Neuroscience 313 (2016) 36–45

ROLE OF SEX HORMONES IN HYPERCAPNIA-INDUCED ACTIVATION OF THE LOCUS COERULEUS IN FEMALE AND MALE RATS

D. DE CARVALHO,[a] D. A. MARQUES,[a] M. P. BERNUCI,[c] C. M. LEITE,[c] R. ARAÚJO-LOPES,[b] J. ANSELMO-FRANCI,[c] K. C. BÍCEGO,[a] R. E. SZAWKA[b] AND L. H. GARGAGLIONI[a]*

[a] *Department of Animal Morphology and Physiology, Sao Paulo State University – UNESP FCAV, Jaboticabal, SP, Brazil*

[b] *Department of Physiology and Biophysics, Institute of Biological Sciences, Federal University of Minas Gerais – UFMG, Belo Horizonte, MG, Brazil*

[c] *Stomatology, Morphology and Physiology, FORP-University of Sao Paulo, Ribeirão Peto, SP, Brazil*

Abstract—The locus coeruleus (LC) has been suggested as a CO_2 chemoreceptor site in mammals. Most of the studies involving the role of the LC in hypercapnic ventilatory responses have been performed in males. Since ovarian steroids modulate the activity of LC neurons and females have a different respiratory response to CO_2 than males, we evaluated the activity of LC noradrenergic neurons during normocapnia and hypercapnia in female and male rats with distinct sex hormone levels. Ovariectomized (OVX), estradiol (E2)-treated ovariectomized (OVX + E2) and female rats on the diestrous day of the estrous cycle were evaluated. Concurrently, males were investigated as gonad-intact, orchidectomized (ORX), testosterone (T)-treated ORX (ORX + T), and E2-treated ORX (ORX + E2). Activation of LC neurons was determined by double-label immunohistochemistry to c-Fos and tyrosine hydroxylase (TH). Hypercapnia induced by 7% CO_2 increased the number of c-Fos/TH-immunoreactive (ir) neurons in the LC of all groups when compared to air exposure. Hypercapnia-induced c-Fos expression did not differ between diestrous females and intact male rats. In the OVX + E2 group, there was attenuation in the c-Fos expression during normocapnia compared with OVX rats, but CO_2 responsiveness was not altered. Moreover, in ORX rats, neither T nor E2 treatments changed c-Fos expression in LC noradrenergic neurons. Thus, in female rats, E2 reduces activation of LC noradrenergic neurons, whereas in males, sex hormones do not influence the LC activity.

Key words: c-Fos, chemosensitivity, estradiol, noradrenaline, sex steroids.

INTRODUCTION

The locus coeruleus (LC) is a noradrenergic nucleus located in the dorsal part of the pons on the lateral border of the fourth ventricle (Jacobs, 1986). It is estimated that about 50% of all the noradrenergic projections in the central nervous system originate in the LC (Aston-Jones et al., 1995; Berridge and Waterhouse, 2003). Thus, this nucleus has been associated with a number of physiological and behavioral functions, including cardiovascular and respiratory control, as well as the sleep–wake cycle, feeding, thermoregulation, nociception, attention and learning (Hobson et al., 1975; Aston-Jones et al., 1985; Oyamada et al., 1998; Putnam et al., 2004; Almeida et al., 2004; Biancardi et al., 2008; De Souza Moreno et al., 2010; Gargaglioni et al., 2010; de Carvalho et al., 2010; Patrone et al., 2014).

