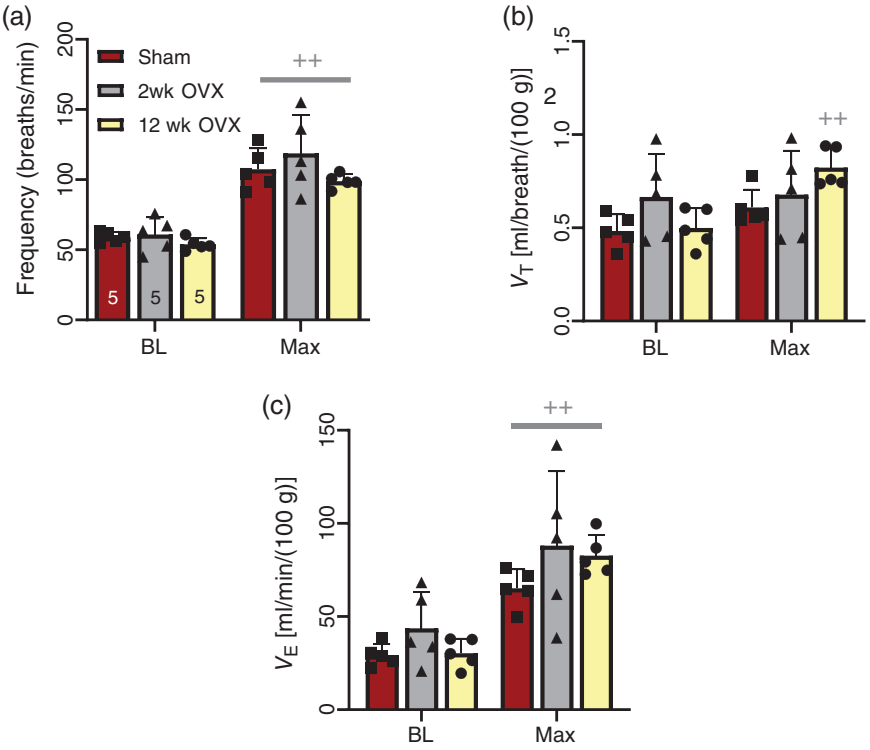

FIGURE 2 Ventilation measured with barometric plethysmography is similar after ovariectomy (OVX). Ventilatory measures were recorded during baseline conditions (BL; 20.9% O_2, balance N_2) and hypercapnic hypoxia (Max; 10% O_2, 5% CO_2, balance N_2) for each treatment: Sham controls ($n = 5$) and female rats 2 weeks (2wk) post-OVX ($n = 5$) and 12 weeks (12wk) post-OVX ($n = 5$). Brething frequency (a), tidal volume (V_T; b) and minute ventilation ($\dot{V}_E$; c) were similar during BL ($P > 0.05$). All groups showed statistical increases in frequency and $\dot{V}_E$ in response to Max challenge (i.e., condition effect; $P < 0.001$), but only the 12wk OVX group showed an increase in V_T with Max relative to BL ($P < 0.001$). Data represent two-way ANOVA with repeated measures and Bonferroni post hoc tests. $^{++}P \leq 0.001$ compared with BL

were significantly heavier than Sham rats ($P = 0.009$; Table 1); 2wk and 12wk OVX rats were similar in weight ($P = 0.16$). Plethysmographic testing resulted in a decrease in core body temperature in all groups. However, one-way ANOVA demonstrated that temperature was equivalent between groups during BL ($P = 0.65$) and Max ($P = 0.39$; Table 1). To account for changes in core temperature, ventilation measures during Max were calculated using the post-experiment core body temperature (Doperalski et al., 2008; Dougherty, et al., 2017; Fuller et al., 2006, 2008, 2009). At BL, all primary respiratory measures (frequency, V_T and $\dot{V}_E$) were similar between groups (Table 1; Figure 2), indicating that OVX had minimal effect on BL breathing function. As expected, a significant condition effect ($P < 0.001$) was noted with two-way ANOVA for each respiratory measure when rats were given a respiratory challenge (hypercapnic hypoxia; Max), indicating that all groups responded appropriately to the Max challenge. Analysis of V_T resulted in an additional treatment × condition interaction ($P = 0.011$). Bonferroni post hoc analyses demonstrated that the response to Max challenge was driven mostly by changes in frequency, because all groups expressed a significant elevation in frequency relative to BL during Max challenge ($P < 0.001$; Figure 2a), whereas only the 12wk OVX group demonstrated a significant increase in V_T with Max challenge relative to BL ($P = 0.0005$; Figure 2b). All groups expressed significantly elevated $\dot{V}_E$ with respiratory challenge ($P \leq 0.001$; Figure 2c). Notably, no treatment effects were observed during respiratory challenge for any of these primary measures; all groups showed similar frequency, V_T and $\dot{V}_E$ within the Max challenge (Figure 2).

