

Participation of locus coeruleus in breathing control in female rats



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ABSTRACT

Several evidences indicate that the locus coeruleus (LC) is involved in central chemoreception responding to CO₂/pH and displaying a high percentage of chemosensitive neurons (> 80%). However, there are no studies about the LC-mediated hypercapnic ventilation performed in females. Therefore, we assessed the role of noradrenergic LC neurons in non-ovariectomized (NOVX), ovariectomized (OVX) and estradiol (E2)-treated ovariectomized (OVX + E2) rats in respiratory response to hypercapnia, using a 6-hydroxydopamine (6-OHDA) – lesion model. A reduction in the number of tyrosine hydroxylase (TH) immunoreactive neurons (51–90% in 3 animals of NOVX group, 20–42% of lesion in 5 animals of NOVX females, 61.3% for OVX and 62.6% for OVX + E2 group) was observed seven days after microinjection of 6-OHDA in the LC. The chemical lesion of the LC resulted in decreased respiratory frequency under normocapnic conditions in OVX and OVX + E2 group. Hypercapnia increased ventilation in all groups as consequence of increases in respiratory frequency (fR) and tidal volume (V_T). Nevertheless, the hypercapnic ventilatory response was significantly decreased in 6-OHDA-NOVX > 50% rats compared with SHAM-NOVX group and with females that had 20–42% of LC lesion. In OVX and OVX + E2 lesioned groups, no difference in CO₂ ventilatory response was observed when compared to SHAM-OVX and SHAM-OVX + E2 groups, respectively. Neither basal body temperature (T_b) nor T_b reduction in response to hypercapnia were affected by E2 treatment, ovariectomy or LC lesion. Thus, our data show that LC noradrenergic neurons seem to exert an excitatory role on the hypercapnic ventilatory response in female rats, as evidenced by the results in NOVX animals with LC lesioned more than 50%; however, this modulation is not observed in OVX and OVX + E2 rats. In addition, LC noradrenergic neurons of OVX females seem to provide a tonic excitatory drive to maintain breathing frequency in normocapnia, and this response may not be functionally influenced by E2.

1. Introduction

Locus coeruleus (LC) has the largest noradrenergic group in central nervous system (CNS), the A6 region (Moore and Bloom, 1979; Dahlström and Fuxe, 1964), and it has been associated with a number of physiological and behavioral functions including cardiovascular, respiratory control, sleep–wake cycle, feeding, thermoregulation, nociception, attention and learning (De Carvalho et al., 2010; De Souza Moreno et al., 2010; Biancardi et al., 2008; Almeida et al., 2004; Putnam et al., 2004; Oyamada et al., 1998; Aston-Jones et al., 1985; Hobson et al., 1975).

LC has been shown to exert an important excitatory role in the ventilatory response to hypercapnia (Silva et al., 2017; Patrone et al.,

2014; Taxini et al., 2013; De Carvalho et al., 2010; Biancardi et al., 2008). Indeed, some studies suggest that LC neurons act directly as CO₂/pH chemosensors and that 80% of these neurons are chemosensitive responding to hypercapnia with increased firing rate (Filosa et al., 2002; Oyamada et al., 1998; Pineda and Aghajanian, 1997; Coates et al., 1993).

In addition to modulating physiological functions, LC is involved in several disorders, including panic disorder, post-traumatic stress and depression (Singewald and Philippu, 1998) which are prevalent in women (Andrade et al., 2006). This prevalence appears to be linked to hormonal and reproductive events (Rapkin et al., 2002). It is known that estrogen receptors alpha (ER α) and beta (ER β) are expressed in noradrenergic neurons of the LC in female mice (Pendergast et al.,

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2008) and that high levels of estradiol (E2) reduce the activity of LC neurons during normocapnia, suggesting an inhibitory role for E2 in this nucleus (De Carvalho et al., 2016).

Previous studies have shown that female rats need a continuous presence of E2 for the development of LC, since the absence of this modulation promotes a decrease in the volume and in the number of LC neurons (De Blas et al., 1990; Segovia et al., 1990; Guillamóm et al., 1988). Moreover, the activity of LC neurons is modulated by this hormone (Szawka et al., 2009) and E2 administration in ovariectomized rats (OVX) produces a dose-dependent elevation in tyrosine hydroxylase (TH) mRNA levels in LC (Serova et al., 2002).

As LC is an important nucleus in chemoreception in males and there are no studies about the LC-mediated hypercapnic ventilation performed in females, we investigated the role of LC in the respiratory response to CO₂ in female rats. Our hypothesis is that LC noradrenergic neurons exert an excitatory control in the hypercapnic ventilatory response in females and E2 modulates this response. Therefore, we assessed the role of these neurons in non-ovariectomized (NOVX), ovariectomized (OVX) and E2-treated ovariectomized (OVX + E2) rats in the respiratory response to hypercapnia by using 6-hydroxydopamine (6-OHDA), a neurotoxin that destroys selectively catecholaminergic neurons in the brain.

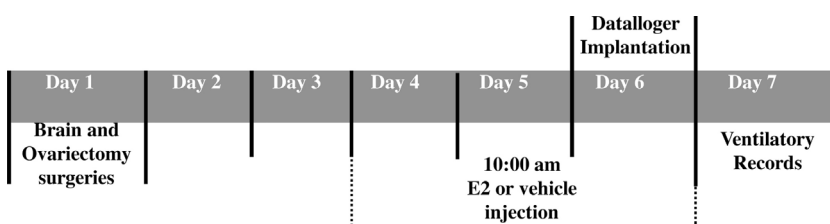
2. Methods

2.1. Animals

Experiments were performed on unanesthetized adult female Wistar rats weighing 250–310 g. The animals had free access to water and food and were housed in controlled temperature room (25 ± 1 °C) with a 12:12 h light:dark cycle (lights on at 6:00 am). Estrous cycle regularity was assessed in females and only rats showing at least three consecutive regular 4-day cycles were subject to surgery and then to the ventilatory experiment. The study was conducted in compliance with the guidelines of the National Council for the Control of Animal Experimentation (CONCEA, MCT, Brazil) and with the approval of the local Animal Care and Use Committee (CEUA, FACV-UNESP Jaboticabal; # 000222-09).

