Neonatal stress affects the aging trajectory of female rats on the endocrine, temperature, and ventilatory responses to hypoxia

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Submitted 6 October 2014; accepted in final form 28 January 2015

Fournier S, Gulemetova R, Baldy C, Joseph V, Kinkead R. Neonatal stress affects the aging trajectory of female rats on the endocrine, temperature, and ventilatory responses to hypoxia. Am J Physiol Regul Integr Comp Physiol 308: R659–R667, 2015. First published February 4, 2015; doi:10.1152/ajpregu.00418.2014.—Human and animal studies on sleep-disordered breathing and respiratory regulation show that the effects of sex hormones are heterogeneous. Because neonatal stress results in sex-specific disruption of the respiratory control in adult rats, we postulate that it might affect respiratory control modulation induced by ovarian steroids in female rats. The hypoxic ventilatory response (HVR) of adult female rats exposed to neonatal maternal separation (NMS) is ∼30% smaller than controls (24), but consequences of NMS on respiratory control in aging female rats are unknown. To address this issue, whole body plethysmography was used to evaluate the impact of NMS on the HVR (12% O2, 20 min) of middle-aged (MA; ∼57 wk old) female rats. Pups subjected to NMS were placed in an incubator 3 h/day for 10 consecutive days (P3 to P12). Controls were undisturbed. To determine whether the effects were related to sexual hormone decline or aging per se, experiments were repeated on bilaterally ovariectomized (OVX) young (∼12 wk old) adult female rats. OVX and MA both reduced the HVR significantly in control rats but had little effect on the HVR of NMS females. OVX (but not aging) reduced the anapyrexic response in both control and NMS animals. These results show that hormonal decline decreases the HVR of control animals, while leaving that of NMS female animals unaffected. This suggests that neonatal stress alters the interaction between sex hormone regulation and the development of body temperature, hormonal, and ventilatory responses to hypoxia.

control of breathing; development; sexual dimorphism; sleep disordered breathing

IN WOMEN, AGING IS ASSOCIATED with a natural decline in circulating levels of ovarian hormones (e.g., estrogens and progesterone), as well as reproductive functions. The onset of menopause constitutes a significant risk factor for several disorders, such as arteriosclerosis and osteoporosis (66). Clinical data for sleep-disordered breathing are striking, as the prevalence for this disease almost doubles at menopause, independently of body mass and other coexisting risk factors (1, 3, 52, 62, 68, 71). Along with sleep-disordered breathing, other menopausal-like symptoms (e.g., hot flashes, mood alteration, and vaginal dryness) have also been observed in premenopausal women undergoing clinical ovariectomy (6, 21). Because hormone replacement therapy prevents and/or alleviates these manifestations (4, 49, 61, 71), ovarian hormones appear to “protect” against many menopause-related symptoms. For most women, however, the age-related decline in ovarian hormones does not result in health-threatening disorders. Therefore, decline in ovarian hormones may not be solely responsible for menopause-related disorders, yet the factors underlying such divergent aging trajectories remain poorly understood.

Results from human and animal research consistently show that early-life exposure to stress affects the normal regulation of the neuroendocrine response to stress and thus represents an important risk factor for a broad range of pathologies, including cardiorespiratory disorders (24, 29, 34, 48, 54, 58). Although most research is performed on male subjects, studies from our laboratory have consistently shown that neonatal stress has sex-specific effects on respiratory control development. Specifically, adult male rats previously subjected to neonatal maternal separation (NMS; 3 h/day from postnatal days 3–12) are hypertensive, present high levels of plasma corticosterone and ACTH, and show a hypoxic ventilatory response (HVR) ∼25% greater than that of controls (23, 24, 27). In females, NMS decreases the HVR by ∼30% (24). Although the physiological consequences of reduced HVR in rodents is uncertain, an exaggerated O2 chemoreflex predisposes to respiratory instability during sleep in both humans and rats (8, 24, 31, 70).

The mechanisms explaining the sex-specific effects of neonatal stress remain to be elucidated; however, results indicate that stress affects the interaction between hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes (11, 18, 55, 64). Simply stated, the functioning of each axis depends on the other and is usually indirectly proportional, such that chronic activation of the HPA axis will blunt gonadal functions. In female rats, estradiol stimulates, whereas progesterone attenuates basal activity of the HPA axis. However, a decrease in ovarian hormones by gonadectomy generally reduces HPA axis activity (25). Although limited, current evidence in humans suggests that the transition to menopause is associated with a rise in cortisol (69).