Next, we assessed the long-term effect of OVX on neural output of the phrenic nerve and the expression of pLTF. Phrenic long-term facilitation was defined as a statistically significant increase in phrenic nerve amplitude from BL to 60 min after the last period of hypoxia, while maintaining steady blood gas levels (Hayashi et al., 1993; Mitchell et al., 2001). The analyses of our phrenic nerve recording data are shown in Figure 3. The amplitude of BL phrenic activity was statistically similar in all groups ($P = 0.09$; one-way ANOVA; Figure 3a). As in previous reports, OVX resulted in a loss of AIH-induced pLTF in our female rats (Dougherty, et al., 2017), and this impact persisted for 12 weeks. Two-way ANOVA demonstrated significant effects of time ($P < 0.001$) and treatment ($P = 0.018$), and a significant time × treatment interaction ($P = 0.018$) in the amplitude of phrenic nerve output. At 60 min post-AIH, the Sham group exhibited a significant increase in phrenic nerve amplitude above BL (ΔmV, 2.41 mV; 89 ± 42%; $P < 0.001$) and relative to both OVX groups ($P < 0.01$; Figure 3b). Although a modest elevation in mean amplitude was observed, neither 2wk (ΔmV, 2.96 mV; 29 ± 13%) nor 12wk (ΔmV, 1.693 mV; 22 ± 33%) OVX rats showed a significant increase in phrenic amplitude above BL at 60 min post-AIH (i.e., no expression of pLTF).

Phrenic nerve amplitudes during hypoxia were compared to determine whether OVX had a persistent impact on the neural response to hypoxia (Figure 4). Overall, the raw amplitudes of phrenic nerve activity were similar between groups during hypoxic challenge ($P = 0.29$; one-way ANOVA; Figure 4a). Analysis of the hypoxic response as a percentage change from BL using a one-way ANOVA indicated a significant difference between groups ($P = 0.022$; one-way ANOVA). Bonferroni post hoc analysis showed that 2wk OVX rats exhibited a significantly reduced hypoxic response relative to Sham ($P = 0.03$), but this difference was lost by 12wks post-OVX ($P = 0.084$; Figure 4b).