2.2. Experimental design

Fig. 1 shows a schematic diagram of experimental design. The selective chemical lesions of LC neurons were performed in female rats seven days before the ventilatory experiment. Rats were stereotaxically injected with 6-OHDA in the LC and after brain surgery some animals were submitted to ovariectomy. A week later, each animal was individually placed in a plexiglass chamber (5 L) and allowed to move freely while the chamber was flushed with humidified air. After approximately 30 min of adaptation, control ventilation was measured. Next, a hypercapnic gas mixture (7% CO₂ in air breathing) was flushed through the chamber for 45 min and Ve was measured at the end of CO₂ exposure. Tb was continuously recorded throughout the experiment. All ventilatory experiments were conducted between 8:00 am and 13:00 pm.



2.3. Anesthesia and post-surgical treatment

All surgical procedures were performed under anesthesia with ketamine (100 mg/Kg, i.p.; Agener, SP, Brazil) and xylazine (10 mg/Kg, i.p.; Coopers, SP, Brazil), antibiotic protection (10 mg/kg, s.c.; Enrofloxacin, Flotril, Schering-Plough, SP, Brazil) and analgesic (2.5 mg/kg, s.c.; Flunixin meglumine, Banamine; Schering-Plough, SP, Brazil).

2.4. Brain surgery

For selective chemical lesions rats were fixed to a stereotaxic frame (David Kopf Instruments, Kent, England) and the coordinates to locate LC (−3.1, −3.4 and −3.7 mm from lambda; ± 1.2 mm from midline; −7.0 mm from the skull surface; incisive bar at 0 mm; inclination of vertical stereotaxic bar at 15°) were based on the stereotaxic atlas for rats (Paxinos and Watson, 1998). Aiming to achieve the full extent of the LC (rostral, medial and caudal nucleus portions) 3 bilateral micro-injections with a distance of 0.3 mm between each point were performed. The tip of a needle was inserted into LC for injection of 6-OHDA (Sigma Aldrich, St Louis, MO, USA) solution of 8 µg/1 µL of vehicle as previously described (Biancardi et al., 2008). Sham rats were injected with vehicle (1 µg ascorbic acid – Sigma Aldrich, St Louis, MO, USA- in 0.1 µL of saline). At each point, it was microinjected 0.250 µL (6-OHDA or vehicle) over 6 min each microinjection, totaling 0.750 µL for each side of the LC.

2.5. Ovariectomy and hormone treatment

A group of animals was submitted to ovariectomy by midline laparotomy. Three consecutive days prior to the experiment the females were treated with vehicle (corn oil, OVX group; 0.2 mL/rat, s.c.) or with 17β-estradiol (OVX + E2 group; 10 µg/0.2 mL/rat, s.c., oestradiol cypionate; Pfizer, SP, Brazil; Szawka et al., 2009; De Carvalho et al., 2016) at 10:00 am On the fourth day, rats were tested in the ventilatory experiment.

2.6. Datalogger implantation

For body core temperature (Tb) measurements, a temperature datalogger (SubCue, Calgary, AB, Canada) was implanted in the abdominal cavity through a midline laparotomy one day before experiments. The datalogger was programmed to acquire data at every 5 min.

2.7. Experimental groups

In the present study, the term SHAM is referring to the animals that had ascorbic acid (vehicle) microinjected in the LC. Our animals were divided in seven groups: 1) non-ovariectomized females that vehicle was microinjected in the LC (SHAM-NOVX, n = 5); 2) non-ovariectomized females that 6-OHDA was microinjected in the LC and promoted a lesion of noradrenergic neurons larger than 50% (6-OHDA-NOVX > 50%, n = 3); 3) non-ovariectomized females that 6-OHDA was microinjected in the LC and promoted a lesion of noradrenergic neurons between 20 and 42% (6-OHDA-NOVX 20–42%, n = 5); 4) ovariectomized females that vehicle was microinjected in the LC and

Fig. 1. Schematic diagram of experimental design.