On the basis of the notion that ovarian hormones protect females against menopause-related symptoms and the important modulatory influence of sex hormones on respiratory regulation (2), we hypothesized that early-life exposure to stress affects age-dependent changes in HVR. To evaluate this, whole body plethysmography was used to measure the HVR of middle-aged female rats (MA; ∼57 wk old) that were either subjected to NMS or undisturbed during early life. We then determined whether the effects observed were related to hormonal decline or aging by comparing the impact of NMS on the HVR of bilaterally ovariectomized (OVX) young (∼12 wk old) adult female rats. In all experiments, a standard assay was used to obtain the hormonal profile of each group at rest and following hypoxic stress. This research aims to provide a
plausible explanation to menopause-related heterogeneity in symptoms and sleep-disordered breathing.

METHODS

Experimental Animals

Experiments were performed on 112 Sprague-Dawley female rats. Manipulation of ovarian hormones involved three groups: young adult rats with intact gonads (reference group), young ovariectomized (OVX) adult rats, and middle-aged rats (MA). Details on group size, age, and body weights are presented in Table 1. All rats were born and raised in our animal care facility. Animals were supplied with food and water ad libitum and maintained in standard care conditions (21°C, 12:12-h dark-light cycle: lights on at 0600 and lights off at 1800). Laval University Animal Care Committee approved the experimental procedures, and the protocols were in accordance with the regulations of the Canadian Council on Animal Care.

Mating and Neonatal Stress Procedures

Nulliparous females were mated and typically delivered between 12 and 18 pups; 2 days after delivery, litters were normalized to 12 pups, when necessary, with an equal number of males and females whenever possible. Litters exposed to neonatal stress were subjected to our standard NMS protocol (18, 24, 34). Briefly, maternal separation occurred from 0900 to 1200 on postnatal days 3 through 12; the entire litter was subjected to the NMS protocol and was separated from the dam and placed in a temperature- (35°C) and humidity- (45%) controlled incubator. Pups were isolated from each other by individual compartments to reduce stimulation between pups and standardizing external conditions influencing body temperature (24, 57, 67). During that period, control pups were left undisturbed. The choice of a control group to which NMS rats are compared is a matter of debate (39, 40), and our rationale for choosing this control group has been discussed previously (28). Briefly, most procedures disturbing the nest or the pups can influence development. For instance, exposing pups to short periods of handling (i.e., less than 15 min) during the neonatal period is sufficient to attenuate the stress response measured at adulthood. Handling is, therefore, considered a form of enrichment (44), which is a distinct treatment on its own (40). In the present context, animals maintained under standard rearing conditions are considered the most desirable and appropriate control group for studies investigating the effects of maternal separation (37).

The control litter was placed in a larger cage where they could remain unattended by animal care technicians for 10 consecutive days, allowing animals to be completely free from human interactions. After weaning on postnatal day 21, animals were housed in pairs until adulthood when surgical interventions and ventilatory measurements were performed. A minimum of two separate litters with different dams was used to avoid potential “litter effects”.

Anesthesia and Surgical Procedures

Ovariectomy. Rats were anesthetized with isoflurane (3% in air). A 1.5-cm abdominal incision was made vertically above the urinary bladder. On each flank, a long strip of pale adipose tissue was pulled out of the incision. This tissue links to the Fallopian tubes and the ovaries. Sham animals had their ovaries pulled entirely out of the abdomen, exposed to room air, and returned inside. Ovariectomized rats had both ovaries clamped, sutured, and completely removed. Controls received surgery solely to implant a telemetric probe (see procedure below). Contrary to shams, controls had no manipulation of internal organs. Preliminary experiments showed no differences between controls and shams; therefore, data from both groups were pooled and arbitrarily called “intact gonads”. This approach augmented statistical power, while optimizing use of experimental animals.