Several lines of evidence support the role of the LC as a central chemosensor in mammals and amphibians (Elam et al., 1981; Haxhiu et al., 1996; Oyamada et al., 1998; Stunden et al., 2001; Filosa et al., 2002; Noronha-de-Souza et al., 2006; Biancardi et al., 2008; Gargaglioni et al., 2010; de Carvalho et al., 2010; Santin and Hartzler, 2013; Patrone et al., 2014), and 80% of LC neurons in mammals were found to be chemosensitive, responding to hypercapnia with an increased firing rate (Coates et al., 1993; Pineda and Aghajanian, 1997; Oyamada et al., 1998; Filosa et al., 2002). Additionally, CO_2 stimulation increases c-Fos expression in the LC of male rats (Haxhiu et al., 1996; Teppema et al., 1997). Interestingly, the LC is sexually dimorphic, such that the LC of females has a larger volume, greater dendritic fields, more neurons and dopamine-β-hydroxylase (DβH)-immunoreactive (ir) cells than the LC of male rats (Guillamóm et al., 1988; Luque et al., 1992; Bangasser et al., 2011), suggesting that the female LC could differentially affect the CO_2 chemosensitivity.

Sexual dimorphism is also observed in the ventilatory control, and this fact may contribute to gender differences in the prevalence of breathing disorders (Saaresranta and Polo, 2002; Jensen et al., 2005). For instance, sleep-disordered breathing (SDB) is more prevalent in men compared to premenopausal women (Lin and Eric, 2008), however, at postmenopausal ages, the prevalence increases in women (Dancey et al., 2001). The occurrence of SDB almost doubles at menopause,

*Corresponding author. Address: Departamento de Morfologia e Fisiologia Animal, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista Júlio de Mesquita Filho, Via de acesso Paulo Donato Castellane s/n, 14870-000 Jaboticabal, SP, Brazil. Tel: +55 16 32092656; fax: +55 16 32024275.
E-mail address: lucihel@fcav.unesp.br (L. H. Gargaglioni).

Abbreviations: DβH, dopamine-β-hydroxylase; E2, estradiol; ER, estrogen receptors; GFP, green fluorescent protein; ir, immunoreactive; LC, locus coeruleus; ORX, orchidectomized; OVX, ovariectomized; RIA, radioimmunoassay; SDB, sleep-disordered breathing; TH, tyrosine hydroxylase.

http://dx.doi.org/10.1016/j.neuroscience.2015.11.026
0306-4522/

independently of body mass and other coexisting risk factors (Bixler et al., 2001; Skegg, 2001; Anttalainen et al., 2006). Hormonal replacement with E2 and P has been reported to be effective in SDB treatment (Hensley et al., 1980; Block et al., 1981; Pickett et al., 1989). In addition, a recent study demonstrated that the difference in the propensity to develop SBD may be due to the destabilizing effect of testosterone rather than the stabilizing effect of progesterone (Chowdhuri et al., 2013). Therefore, it is apparent that sexual hormones affect breathing control, but the underlying mechanisms of their action remain uncertain.

Ovarian steroids modulate the activity of LC neurons, wherein estradiol (E2) inhibits, while progesterone, after E2 pre-treatment, stimulates LC neuronal activity (Szawka et al., 2009). Additionally, E2 administration in ovariectomized (OVX) female rats elicits a dose-dependent elevation in mRNA levels of tyrosine hydroxylase (TH) in the LC (Serova et al., 2002). In this regard, Pendergast et al. (2008) demonstrated that estrogen receptors (ER) α and β are expressed in TH-ir neurons of the LC in male and female mice. In the female LC, ERα mRNA is present at similar levels compared to males, whereas ERβ mRNA expression is significantly lower than in males. In male rats, androgen receptors are highly expressed in the LC (Hamson et al., 2004) and are important for sexual differentiation of this nucleus (Garcia-Falgueras et al., 2005). Accordingly, the LC in male rats lacking functional androgen receptors has more neurons and a larger volume than control littermates (Garcia-Falgueras et al., 2005).