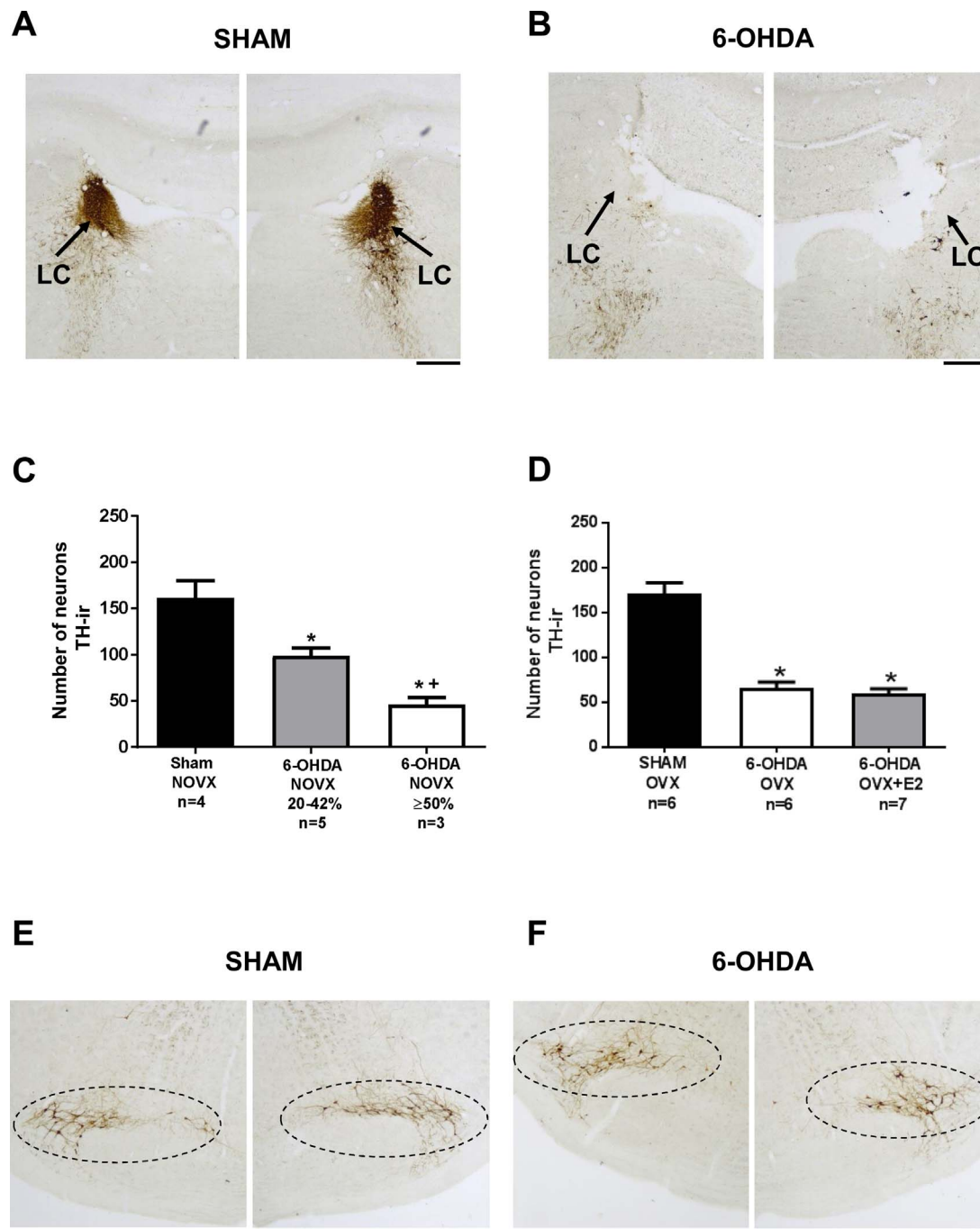


Fig. 2. (A and B) Representative photomicrographs of the immunohistochemistry staining for tyrosine hydroxylase (TH) in the LC in sham (A; Ascorbic Acid) and bilaterally lesioned (B; 6-OHDA) groups. LC: *Locus coeruleus*. Calibration bar: 100 μ m. (C) Number of LC TH-ir neurons of non-ovariectomized animals (NOVX). (D) Number of LC TH-ir neurons of ovariectomized animals treated with oil (OVX) or estradiol (OVX + E2). (E-F) A5 region (dashed circles) marked for TH-ir neurons in sham (E) and lesioned rats (F). Calibration bar: 100 μ m. *Difference between sham and lesioned groups ($P < 0.0001$; One-way ANOVA). +Difference between 6-OHDA-NOVX $> 50\%$ and 6-OHDA-NOVX 20–42% ($P < 0.0001$; One-way ANOVA).

received corn oil subcutaneously (SHAM-OVX, $n = 6$); 5) ovariectomized females that 6-OHDA was microinjected in the LC and received corn oil subcutaneously (6-OHDA-OVX, $n = 7$); 6) ovariectomized females that vehicle was microinjected in the LC and received E2 subcutaneously (SHAM-OVX + E2, $n = 7$); 7) ovariectomized females that 6-OHDA was microinjected in the LC and received E2 subcutaneously (6-OHDA-OVX + E2, $n = 7$).

2.8. Determination of pulmonary ventilation

Measurements of pulmonary ventilation (V_E) were performed using

the whole body plethysmograph method (Bartlett and Tenney, 1970) as previously described (for details see De Carvalho et al., 2010; Biancardi et al., 2008). Freely moving rats were kept in a 5-L chamber ventilated with humidified room air or with a mixture containing 7% CO_2 in air (7% CO_2 , 21% CO_2 and N_2 balance – White Martins, Sertãozinho, SP, Brazil). The animal chamber operates with inflow of humidified gas, and the flow rate was maintained in 0.8–1.0 L/min by a flowmeter (model 822-13-OV1-PV2-V4, Sierra Instruments, Monterey, CA, USA). The air temperature in the chamber was monitored by datalogger (SubCue, Calgary, AB, Canada) and the chamber was constantly humidified and humidity was settled as 100%. The results were analysed