Table 1. Selected respiratory and metabolic variables measured under resting (normoxic) conditions

<table>
<thead>
<tr>
<th></th>
<th>Intact</th>
<th>Ovariectomy</th>
<th>Middle Age</th>
<th>Intact</th>
<th>Ovariectomy</th>
<th>Middle Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>315 ± 12</td>
<td>349 ± 12*</td>
<td>480 ± 18#</td>
<td>305 ± 11</td>
<td>348 ± 13*</td>
<td>497 ± 22#</td>
</tr>
<tr>
<td>Age, wk</td>
<td>12.6 ± 0.2</td>
<td>12.3 ± 0.2</td>
<td>56.8 ± 0.4#</td>
<td>13.9 ± 0.4</td>
<td>12.2 ± 0.5*</td>
<td>57.1 ± 0.3#</td>
</tr>
<tr>
<td>Tm, °C</td>
<td>38.6 ± 0.3</td>
<td>38.2 ± 0.2</td>
<td>37.3 ± 0.2*</td>
<td>38.1 ± 0.1</td>
<td>38.3 ± 0.2</td>
<td>37.4 ± 0.2#</td>
</tr>
<tr>
<td>Frequency, breaths/min</td>
<td>74 ± 5</td>
<td>87 ± 3</td>
<td>78 ± 3</td>
<td>77 ± 3</td>
<td>91 ± 3*</td>
<td>78 ± 4#</td>
</tr>
<tr>
<td>Allometric frequency, breaths/min</td>
<td>129 ± 8</td>
<td>143 ± 6</td>
<td>111 ± 4#</td>
<td>135 ± 4</td>
<td>149 ± 5</td>
<td>111 ± 5#</td>
</tr>
<tr>
<td>Tidal volume, ml BTPS/100 g</td>
<td>0.68 ± 0.05</td>
<td>0.70 ± 0.04</td>
<td>0.38 ± 0.06#</td>
<td>0.76 ± 0.1</td>
<td>0.61 ± 0.04</td>
<td>0.48 ± 0.06*</td>
</tr>
<tr>
<td>Vt, ml BTPS-min⁻¹·100 g⁻¹</td>
<td>50 ± 4</td>
<td>61 ± 5</td>
<td>29 ± 5#</td>
<td>60 ± 10</td>
<td>56 ± 3</td>
<td>37 ± 5#</td>
</tr>
<tr>
<td>Allometric Vt, ml BTPS-min⁻¹</td>
<td>29 ± 3</td>
<td>29 ± 2</td>
<td>9 ± 2#</td>
<td>35 ± 5</td>
<td>27 ± 3</td>
<td>12 ± 2#</td>
</tr>
<tr>
<td>V0₂, ml STPD-min⁻¹·100 g⁻¹</td>
<td>2.8 ± 0.2</td>
<td>3.0 ± 0.3</td>
<td>1.9 ± 0.1#</td>
<td>2.6 ± 0.2</td>
<td>3.3 ± 0.2*</td>
<td>1.8 ± 0.1#</td>
</tr>
<tr>
<td>Allometric V0₂, ml STPD/min</td>
<td>15.2 ± 0.8</td>
<td>17.4 ± 2.1</td>
<td>12.9 ± 0.7#</td>
<td>13.9 ± 1.1</td>
<td>19.0 ± 1.0*</td>
<td>11.8 ± 0.8#</td>
</tr>
<tr>
<td>Ve/V0₂</td>
<td>18 ± 2</td>
<td>22 ± 3</td>
<td>15 ± 3</td>
<td>26 ± 6</td>
<td>17 ± 1</td>
<td>23 ± 4#</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. The number measurement for each mean is indicated in parentheses. Note that the number of animals for some variables may differ since blood samples were sometimes taken for hormone analysis without measuring all parameters. Tm, body temperature; Vt, minute ventilation; V0₂, O2 consumption; Ve/V0₂, convective requirement ratio. Measurements were performed in rats subjected to neonatal maternal separation or undisturbed (control) over the same time period. At adulthood, rats either remained intact, were ovariectomized, or were raised until middle age. *Significantly different (P < 0.05) from intact adults. #Significantly different (P < 0.05) from ovariectomy. Allometric correction factors were obtained from Mortola et al. (42): Vt: baseline = Vt ml BTPS·min⁻¹·W⁰·⁶⁷⁻¹; V0₂ baseline = V0₂ ml STPD·min⁻¹·W⁰·⁵²⁻¹, where W refers to the weight of the animal expressed in kilograms. Allometric correction for breathing frequency was obtained by allometric Vt by tidal volume (corrected for BTPS but not body weight).
Telemetric probe insertion. A telemetric probe transponder (E-mitter, Mini Mitter, Bend, OR) was implanted surgically in each animal to measure real-time body temperatures (Ts) throughout the experiments. While rats were still under isoflurane anesthesia (3% in air), the probe was inserted in the peritoneum and sutured behind the internal wall of the abdominal cavity, according to our standard procedure (16, 18, 23, 45).