Considering that: (1) the LC is an important site for CO_2 chemoreception, (2) the effects of sex hormones have been reported in the LC and (3) the LC is sexually dimorphic in rats, the present study aimed to evaluate the effect of sex steroids on the activation of LC noradrenergic neurons during CO_2 challenge in female and male rats using double-label immunohistochemistry to c-Fos and TH. OVX and E2-treated OVX (OVX + E2) female rats were evaluated on the diestrous day of the estrous cycle. Gonad-intact, orchidectomized (ORX), testosterone (T)-treated ORX (ORX + T), and E2-treated ORX (ORX + E2) males were also investigated.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on conscious adult female and male Wistar rats weighing 250–310 g. The animals had free access to water and food and were housed in a controlled temperature room (25 ± 1 °C) with a 12:12-h light–dark cycle (lights on at 6:00 AM). All experimental procedures were done in compliance with the Brazilian College of Animal Experimentation (COBEA) guidelines and approved by the local Animal Care and Use Committee (CEUA-FCAV # 000222-09).

Surgery

All surgical procedures were performed under anesthesia with ketamine (100 mg/kg, i.p.; Agener, Sao Paulo, Brazil) and xylazine (10 mg/kg, i.p.; Coopers, Sao Paulo, Brazil), antibiotic protection (10 mg/kg, s.c.; Enrofloxacina, Flotril, Schering-Plough, Sao Paulo, Brazil) and analgesic (2.5 mg/kg, s.c.; Flunixina meglumina, Banamine; Schering-Plough, Sao Paulo, Brazil).

Ovariectomy and hormone treatment. Ten days before experiments, female rats were submitted to ovariectomy by midline laparotomy. On three consecutive days prior to the experiment, females were treated with vehicle (corn oil, OVX group; 0.2 mL/rat, s.c., Liza; Cargill, Sao Paulo, Brazil) or 17β-estradiol (OVX + E2 group; 10 μg/0.2 mL/rat, s.c., E2 cypionate; Pfizer, Sao Paulo, Brazil) at 10:00 AM. Estrous cycle regularity was assessed daily, and only rats showing at least three consecutive, regular four-day cycles were subjected to surgery and oil or E2 treatment. The hormone treatment regimen used yielded physiological levels of plasma E2 (Szawka et al., 2009; Marques et al., 2015).

Orchidectomy and hormone treatment. Ten days before experiments, male rats were submitted to orchidectomy by incision in the scrotum. Male rats were treated daily at 10:00 AM with vehicle (corn oil, ORX group; 0.2 mL/rat, s.c., Liza; Cargill, Sao Paulo, Brazil) for seven consecutive days prior to the experiment, 17β-estradiol (ORX + E2 group; 10 μg/0.2 mL/rat, s.c., E2 cypionate; Pfizer, Sao Paulo, Brazil) for three consecutive days prior to the experiment (Szawka et al., 2009; Marques et al., 2015), or T (ORX + T group; 0.25 mg/0.2 mL/rat, s.c., testosterone propionate) for seven consecutive days prior to the experiment, according to Kalil et al. (2013).

Hormone assay

After exposure to hypercapnia or normocapnia, rats were anesthetized with ketamine and xylazine, and a blood sample of approximately 1 ml was collected from the heart in 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 and T levels by radioimmunoassay (RIA). Plasma E2 and T concentrations were determined by double-antibody RIA with MAIA kits provided by Biochem Immunosystem (Bologna, Italy). The lower limits of detection for E2 and testosterone were 5.0 pg/mL. The intra-assay coefficient of variation was 4.3% for E2 and 4% for testosterone.