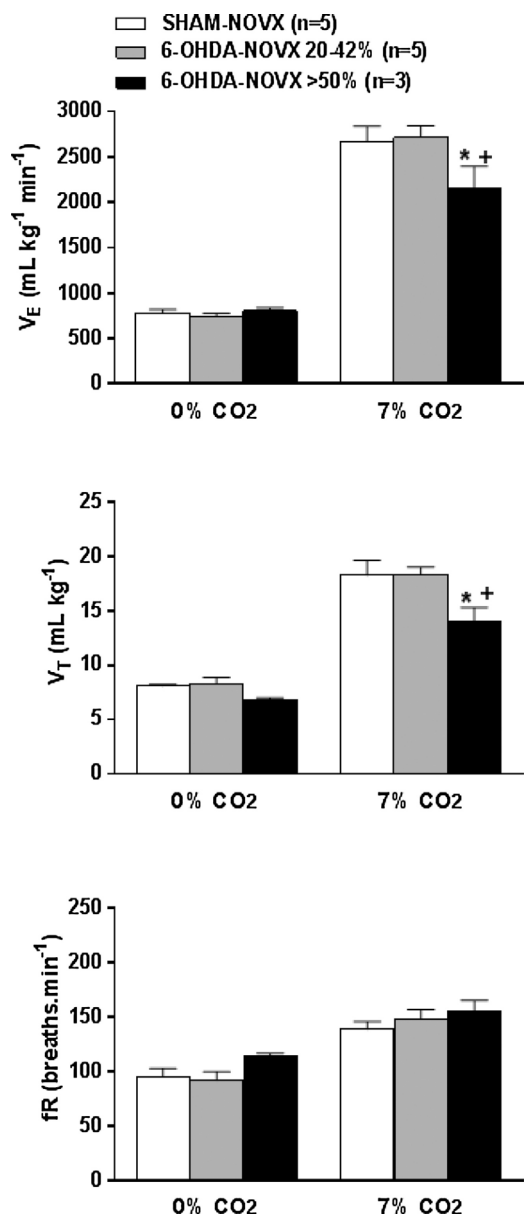


Fig. 3. Ventilation (V_E), tidal volume (V_T) and respiratory frequency (fR) in sham non-ovariectomized females (SHAM-NOVX), non-ovariectomized females that 6-OHDA promoted a LC lesion larger than 50% (6-OHDA-NOVX > 50%) and non-ovariectomized females that 6-OHDA promoted a LC lesion between 20 and 42% (6-OHDA-NOVX 20–42%) exposed to normocapnia and hypercapnia (7% CO₂). *Difference between SHAM-NOVX and 6-OHDA-NOVX > 50%. + Difference between 6-OHDA-NOVX > 50% and 6-OHDA-NOVX 20–42%.

with the data-analysis software Acqknowledge (v3.8.1 data acquisition system, Biopac Systems Inc., Santa Barbara, CA, USA).

2.9. Hormone assay

After ventilatory experiments, OVX and OVX + E2 rats were anesthetized with ketamine and xylazine and a blood sample of approximately 1 mL was collected from the heart into heparinized syringes. Plasma was separated by centrifugation at 3000 rpm for 20 min at 4 °C and stored at –20 °C for posterior analyses of E2 levels by radioimmunoassay (RIA). E2 plasma concentrations were determined by double-antibody RIA with MAIA kits provided by Biochem Immunosystem (Bologna, Italy). The lower limits of detection for E2 were 5.0 pg/mL and the intra-assay coefficient of variation was 4.3%.

2.10. Assessment of 6-OHDA chemical lesion effectiveness and placement

Rats under deep ketamine and xylazine anesthesia were transcardially perfused with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Frontal sections of 25 μm were cut through the LC region in a cryostat (Microm, Model HM500 OM, Germany). To verify the correct placement and effectiveness of the chemical lesions, tyrosine hydroxylase (TH) immunoreactivity was assessed as a marker of catecholaminergic neurons (Xu et al., 2003). To this purpose, the sections were incubated for 48 h with monoclonal mouse anti-TH antibody (Sigma, MO, USA) at 1:10000 followed by 2 h incubation with a biotinylated rabbit polyclonal anti-mouse (Dako, Denmark) at 1:1000. The biotinylated antibody was complexed with avidin DH–biotinylated horseradish peroxidase (PK-4001, Vector Laboratories, Burlingame, CA, USA), and the complex was developed by addition of the peroxidase substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB) according to manufacturer's instructions (Sigma, MO, USA). Sections were mounted on gelatin-coated glass slides. Photomicrographs were captured using AxioVision software (from Carl Zeiss, Munich, Germany). To estimate the ablation of TH-ir neurons caused by 6-OHDA, we counted the number of TH-immunoreactive (ir) neurons bilaterally in 25-μm sections (9.16–10.32 mm from bregma). Analyses were performed using a computerized image analysis system Image J (US National Institutes of Health).

2.11. Data processing and analysis

The results are reported as means ± SEM. Ventilatory and temperature data were analysed using Two-way ANOVA followed by the Bonferroni post-hoc test. The reduction of LC noradrenergic neurons was determined by One-way ANOVA. Statistical analyses were performed using GraphPad Prism (La Jolla, CA, USA). Values of $P < 0.05$ were considered to be significant. In the graphs, the differences between normocapnia and hypercapnia are not represented, only differences between groups (sham and 6-OHDA) with statistical significance.

3. Results

3.1. Assessment of 6-OHDA chemical lesion effectiveness and placement

The immunohistochemistry for TH shows the effectiveness of chemical lesions of the LC with 6-OHDA (Fig. 2). The LC of SHAM groups was intact and typically appeared as a compact cluster of intensely stained cells (Fig. 2A). Successful bilateral 6-OHDA-lesions of the LC were revealed mostly by the decrease of TH-positive cells (Fig. 2B). TH-ir neurons were quantified after 6-OHDA microinjection in NOVX, OVX and OVX + E2 groups. In NOVX animals, 3 females presented a reduction of TH-ir neurons larger than 50% compared to SHAM-NOVX group, whereas in other 5 females, 6-OHDA decreased TH-ir LC neurons from 20% to 42% (Fig. 2C; $P < 0.0001$; One-way ANOVA). In OVX and OVX + E2 groups, chemical lesion caused a reduction of TH-ir neurons higher than 50%. Injections of 6-OHDA into the LC reduce TH-ir cells in the LC of OVX (61.3% of reduction, $P < 0.0001$; One-way ANOVA) and OVX + E2 rats (62.6% of reduction, $P < 0.0001$; One-way ANOVA) with no difference between OVX and OVX + E2 rats (Fig. 2D). We can observe that lesion was selective to the LC since the noradrenergic population of neurons in the A5 region, remained preserved after the microinjections of 6-OHDA (Fig. 2E–F).

3.2. Estrous cycle stages, pulmonary ventilation and body temperature of non-ovariectomized females

In SHAM-NOVX group 3 rats were in diestrus and 2 in estrus. After LC lesions, all rats remained in diestrus (data not shown).