Post-surgical care and recovery period. At the end of the surgery, rats received subcutaneous injections of an antibiotic (Baytril; 5 mg/kg), an analgesic (buprenorphine; 0.02 mg/kg), and fluids (5 cc lactated Ringer). All animals were housed individually to avoid playing and fighting, which could interfere with the healing process. Animals were allowed 2 wk of recovery prior to ventilatory measurements.

Ventilatory and Metabolic Measurements

Ventilatory measurements were performed using a whole body, flow-through plethysmographic system (model PLY3223; Buxco Electronics, Sharon, CT), according to our standard protocol (16, 18, 23, 33, 45). Briefly, the rat was placed unrestrained in a 4.5-liter Plexiglas experiment chamber and allowed to calm and acclimate before launching measurements. This period typically lasted between 30 and 60 min. The breathing frequency (f), tidal volume (VT), minute ventilation (VE), and oxygen consumption (VO2) were all recorded using a data acquisition software (IOX, EMKA Technologies, Falls Church, VA). During the acquisition, parameters were adjusted to eliminate “nonrespiratory” signals related to movement artifacts or sniffing. The flow rate of air going in and out of the chamber was maintained between 2.0 and 2.5 l/min using a push-flow regulator pump (PLY 1020; Buxco Research, Wilmington, NC). Inflowing and outflowing O2 levels were monitored using an oxygen analyzer (model S-3A; Ametek, Pittsburg, PA) and portable O2 analyzer (TED-60-T; Teledyne Analytical Instruments, City of Industry, CA), respectively.

In all animals, basal ventilatory activity was first recorded while the rat was breathing room air (normoxia) for 10 min immediately followed by a 20-min period of moderate hypoxia (FIO2 0.12). When expressing results as a percent change from baseline, the last 5 min of hypoxic recording were averaged and compared with the averaged total baseline. Recordings were made between 0900 and 1300 to minimize fluctuations associated with circadian rhythms. Also during ventilatory measurements, the barometric pressure, body temperature, chamber temperature, and humidity were all recorded for subsequent calculation of VT expressed in milliliters (BTPS) per 100 g of body wt (Wb), according to the equations provided by Drorbough and Fenn (10). Because this study deals with animals of different ages and body weights, minute ventilation, breathing frequency, and oxygen consumption were corrected according to each animal’s own body weight following allometric calculations provided by Mortola et al. (46). Because there are several allometric correction factors for breathing frequency (20, 51), allometric frequency was calculated by dividing allometric VE by VT (expressed in BTPS but not corrected for Wb). Differences in calculations from standard (per 100 g) to allometric corrections are shown in Table 1. Differences between corrected and uncorrected results being typically minor, all figures report results with allometric corrections for conciseness.

Blood Sampling and Hormone Analyses

Blood samples for hormone analysis were obtained by terminal samples either under baseline conditions (normoxia) or at the end of hypoxia exposure. Prior to sampling, rats were placed in the plethysmography chamber, according to the protocol described previously. Following this acclimatization procedure (normoxia) or at the end of ventilatory recording (hypoxia), animals were deeply anesthetized with ketamine (Rogarsetic; 80 mg/kg) and xylazine (Rompun; 10 mg/kg), and blood was obtained by intracardiac puncture. The sample was placed in a serum-gel clotting activator microtube (Sarstedt, Nümbrecht, Germany) for analysis of progesterone and estradiol. Serum-gel tubes were kept at room temperature for 30 min before centrifugation (13,000 rpm, 4°C for 5 min). After centrifugation, blood serum was collected and placed in a −80°C freezer until assayed. Analyses of estradiol and progesterone were performed by the clinical biochemistry laboratory of our hospital using an electrochemiluminescence immunoassay test and read by the Elecsys 1010/2010 modular analyzer (Roche Canada, Mississauga, ON, Canada).

Statistical Analysis

The effects of hormonal decline (OVX vs. MA) and treatment (control vs. NMS) on body weights, as well as normalized ventilatory data, were compared using a two-way ANOVA. Absolute (nonnormalized) respiratory data and hormone levels were also analyzed using a three-way ANOVA (hypoxia, hormones, and stress). All statistical analyses were done using StatView 5.0 (SAS Institute, Cary, NC). A repeated-measures design was used when appropriate. ANOVAs were followed by Fisher’s post hoc test when P ≤ 0.05. ANOVA results are mainly reported in the figures, but not in Table 1. ANOVA results for some ventilatory variables (nonnormalized data) are reported in the text. Note that whenever main effects and factorial interactions were significant, only the results of the interaction are reported for clarity and conciseness. Results from post hoc tests are displayed with symbols in figures. Data are reported as means ± SE.