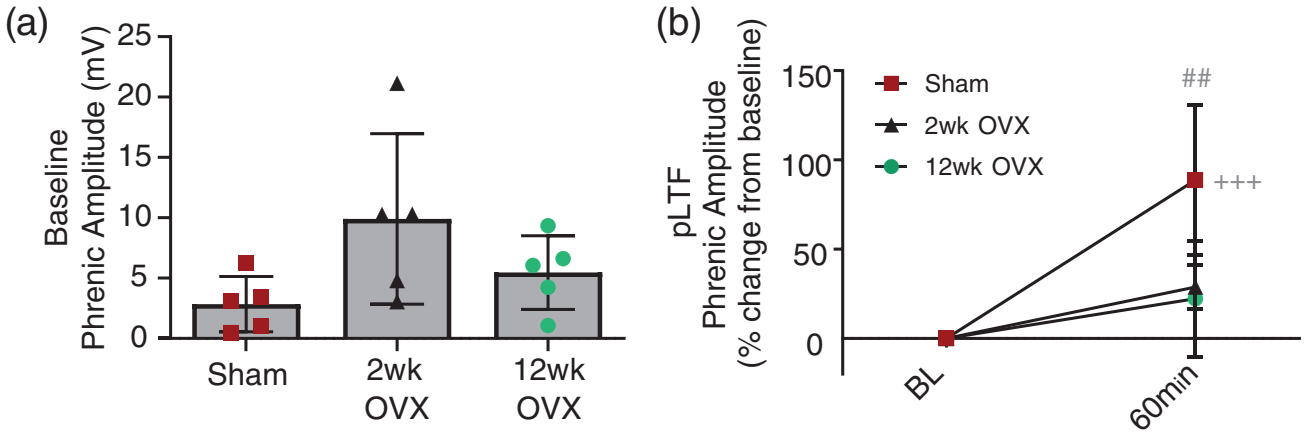

FIGURE 3 Phrenic long-term facilitation (pLTF) is lost for 12 weeks (12wk) after ovariectomy (OVX). Phrenic nerve recordings were assessed in anaesthetized, mechanically ventilated rats ($n = 5$ per group). (a) Raw phrenic amplitudes in baseline (BL) recording conditions were similar between experimental groups (one-way ANOVA; $P = 0.09$). (b) To discern expression of pLTF, phrenic amplitudes 60 min post-acute intermittent hypoxia (AIH) were expressed relative to BL and compared using two-way ANOVA. Significant effects of time ($P < 0.001$) and treatment ($P = 0.018$) and a significant time × treatment interaction ($P = 0.018$) were identified. Sham control rats showed a persistent increase in phrenic amplitude above BL (ΔmV, 2.41 mV; 89 ± 42%; $P < 0.001$) indicative of pLTF, whereas OVX groups showed no evidence of pLTF (2wk: ΔmV, 2.96 mV, 29 ± 13%; 12wk: ΔmV, 1.693 mV, 22 ± 33%; both $P > 0.05$). Phrenic amplitude in Sham rats 60 min post-AIH was also elevated significantly above that of both OVX groups ($P < 0.01$). $^{+++}P < 0.001$ compared with BL; $^{\#\#}P < 0.01$ compared with both OVX groups

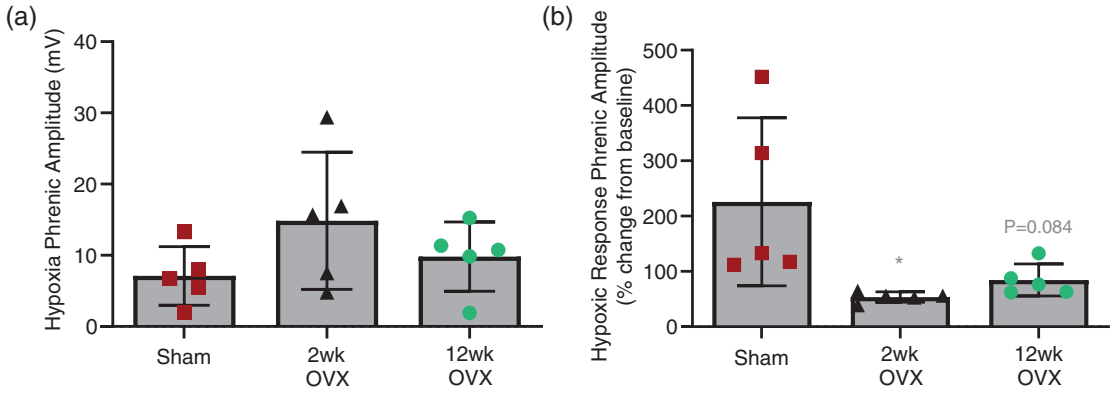

FIGURE 4 Phrenic hypoxic responses are modestly impacted by ovariectomy (OVX). (a) We compared the phrenic neural response to hypoxia between experimental groups ($n = 5$ per group). Raw phrenic nerve amplitudes during hypoxia were similar between experimental groups (one-way ANOVA; $P = 0.29$). (b) When compared as a relative increase above baseline (BL) amplitudes, 2 week (2wk) OVX rats exhibited a significantly reduced hypoxic response relative to Sham ($P = 0.03$), but this difference was lost by 12 weeks (12wk) post-OVX (one-way ANOVA; $P = 0.084$). $^{*}P < 0.05$ compared with Sham