Fig. 3 presents the V_E , V_T and fR values of SHAM (Ascorbic acid) and LC-lesioned (6-OHDA-NOVX > 50% and 6-OHDA-NOVX 20–42%)

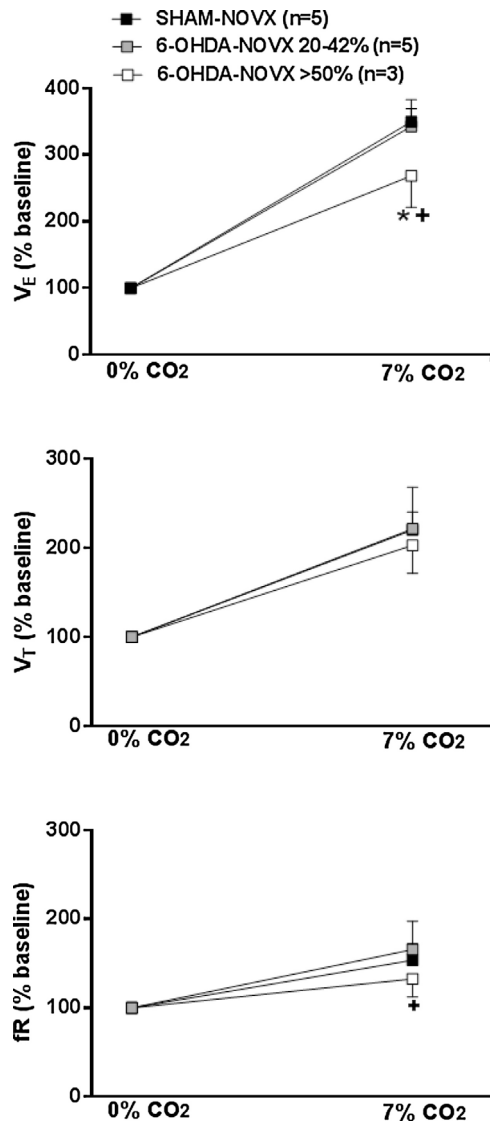


Fig. 4. The relative changes in ventilation (V_E), tidal volume (V_T) and respiratory frequency (fR) in sham non-ovariectomized females (SHAM-NOVX), non-ovariectomized females that 6-OHDA promoted a LC lesion larger than 50% (6-OHDA-NOVX > 50%) and non-ovariectomized females that 6-OHDA promoted a LC lesion between 20 and 42% (6-OHDA-NOVX 20–42%). Data are expressed relative (%) to levels before CO_2 administration as means \pm SEM. *Difference between SHAM-NOVX and 6-OHDA-NOVX > 50%. +Difference between 6-OHDA-NOVX > 50% and 6-OHDA-NOVX 20–42%.

rats during normocapnia and hypercapnia (7% CO_2). Under normocapnic conditions, the lesion did not change the values of V_E , V_T and fR. Hypercapnia caused an increase in V_E ($P < 0.0001$; Two-way ANOVA) due to an increase in both V_T ($P < 0.0001$; Two-way ANOVA) and fR ($P < 0.0001$; Two-way ANOVA) in all groups. The hypercapnic ventilatory response was significantly decreased in 6-OHDA-NOVX > 50% rats compared with SHAM-NOVX ($P < 0.05$; Two-way ANOVA) and 6-OHDA-NOVX 20–42% ($P < 0.05$, Two-way ANOVA). During CO_2 exposure, V_T was lower in 6-OHDA-NOVX > 50% rats compared to SHAM and 6-OHDA-NOVX 20–42% ($P < 0.05$ for SHAM-NOVX and $P < 0.01$ for 6-OHDA-NOVX 20–42%, Two-way ANOVA).

To emphasize the effect of 6-OHDA lesion on the sensitivity to CO_2 , we expressed the hypercapnic responses as percent changes from baseline (Fig. 4). The CO_2 ventilatory response of 6-OHDA-NOVX > 50% animals was lower compared to SHAM and 6-OHDA-NOVX 20–42% ($P < 0.01$ for both groups, Two-way ANOVA), and at least compared to 6-OHDA-NOVX 20–42%, this effect was due to a reduced

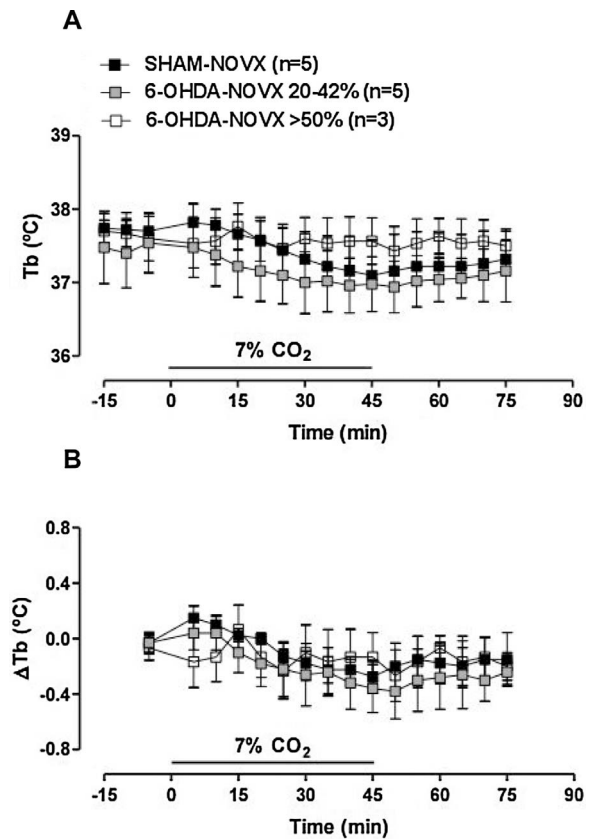


Fig. 5. (A) Body core temperature (Tb) in sham non-ovariectomized females (SHAM-NOVX), non-ovariectomized females that 6-OHDA promoted a LC lesion larger than 50% (6-OHDA-NOVX > 50%) and non-ovariectomized females that 6-OHDA promoted a LC lesion between 20 and 42% (6-OHDA-NOVX 20–42%) during normocapnia and hypercapnia (7% CO_2). (B) Variation in body core temperature (Tb) in SHAM-NOVX, 6-OHDA-NOVX > 50%, and 6-OHDA-NOVX 20–42%.

fR ($P < 0.05$, Two-way ANOVA).