RESULTS

Age and body weights. Middle-aged female rats were on average 44 wk older than intact and OVX adults (Table 1; age effect: P < 0.0001). Body weights recorded on the day of experimentation were significantly higher in MA rats (age effect: P < 0.0001) but were unaffected by NMS (P = 0.27). Also, the body weights of OVX were significantly higher than age-matched intact rats (Table 1, P < 0.0001).

Estradiol. Ovariectomy and aging similarly decreased basal (normoxic) levels of estradiol (Fig. 1A). Basal levels of estradiol were not affected by neonatal stress. Statistical analysis suggests that all three factors (hypoxia, stress, and hormonal status) influenced estradiol; following hypoxia, estradiol levels of intact NMS females were higher than baseline and the corresponding control value (Fig. 1B).

Progesterone. Resting levels of progesterone were significantly reduced following OVX and MA independently of the NMS treatment (Fig. 2A). Hypoxia had no effect on progesterone levels (Fig. 2B).

Tidal volume. When normalized to body weight, basal tidal volume of MA rats were lower than intact adults and OVX (hormonal effect: P < 0.0001) but were unaffected by NMS (P = 0.53). Tidal volume (VT) increased during hypoxia (P = 0.0003; absolute data not shown), and the response was influenced by hormonal treatment and stress. While the VT response of intact rats did not differ between groups, OVX and MA augmented the response significantly in NMS but not controls; this effect was most noticeable in OVX females (Fig. 3A).

Breathing frequency. During normoxia, the breathing frequency (f) of OVX animals was generally higher than intact adults and MA (Table 1: P = 0.002); this effect was unaffected by NMS (P = 0.53). Following allometric correction, f values were lowest in MA females (hormonal effect: P < 0.0001); this variable was not influenced by NMS even after correction (NMS effect: P = 0.37). Hypoxia significantly increased both f and allometric f in all animals (hypoxia effect: P < 0.0001 for
both; data not shown); however, the phenotype of the response was influenced by hormonal status and neonatal stress (Fig. 3B). Ovariectomy and aging both decreased the allometric frequency response to hypoxia significantly in controls but not NMS rats (Fig. 3B).

**Minute ventilation.** Resting allometric minute ventilation ($V_e$) was significantly reduced by aging (Table 1: hormonal effect: $P < 0.0001$) but was not affected by NMS (Table 1; $P = 0.13$). Hypoxia significantly increased the allometric $V_e$ in all groups ($P < 0.0001$; absolute data not shown). Expressing allometric $V_e$ as a percentage change from baseline showed that aging and OVX reduced the ventilatory response of controls. This effect was not observed in NMS rats (Fig. 3C).

Performing this analysis on nonnormalized data (i.e., without allometric correction and not expressed as % change from baseline) yielded similar results (hypoxia × hormonal treatment × NMS: $P = 0.04$; data not shown).

**Body temperature.** Basal body temperature ($T_b$) was significantly lower in MA animals compared with intact adults and OVX (Table 1: $P < 0.0001$) but was not affected by NMS ($P = 0.60$). Anapyrexia was observed in all rats except OVX females (hypoxia effect: $P < 0.0001$; absolute data not shown); MA females showed the largest response (Fig. 4A). This response was not affected by NMS.

**Allometric oxygen consumption.** Basal allometric oxygen consumption ($\dot{V}O_2$) was lower in MA females. Ovariectomy

![Diagram A](image1.png)

![Diagram B](image2.png)

Fig. 1. Effects of ovariectomy (OVX), aging, and neonatal stress on total plasma estradiol levels measured at rest and following exposure to moderate hypoxia. Blood samples were taken after the rat was allowed to acclimatize to the plethysmography chamber under normoxic conditions for 1 h (A) or following ventilatory measurements under moderate hypoxia (12% O₂; 20 min) (B). Histograms compare the effects of OVX and aging (middle age) in rats previously subjected to neonatal maternal separation (NMS; black bars) vs. animals maintained under control conditions over the same period (CTR; white bars). Data are reported as means ± SE. *Significantly different from corresponding intact group at $P < 0.05$. †Significantly different from corresponding control value at $P < 0.05$.