Table 2 lists the physiological variables monitored during phrenic nerve recordings. No significant differences were noted in body temperature, pH, P_{aCO_2}, P_{aO_2}, standard base excess or blood pressure ($P > 0.05$; one-way ANOVA). Consistent with our previous work, we measured serum E_2 concentrations in all rats with an ELISA (Figure 5; Dougherty, et al., 2017). One-way ANOVA indicated a significant difference in serum E_2 concentrations between experimental groups ($P = 0.0009$). Ovariectomy resulted in a significant reduction in serum E_2 that persisted for 12 weeks. Ovariectomy caused a persistent reduction in serum E_2 concenrations at both 2wks ($P < 0.001$) and 12wks ($P < 0.01$) compared with Sham rats (Figure 5a). Linear regression analysis demonstrated a significant relationship between the serum oestradiol concenration and the magnitude of pLTF ($P = 0.039$; $r^2 = 0.36$; $F = 5.618$; degrees of freedom numerator, degrees of freedom denominator [1,10]; Figure 5b). This is consistent with our previous data (Dougherty, et al., 2017).

4 | DISCUSSION

Ovariectomy rapidly reduces circulating sex hormone concentrations in females and is frequently used to determine the impact of sex hormones on CNS structure and function. We demonstrated previously that OVX eliminates the expression of AIH-induced pLTF, a form of respiratory neuroplasticity (Dougherty et al., 2017a). Although other hormones are impacted by OVX, E_2 is probably the hormone

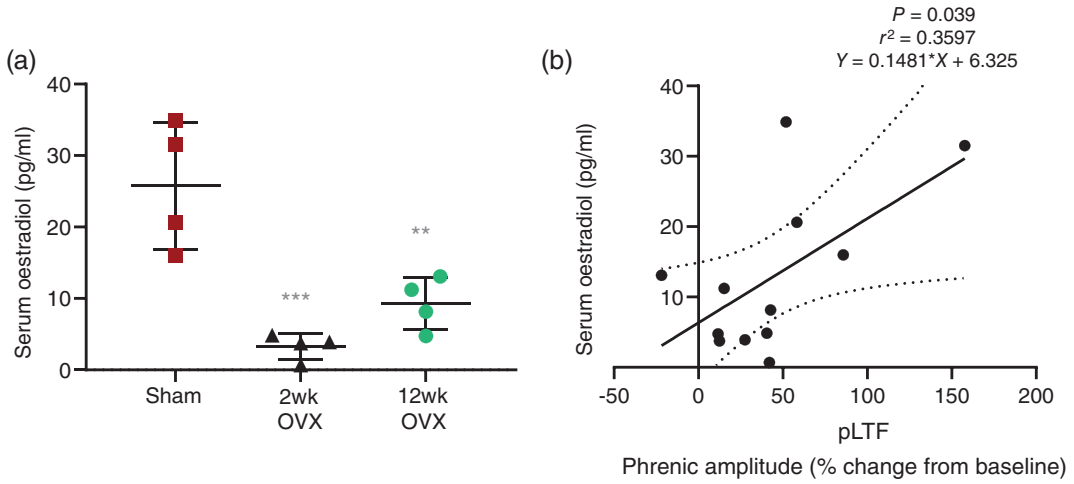

FIGURE 5 Serum oestradiol concentrations are chronically reduced after ovariectomy (OVX) and are correlated with phrenic long-term facilitation (pLTF). (a) One-way ANOVA indicated a significant difference in serum oestradiol concentrations between experimental groups ($P < 0.001$). Ovariectomy caused a persistent reduction in serum oestradiol concentrations at both 2 weeks (2wk; $P < 0.001$) and 12 weeks (12wk; $P < 0.01$) compared with Sham rats. (b) Linear regression analysis demonstrated a significant relationship between serum oestradiol and the magnitude of pLTF expression across groups [$P = 0.039$, $r^2 = 0.36$, $F = 5.618$; degrees of freedom numerator, degrees of freedom demoninator (1,10); shown with 95% confidence intervals]. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared with Sham