Fig. 5A presents the effects of the LC lesion on Tb in NOVX rats during normocapnia and hypercapnia. Hypercapnia reduced Tb in all groups ($P < 0.05$; Two-way ANOVA) and ablation of LC neurons had any effect on Tb. The graphic of variation in Tb (Fig. 5B) shows that a fall in Tb caused by hypercapnia was similar in all groups ($P < 0.0001$; Two-way ANOVA).

3.3. Plasma hormone concentrations, pulmonary ventilation and body temperature of ovariectomized females

Table 1 shows plasma E2 concentrations in SHAM and 6-OHDA groups after ovariectomy (OVX) and ovariectomy with E2 replacement (OVX + E2). The OVX + E2 group showed higher plasma E2 levels than OVX rats ($P < 0.001$) in both SHAM and 6-OHDA animals.

During room air conditions, the lesion did not change the values of V_E and V_T , but reduced fR in both OVX and OVX + E2 group ($P < 0.05$; Two-way ANOVA) (Fig. 6). Hypercapnia enhanced V_E in all groups ($P < 0.0001$; Two-way ANOVA) due to an increase in both V_T

Table 1

Plasma estradiol levels in 6-OHDA and sham groups after ovariectomy (OVX) and ovariectomy with E2 replacement (OVX + E2).

	ESTRADIOL (pg/mL)	
	SHAM	6-OHDA
OVX	72.4 \pm 8.9 (n = 6)	64.7 \pm 2.9 (n = 7)
OVX + E2	123.7 \pm 8.9 (n = 7)	112.7 \pm 8.8 (n = 7)

Results are reported as mean \pm SEM.

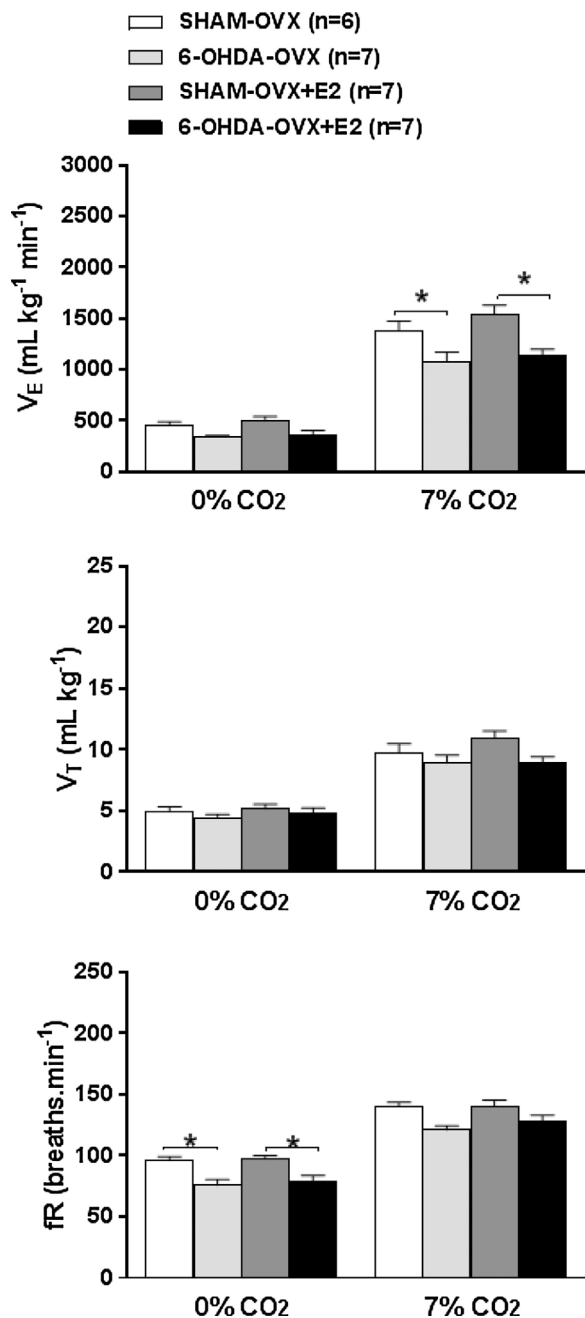


Fig. 6. Ventilation (V_E), tidal volume (V_T) and respiratory frequency (fR) in sham (Ascorbic acid) and lesioned (6-OHDA) groups in ovariectomized rats treated with vehicle (OVX) or estradiol (OVX + E2) exposed to normocapnia and hypercapnia (7% CO₂). *Significant differences between sham and lesioned groups.

($P < 0.0001$; Two-way ANOVA) and fR ($P < 0.0001$; Two-way ANOVA). Even though the hypercapnic ventilatory response was significantly decreased in 6-OHDA-OVX and 6-OHDA-OVX + E2 groups compared with SHAM groups ($P < 0.05$; Two-way ANOVA) (Fig. 6), the sensitivity to CO₂ was not different among groups (Fig. 7).

Fig. 8A presents the effects of the LC lesion on Tb in OVX and OVX + E2 rats during normocapnia and hypercapnia. Hypercapnia reduced Tb in all groups ($P < 0.05$; Two-way ANOVA) and neither ablation of LC neurons nor E2 treatment had any effect on Tb. The fall in Tb caused by hypercapnia was similar in all groups ($P < 0.0001$; Two-way ANOVA; Fig. 8B).