![Diagram C](image3.png)

Fig. 2. Effects of OVX, aging, and neonatal stress on total plasma progesterone levels measured at rest and following exposure to moderate hypoxia. Blood samples were taken after the rat was allowed to acclimatize to the plethysmography chamber under normoxic conditions for 1 h (A) or following ventilatory measurements under moderate hypoxia (12% O₂; 20 min) (B). Histograms compare the effects of OVX and aging (middle age) in rats previously subjected to neonatal maternal separation (NMS; black bars) vs. animals maintained under control conditions over the same period (CTR; white bars). Data are reported as means ± SE. *Significantly different from corresponding intact group at $P < 0.05$. Numbers in parentheses indicate the number of animals for each measurement.
increased \( \dot{V}O_2 \) above levels measured in intact females; however, this effect was significant only in NMS rats (Table 1; hormonal effect: \( P < 0.001 \)). Hypoxia significantly decreased \( \dot{V}O_2 \) (hypoxia effect: \( P < 0.0001 \) for both corrected and uncorrected measurements; data not shown); however, the allometric response (% \( \dot{V}O_2 \)) was not affected by hormonal conditions and NMS treatment (Fig. 4B).

**Convective requirement ratio.** The basal convective requirement ratio (\( \dot{V}E/\dot{V}O_2 \)) was unaffected by OVX, aging or NMS (Table 1: hormonal effect: \( P = 0.61 \); NMS effect: \( P = 0.21 \)). Hypoxia increased the allometric convective requirement ratio \( \dot{V}E/\dot{V}O_2 \) in all groups (\( P < 0.0001 \); absolute data not shown). During hypoxia, \( \dot{V}E/\dot{V}O_2 \) was influenced by hormonal status and NMS. By comparison with intact animals, the \( \dot{V}E/\dot{V}O_2 \) of MA females was reduced in controls but not in NMS (Fig. 5). As a result, the hypoxic \( \dot{V}E/\dot{V}O_2 \) of MA females was enhanced in NMS females compared with controls (Fig. 5).

**DISCUSSION**

At menopause, the emergence of sleep-disordered breathing is not solely caused by a decline in circulating levels of ovarian hormones, since a significant number of menopausal women remain symptom-free. Research has shown that early-life perturbations affect the normal regulation of the neuroendocrine response to stress and its interaction with the HPG axis. Consequently, this represents an important risk factor for a broad range of diseases, including cardiorespiratory disorders. The present series of experiments examined the consequences of neonatal stress (maternal separation) on functions of the respiratory control system during middle-age (MA) in female rats. Also, to investigate the impact of ovarian hormones, experiments were repeated on bilaterally ovariectomized (OVX) adult females. The main finding of this study is that NMS affects the impact of hormonal decline on thermoregu-
Critique of methods. The hormone levels reported here are comparable to those we have obtained previously (12, 19), but they are slightly lower than those reported by others (15, 72). The factors explaining these discrepancies are unknown; however, ovarian hormones fluctuate significantly throughout the day and the estrous cycle. Despite their importance, these variables are difficult to control and are rarely considered in experimental designs, a situation that likely contributes to differences in hormone levels reported between studies. Here, the estrous cycle was not determined because its evaluation (vaginal smears) is stressful, and preliminary work performed in our laboratory has shown that this procedure influences experimental results, especially in NMS females.

In female rats, irregularities in estrous cycles and in secretion of gonadotropin and ovarian steroids become apparent as early as 10–12 mo old (47), and menopause is established between 64 and 77 wk of age (13). As our MA females were slightly younger, their precise stage is uncertain, but the reduction in hormone levels indicate that these animals were at the “premenopausal” stage.

Unlike castration in males (17), OVX did not reduce gonadal hormone levels near zero (Figs. 1 and 2), owing to the synthesis occurring at extra-ovarian sites, such as the adrenal glands, the liver, and adipose tissues (30). Because ovariectomy normally results in large increases in food intake and weight gain (5), the difference in body weight between OVX and intact rats suggests that the surgical procedure was successful. Most importantly, 1) MA and OVX rats both efficiently reduced circulating ovarian hormones to comparable levels (the levels measured did not differ significantly between these groups) and 2) the reductions achieved in OVX and MA were sufficient to reveal substantial effects on key homeostatic systems.