necessary to facilitate pLTF in female rats (Dougherty et al., 2017a), for the following reasons: (1) pLTF expression coincides with changes in E_2 during the normal oestrous cycle; and (2) pLTF is rescued in OVX female rats with E_2 supplementation or spinal activation of E_2 receptors (Dougherty et al., 2017a). The ovaries are the primary source for circulating E_2 in females; however, several extra-ovarian sources of oestradiol production might have the capacity to increase E_2 levels in the weeks and months after OVX. It was unknown whether these other sources of E_2 production would impact respiratory function or restore conditions for AIH-induced pLTF. We addressed this question by assessing ventilation and AIH-induced pLTF in adult female rats 2 and 12 weeks after OVX and comparing them with gonadally intact female rats in the pro-oestrous phase of the oestrous cycle. Pro-oestrus is notable for high concentrations of circulating E_2 and is the only stage when female rats express respiratory neuroplasticity (Dougherty et al., 2017a; McIntosh & Dougherty, 2019). Our results demonstrate that although OVX had minimal impact on overall breathing function, the expression of AIH-induced pLTF was abolished for ≤ 12 weeks. Collectively, these data support the idea that circulating sex hormones, and E_2 in particular, are crucial for the expression of AIH-induced pLTF in females and that alternative sources of E_2 synthesis are insufficient to restore the conditions for pLTF after OVX.

Few studies have examined carefully the impact of circulating sex hormones on respiratory function in awake, freely behaving rats (Gargaglioni et al., 2019; McIntosh & Dougherty, 2019). Marques et al. (2015, 2017) carried out carefully controlled studies in gonadally intact female rats and found that across oestrous cycle phases, and after OVX, female rats generally produced similar $\dot{V}_E$ during room-air breathing. In other words, BL respiratory output was not impacted by changes in circulating sex hormones. Our data confirm these findings and extend them to indicate that BL respiratory function is impacted minimally by OVX for ≤ 12 weeks post-surgery. Furthermore, the response to a robust hypercapnic hypoxia respiratory challenge (Max) was also similar across experimental groups. A significant increase in $\dot{V}_E$ during Max was driven primarily by increased respiratory frequency, because all groups displayed significant increases in frequency during Max challenge relative to BL. Rats in the 12wk OVX group displayed significantly larger V_T production during Max challenge relative to BL. This was the only experimental group to display this increase. An explanation for this finding is not immediately clear; however, the magnitude of V_T between groups during Max challenge was similar (i.e., there was no specific treatment effect). It is attractive to speculate that the augmented V_T response to respiratory challenge over time after OVX is related to the chronic reduction of sex hormones, but this idea is not supported by our phrenic neurophysiology results, because OVX groups showed a reduced neural response to hypoxia. Further studies will be required to determine whether there is functional significance to this result.

The most significant finding of these experiments was that OVX impaired the expression of AIH-induced pLTF for 12 weeks. We demonstrated previously that the significant reduction in circulating E_2 after OVX eliminated pLTF in female rats 1 week post-surgery. In the present study, we expand those findings and demonstrate that chronic OVX reduces the capacity for plasticity for months. This finding is significant, in that non-ovarian sources of E_2 do not appear sufficient to restore either the necessary amounts of circulating E_2 or the focused local release of E_2 needed to enable pLTF. After the menopause in women or after OVX in experimental rodents, increased E_2 production can occur through enhanced aromatase conversion of testosterone in adipose tissue, bone, vascular endothelial cells, aortic smooth muscle and in multiple CNS regions (Simpson, 2003; Zhao et al., 2005). Aromatase activity has been described in the brain

(Fester et al., 2011; Li & Gibbs, 2019; Rossetti et al., 2016) and in the ventral spinal cord of rodents (Ji et al., 2017), supporting region-specific oestrogen production in the CNS. The E_2 produced in these extra-ovarian regions is unique in that it primarily functions locally through paracrine or intracrine signalling mechanisms (Labrie et al., 1997; Simpson, 2003), although it can escape to the general circulation (Simpson, 2003; Simpson et al., 2002). Advancing this idea, Zhao et al. (2005) demonstrated that serum E_2 gradually increased in the 6 months after OVX in female rats through extra-ovarian synthesis, although levels remained below age-matched ovarian-intact control rats. Here, circulating E_2 in 12wk OVX rats was slightly elevated in comparison to 2wk OVX rats, but not significantly so, and remained well below the concentrations measured in ovarian-intact, pro-oestrus rats. Although we cannot discount the possibility that later post-OVX time points might result in higher concentrations of circulating E_2 impacting AIH-induced pLTF, our data indicate that extra-ovarian E_2 production was unable to restore conditions for pLTF expression.