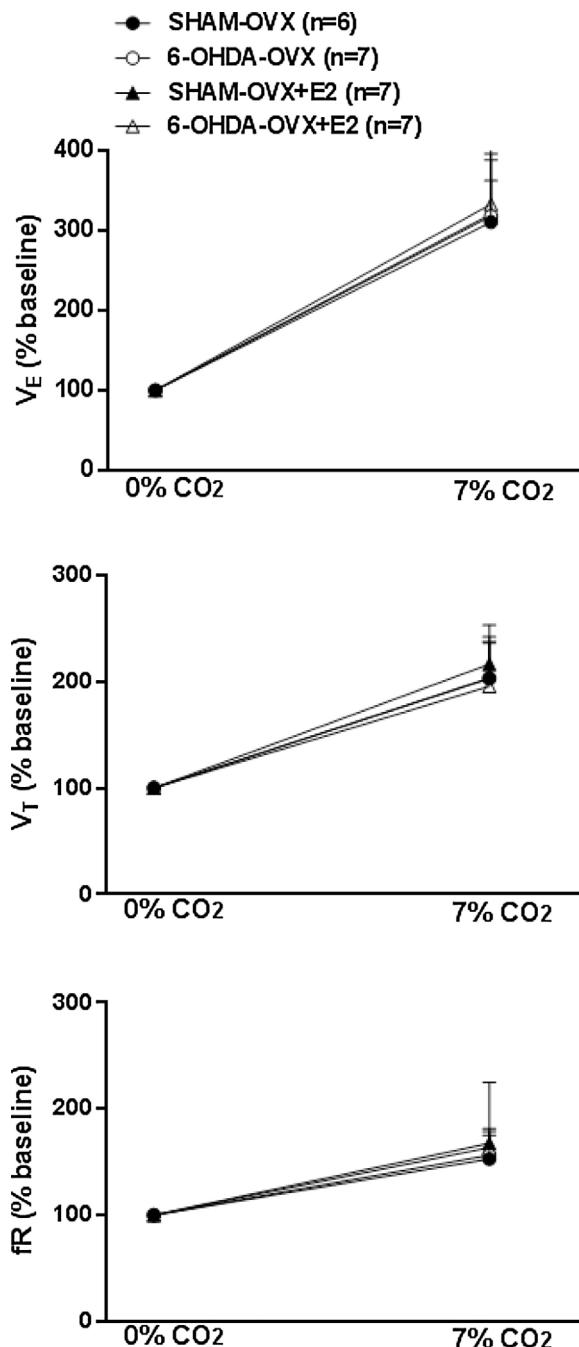


Fig. 7. The relative changes in ventilation (V_E), tidal volume (V_T) and respiratory frequency (fR) in sham (Ascorbic acid) and lesioned (6-OHDA) groups in ovariectomized rats treated with vehicle (OVX) or estradiol (OVX + E2). Data are expressed relative (%) to levels before CO₂ administration as means \pm SEM.

4. Discussion

Abnormalities in the LC is related to disorders such as depression, anxiety, panic disorder and Rett syndrome (Alba-Delgado et al., 2013; Taneja et al., 2009). Alba-Delgado et al. (2013) demonstrated marked alterations in morphological, electrical, and neurochemical properties of LC neurons in Rett syndrome model. Moreover, it is known that panic disorder victims are more vulnerable to the effects of CO₂ (Bailey et al., 2003) whereas mouse models of Rett syndrome are less CO₂ sensitive (Bissonnette et al., 2014; Zhang et al., 2011).

In the current study, LC lesion by 6-OHDA did not affect ventilation under resting conditions, similar to previous studies in males (De Carvalho et al., 2010; Biancardi et al., 2008). However, in OVX females,

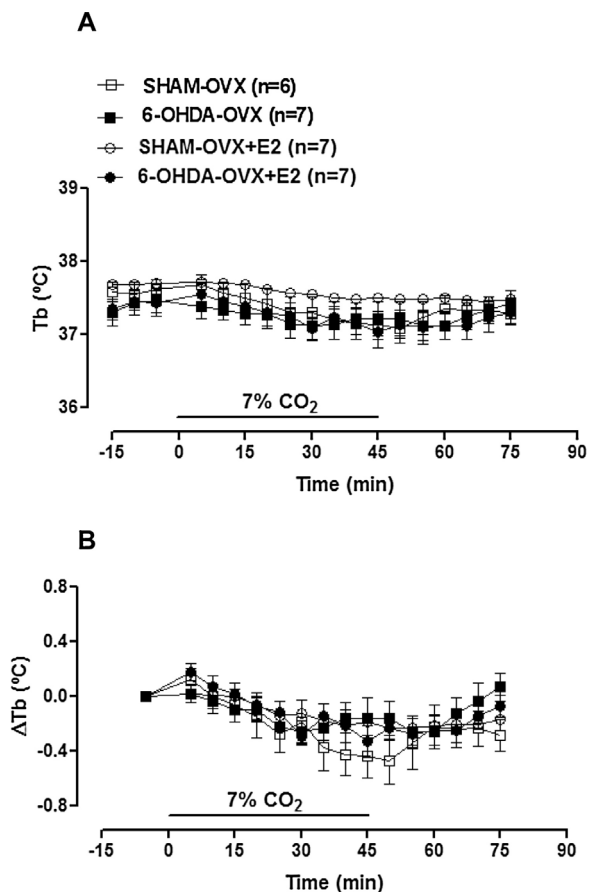


Fig. 8. (A) Body core temperature (Tb) in sham (Ascorbic acid) and lesioned (6-OHDA) groups during normocapnia and hypercapnia (7% CO₂) in females treated with vehicle (OVX) or estradiol (OVX + E2). (B) Variation in body core temperature (Tb) in sham and lesioned animals treated with vehicle or estradiol.