The accuracy of tidal volume (VT) measurements obtained with whole body plethysmography is a matter of debate, especially in neonatal rodents (14, 73). Tidal volume is calculated from the pressure changes in the chamber where the animal is placed, which is why temperature, barometric pres-
sure, and humidity must be considered in these measurements. Originally, the barometric method was elaborated using a closed chamber (9); however, the need to perform ventilatory measurements over prolonged periods while ensuring constant (and controlled) $O_2$ and $CO_2$ levels in the chamber led to the development of an “open-flow” system, such as the one used here. Although convenient, this system implies a decay in the pressure waveform, and consequently, it could be argued that the signal no longer provides a direct measurement of $V_T$. As a result, the values obtained could reflect a qualitative rather than a quantitative assessment of this variable. Regardless of the theoretical arguments, validation of any experimental technique is ultimately based on comparisons with other methods. We recently performed this exercise in mice, and data show that the open flow plethysmograph produces $V_T$ values that are very similar to those obtained with more direct methods such as “head-out” plethysmography (53). Placing a pneumotachograph on the trachea of an anesthetized animal is arguably the most direct (and accurate) method to measure $V_T$, and in adult rats, the baseline values obtained with this method [$0.7 \pm 0.13\text{ mL/100 g;}$ (22)] are similar to those reported in Table 1. Clearly, accurate $V_T$ measurements with an open-flow plethysmograph has its limitations and can be complex, but the favorable comparisons with other (more direct) approaches validate the accuracy of our results.

Sleep-wake cycles have a profound effect on breathing (50). Here, ventilatory recordings were initiated when the rat appeared calm and breathing was stable, assuming the rat was in “quiet wakefulness”. Although rats were monitored visually (and sometimes aroused by gently tapping on the box) to ensure that they were not asleep, the lack of proper instrumentation may have led us to assume that results were always obtained in the same arousal state. Instrumentation for sleep/wake monitoring requires an invasive surgical procedure. Given the limited number of MA females available, we were concerned about the potential impact/mortality risk associated with this procedure. Moreover, we previously showed that the effects of NMS on respiratory regulation during wakefulness and non-REM sleep are similar (34). Although the lack of sleep/wake monitoring is a limitation of this study, the present study reveals significant effects of neonatal stress on the physiology of aging females. As we discuss below, the hormonal profile and the use of OVX provides valuable mechanistic insight.

**Impact of neonatal stress, ovarectomy, and aging on circulating levels of ovarian hormones at rest and during hypoxia.** Neonatal stress did not affect basal levels of ovarian hormones in any of the three experimental groups. Following hypoxia, however, estradiol levels measured in NMS females with intact gonads were higher than baseline and the corresponding hypoxic control values (Fig. 1B); this increase was not observed in OVX and MA. To the best of our knowledge, this is the first report, suggesting a rapid increase in circulating levels of estradiol in response to hypoxia. Despite substantial differences, the marginal statistical interaction supporting this effect of hypoxia ($P = 0.08$) advises caution with data interpretation. Nevertheless, this result is consistent with the concept that neonatal stress disrupts the development and functions of the HPG axis (64). The source of estradiol is uncertain, but considering that this hypoxic response was not observed in OVX females, it suggests that the ovaries play a prominent role. Given that the response was relatively rapid (within 20 min of hypoxic challenge), it would appear that a strictly endocrine pathway is unlikely and point to a more direct (neural) communication between the hypothalamus and the gonads, which bypasses the pituitary and release of the luteinizing hormone-releasing hormone (41, 59, 60, 64). Hence, we propose that hypoxia stimulates the hypothalamic and/or brain stem regions, which then activates the ovaries neurally. Stress usually blunts reproductive functions (64), so the reason why stress-induced stimulation of the ovaries occurs in NMS females remains poorly understood but suggests a hypersensitivity of this circuitry. We must keep in mind, however, that sex hormone-binding globulins (SHBG) are important modulators of bioavailable (and thus detectable) levels of hormones in the circulation (30). Because many stressors (including hypoxia) can influence hepatic SHBG production (38), it is, therefore, possible that these proteins contributed to changes in hormone levels reported here. It is noteworthy that ovariectomy does not affect corticosteroid-binding globulin (CBG) levels in female rats (25).