Most studies of AIH-induced pLTF use rats in the age range of 3–4 months (e.g., Dougherty et al., 2015, 2017a). Our experimental design aimed to test female rats at 6 months of age, which is still considered 'young-adult'. Nevertheless, age might have had a modest impact on our results. Unlike male rats, which experience a steady age-dependent decline in pLTF expression (Zabka et al., 2001a), female rats demonstrate enhanced pLTF in early 'middle-age' (13 months; Frick, 2009; Zabka et al., 2001b) and even into early 'geriatric' ages (20–22 months; Zabka et al., 2003) relative to young-adult female rats (Zabka et al., 2001b). The elevation in pLTF expression during this period might be related to a characteristic surge in systemic E_2 as middle-aged female rats transition to senescence (Frick, 2009). The elevation in E_2 remains somewhat steady and can last for an extended period of time (Morrison et al., 2006). An elevated state of circulating E_2 is supported by the work of Zabka et al. (2003), who measured equivalent serum E_2 concentrations in their young and geriatric rats. Our results, based on the magnitude of pLTF in our Sham rats, appear to fall between those of young-adult rats in our previous work (Dougherty et al., 2017a) and the enhanced pLTF magnitudes reported by Zabka et al. (2001b). We attempted to mitigate the influence of age in our experimental design by performing OVX surgery at different time points to ensure that all rats were the same age for our experiments as opposed to performing OVX at the same age and then studying the rats at different post-OVX ages. In addition, our serum E_2 concentrations measured at 6 months were similar to levels in young-adult rats during pro-oestrus (Dougherty et al., 2017a), reducing the likelihood of age-related effects in the present study.

We recognize that animal numbers within each experimental group ($n = 5$) might have reduced the overall statistical power of our studies. Group numbers were determined based on *a priori* power analysis of preliminary phrenic pLTF studies using G*Power Suite v.3.1.9.2. After completion of data collection, we confirmed statistical power with post hoc power analysis and concluded that our study was appropriately powered to detect significant differences in pLTF magnitude with $n = 5$ per group (power = 0.865). However, low group numbers open up the potential for type II statistical errors that would result in 'false-negative' conclusions or inappropriate acceptance of the null hypothesis. Hence, although we are confident that meaningful differences in pLTF magnitude would remain with larger group sizes, we cannot discount the possibility that our OVX groups might have demonstrated small, but significant pLTF expression with larger group numbers. Here, the 2wk OVX group showed a pLTF magnitude of 29 ± 13% above baseline, which was larger than 12wk OVX females (22 ± 33%). Our previous studies used female rats ~1 week post-OVX to demonstrate loss of pLTF expression (7 ± 5% above baseline; Dougherty et al., 2017a). Given the reported loss of pLTF at 1 and 12 weeks post-OVX, our present findings indicating the loss of pLTF expression 2 weeks post-OVX are consistent despite a lower sample size.

Collectively, these data provide additional support for a significant role of ovarian hormones in the expression of AIH-induced pLTF in female rats. The present report establishes that OVX impairs the capacity for AIH-induced pLTF for extended periods of time and sets the stage for future mechanistic studies aimed at understanding the impact of sex steroid signalling on multiple levels of the respiratory motor control system. Specifically, future directions will focus on how spinal E_2 signalling enables the expression of AIH-induced pLTF. Establishing how gonadal steroids gate respiratory plasticity will be fundamental to optimizing future translational applications of AIH-induced motor plasticity (Gonzalez-Rothi et al., 2015).

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AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the work and to data acquisition, analysis and interpretation. R.B. drafted the manuscript, with revisions and edits provided by J.M.L.G. and B.J.D. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

COMPETING INTERESTS

None declared.

DATA AVAILABILITY STATEMENT

All data supporting the results are reported above and stored in a secure computer on the campus of the University of Minnesota. Data are available from the senior author upon request.

ORCID

Jessica M. L. Grittner https://orcid.org/0000-0002-7806-9564
Brendan J. Dougherty https://orcid.org/0000-0002-5995-8948

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