LC lesion caused a decrease in fR during normocapnia that was not altered by E2 replacement, suggesting that, in the absence of the ovaries, LC noradrenergic neurons provide a tonic excitatory drive to maintain breathing frequency. It is known that ovarian steroids modulate the activity of LC neurons, wherein E2 inhibits LC neuronal activity, while progesterone after E2 pre-treatment stimulates it (Szawka et al., 2009). Since E2 replacement in SHAM group did not affect breathing frequency, it is likely that this hormone is not involved in breathing modulation, at least with the physiological dose used in the present study. Therefore, in the absence of the ovaries, progesterone or even other non-steroidal gonadal hormones, such as anti-Müllerian hormone, activin, inhibin, and follistatin, might act in the LC causing the alteration in respiratory frequency. Nevertheless, further studies are needed to clarify this issue.

Previous studies have indicated a synergistic effect of E2 and progesterone on breathing regulation (Regensteiner et al., 1989); therefore, one could argue that E2 should be replaced together with progesterone to affect ventilation. Unlike E2, progesterone exerts excitatory function in the LC (Szawka et al., 2009) but only after a prior action of E2, since this hormone stimulates synthesis of progesterone receptors in this nucleus (Thornton et al., 1986; MacLusky and McEwen, 1978; Leavitt and Blaha, 1972). Nevertheless, when we performed experiments in females during diestrus (De Carvalho et al., 2016), a phase with relatively stable levels of E2 and progesterone (Smith et al., 1975), no difference in hypercapnia-induced c-Fos expression in the LC was observed. In addition, ventilation in OVX rats under either normocapnia or hypercapnia does not change after administration of progesterone combined with E2 (Marques et al., 2015).

It is known that LC is an important CO₂ chemoreceptor nucleus (De

Carvalho et al., 2016, 2010; Patrone et al., 2014; De Souza Moreno et al., 2010; Biancardi et al., 2008) and brain disorders as depression, anxiety and Rett syndrome (Alba-Delgado et al., 2013; Taneja et al., 2009) are prevalent in women. Thus, we have evaluated the participation of this nucleus in CO₂-drive to breath in females and we determined the effects of ovariectomy and E2 in this response. Our data show that non-ovariectomized females with LC lesioned more than 50% showed a reduction in CO₂-drive to breathing. The reduction in V_E was approximately 20% due to a decrease in fR. In male rats, the micro-injection of 6-OHDA in LC reduced 80% of noradrenergic neurons and decreased the response to CO₂ by approximately 64% due to a lower V_T (Biancardi et al., 2008). Studies have shown that female LC has greater volume and dendritic field than male LC (Bangasser et al., 2011; Guillamóm et al., 1988). Thus, LC being lower in males, the neurotoxin could lesion the majority of LC neurons causing a greater attenuation of CO₂ response. Moreover, there is the possibility of different mechanisms involved in the response to hypercapnia between males and females implying that LC in females could affect differently the respiratory control in response to CO₂ since we observed change in fR and the same was not showed by Biancardi et al. (2008).

The difference in central CO₂ chemosensitivity between males and females has been observed in previous studies. Assessment of the sensitivity of central chemoreceptors to CO₂ in vivo indicates that the CO₂ threshold for the activation of retrotrapezoid neurons (RTN) neurons is higher in female than in male adult mice (Niblock et al., 2012; Niblock et al., 2010). Niblock et al. (2012) have observed that female RTN neurons are activated after 10% CO₂ exposure, but not to 5% CO₂ in vivo. On the other hand, male RTN neurons are activated both when exposed to 5 and 10% CO₂, indicating that there are gender differences in the CO₂ threshold required for the activation of the RTN neurons, in which males seem to be more sensitive (Niblock et al., 2012; Niblock et al., 2010).

The SHAM-OVX animals presented an increase of 250% of the hypercapnic ventilatory response (Fig. 4), whereas in SHAM-OVX groups, the increase of ventilation was approximately 200% in both groups (Fig. 7). Similarly, we found that ovariectomy promoted an attenuation (43%) of the hypercapnic hyperventilation, compared with the response obtained from intact females (Marques et al., 2015). Further, hormonal replacement with E2 did not restore the effects of ovariectomy on CO₂ chemosensitivity. Interestingly, when OVX and OVX + E2 females were lesioned with 6-OHDA, the ventilatory response to CO₂ was similar compared to sham groups. Since CO₂ chemosensitivity is already decreased in OVX animals, LC lesion did not promote further reduction of CO₂ ventilatory response. As suggested before, since E2 treatment in SHAM animals did not affect the ventilatory response to CO₂, similar to our previous data (Marques et al., 2015), it is possible that this hormone is not involved in respiratory regulation during hypercapnic challenge. Therefore, other ovarian hormones might be involved in this regulation.

Regarding LC participation in thermoregulation, there is evidence that catecholaminergic LC neurons are part of a thermoeffector neuronal pathway that is specifically activated by pyrogens (e.g. PGE₂) to induce thermogenesis and produce fever in a subthermoneutral environment (Almeida et al., 2004). The present study was performed in thermoneutral condition and LC noradrenergic neurons lesion did not change Tb corroborating previous studies performed in males (De Carvalho et al., 2010; Biancardi et al., 2008). Therefore, LC seems to participate in some way involved in thermoregulation but only in subthermoneutral condition and not in thermoneutral condition.

Hypercapnia reduced Tb in all groups corroborating our previous study in which exposure to 7% CO₂ caused a drop in Tb in OVX and cycling female rats (Marques et al., 2015). Thus, the reduction in temperature during hypercapnia in females may not be caused by hormone fluctuations since both OVX and regularly cycling females present this alteration.

In conclusion, our data show that LC noradrenergic neurons in

intact females exert an excitatory modulation in the hypercapnic ventilatory response that is not observed in OVX females. In addition, LC noradrenergic neurons of OVX females seems to provide a tonic excitatory drive to maintain breathing frequency, and this response that is not functionally influenced by E2.

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