**Effect of neonatal stress, ovarectomy, and aging on the ventilatory response to hypoxia.** Stress-related attenuation of the HVR in young females with intact gonads is consistent with previous reports published from our laboratory (24). Here, we show that in control rodents, reducing circulating levels of ovarian hormones (whether by OVX or aging) reduces the HVR. This result is consistent with the notion that ovarian hormones are powerful ventilatory stimulants (2, 56). By contrast, the HVR of NMS rats is insensitive to reduction of ovarian hormones. These results are very interesting but remain difficult to explain at this point. Since our previous work showed that NMS can influence expression of androgen receptors in key brain stem regions regulating the HVR (17), a reduction in ovarian hormone receptors in NMS is plausible. Should NMS exert a similar effect in females, a reduction in estradiol (or progesterone) receptors in key regions (e.g., nucleus of the solitary tract) could explain why OVX or aging did not affect the HVR of NMS rats. Nevertheless, data show that the HVR of MA females is greater in NMS rats than controls, and the higher $V_{E}/V_{O_2}$ observed during hypoxia reveals a relative hyperventilation, indicative of an abnormally elevated neural command. It is noteworthy that this effect was not observed in OVX females, which constitutes an important distinction between the two experimental approaches. Consequently, using OVX as a tool to mimic the effect of aging in fundamental research contains limitations, at least regarding respiratory physiology.

At middle age, the respiratory phenotype of NMS females is similar to that observed in young adult males in which the magnitude of the HVR is ~25% greater than control (23, 24) and correlates positively with respiratory instability during non-REM sleep (34). It is likely that the augmented HVR in MA females has a similar pathophysiological impact. However, this remains to be confirmed experimentally. Recent studies on male rats have addressed the numerous and complex mechanisms explaining how NMS interferes with the function of key groups of neurons regulating breathing, and data show that disruption in the balance between inhibitory and excitatory modulation is an important effect of NMS (23, 27, 32). Whether this applies to MA females remains to be determined. While disruption of hormonal secretion appears important in
young adults, the hormonal profile obtained at MA suggests that this mechanism is no longer important.

Effect of neonatal stress, ovariectomy, and aging on the thermoregulatory response to hypoxia. Compared with intact animals, results show that aging (and not OVX) decreases the resting body temperature ($T_b$) of both controls and NMS (Table 1). As it is likely associated to reduced heat production, the lower $V_O_2$ observed in MA females could contribute to this effect. The effect of aging on $T_b$ regulation is observed in most mammals, including humans (7, 42, 43, 63). With aging, it becomes more difficult to regulate core $T_b$ and control heat loss (26). Thermoregulation is managed by neurons of the hypothalamus and is under the control of hormones. For example, injections of estradiol in the median preoptic area of female rats maintain $T_b$ constant during cold exposure (65). However, the drop in resting $T_b$ observed in MA animals occurs despite similar levels of ovarian hormones with OVX rats.

Under hypoxia, animals go through anapnoea, a regulated thermal response that aims at lowering body temperature in the attempt to reduce oxygen demand. Although its related mechanism of action is not fully understood, it includes the participation of various neurotransmitters (e.g., nitric oxide) and hydrogen sulfide (35). Current results show that MA (and not OVX) females still express anapnoea. Recent findings in our laboratory have shown that estradiol supplementation (alone or in combination to progesterone) prevents anapnoea in rat pups (36). Subtle changes in housing conditions that increase levels of estradiol in adult male rats also contribute to prevent anapnoea (18). While estradiol seems to play an important role in the anapnoeic response, levels recorded in OVX females do not exceed that of intact adults and are similar to levels found in MA animals. This alone cannot explain why the anapnoeic response is abolished in OVX and not in MA females. One possible explanation is that levels of ovarian hormones decrease rapidly in OVX females compared with the slowly fading activity of MA. This sudden change likely elicited changes in the brain structure and function (e.g., synaptic plasticity and receptor availability) that may not have occurred in MA females.

Perspectives and Significance

Neonatal stress affects the developmental trajectory of vital homeostatic systems, such as respiration, and may represent an important risk factor for cardiorespiratory diseases. Throughout adulthood, ovarian hormones modulate ventilation and seem to “mask” neonatally induced perturbations in the control of breathing. As women approach menopause, the natural decline in ovarian hormones unveils these deficits; however, some women remain symptom-free. While most affected women respond positively to hormone replacement therapy (61, 71), current results show that ovarian hormone-induced plasticity of the respiratory control system is influenced by the neonatal history of stress of the individual. This result may encourage the evaluation of menopause-related symptoms to include questionnaires about early childhood health, adversity, and parental socio-economic status.

GRANTS

This research was supported by an operating grant from Canadian Institute of Health Research to R. Kinkead (MOP: 81335).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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