Double-label immunohistochemistry to Fos and TH

Sections from the female and male LC were processed separately, and the reactions were adapted from Bernuci et al. (2008) and Szawka et al. (2009). Under deep anesthesia, rats were transcardially perfused with PBS, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Frontal sections of 30 μm were cut through the LC region in a cryostat (Microm, Model HM500 OM, Walldorf, Germany). Sections were incubated with anti-c-Fos rabbit antibody (Ab-5, Calbiochem, Gibbstown, NJ, USA, EUA) at 1:15.000 in PBS containing 0.3% Triton X-100 and 1%

BSA for 40 h. The sections were then incubated with a secondary antibody (anti-horse for males, Kit ABC Elite, PK-6200, Vector, Burlingame, CA, USA; anti-rabbit for females, Kit ABC Elite, BA-1000, Vector, CA, USA) at 1:600 for 2 h (primary and secondary antibodies were diluted in the same solution), and then the sections were incubated with avidin–biotin complex solution (Elite ABC kit; Vector, CA, USA) at 1:400 for 1 h. The antibody-peroxidase complex was visualized with a solution of nickel chloride (25 mg/mL), 3,3′-diaminobenzidine-HCl (0.2 mg/mL; Sigma, St. Louis, MO, USA), and H_2O_2 (1 μL/mL of 30% stock solution) in 0.175 M acetate buffer (pH 7.5). Sections were then incubated with the anti-TH mouse antibody (anti-TH2; Sigma, MO, USA; males at 1:100.000 and females at 1:500.000 dilution) for 40 h, and then with a secondary antibody (anti-horse for males, Kit ABC Elite, PK-6200, Vector, CA, USA; anti-mouse for females, Kit ABC Elite, BA-2001, Vector, CA, USA) at 1:600 for 2 h, and Elite ABC kit for 1 h. TH was immunostained with a solution containing 3,3′-diaminobenzidine-HCl (0.2 mg/mL; Sigma, MO, USA l) and H_2O_2 (1 μL/mL of 30% stock solution) in 0.05 M Tris–HCl buffer (pH 7.6). The sections were then mounted on gelatin-coated glass slides. Sections were blindly analyzed under a light microscope Axio Imager Z2 (Carl Zeiss, Munich, Germany), and Fos-ir neurons, colocalized with TH, were counted in the LC over the entire length (−9.16 to −10.32 mm from bregma; Paxinos and Watson, 1998), and the number of double-labeled neurons was counted bilaterally in approximately 20 sections. Photomicrographs were captured using AxioVision software (Carl Zeiss, Munich, Germany).

Measurement of uterine and seminal vesicle weight

During perfusion, the organs were removed and the fresh weights were measured. The weights of the uterus and the seminal vesicle were verified to confirm the effectiveness of hormone treatment since E2 and T cause trophic effects on these organs. All seminal fluid was discarded before the measurement of weight.

Data processing and analysis

The results are reported as mean ± SEM. The numbers of c-Fos/TH-ir neurons were compared using a two-way ANOVA, followed by the Bonferroni post hoc test. Statistical differences in organ weight were determined by a one-way ANOVA. Plasma hormone concentrations were analyzed by a two-way ANOVA, followed by the Bonferroni post hoc test. Values of $P < 0.05$ were considered to be significant. Statistical analyses were performed using GraphPad Prism (La Jolla, CA, USA).

Protocols

Effect of hypercapnia on Fos immunoreactivity in the LC noradrenergic neurons of intact female and male rats. Vaginal smears were taken daily and only rats showing at least three consecutive, regular four-day estrous cycles were used. We standardized the use of females on diestrus, as defined by the day following metestrus and before proestrus, because this is a long-lasting phase with respect to the vaginal cytology with relatively stable levels of E2 and progesterone (Smith et al., 1975). To reduce the nonspecific c-Fos expression, animals were acclimated for 1 h on three consecutive days prior to experiments during the same hour that actual experiments would occur. On the fourth day, female and male rats were submitted to atmospheric air or hypercapnia (7% CO_2, 21% O_2 and N_2 balance) for 2 h (Teppema et al., 1997). For each animal submitted to hypercapnia, another animal from same group was submitted to atmospheric air at the same time. Vaginal smears were taken before and after the experiment to ensure a diestrous cytology throughout. After the experiments, rats were anesthetized and blood samples were collected. Rats were transcardially perfused, the uterus or seminal vesicle was collected and brains were processed for immunohistochemical analysis.

Effect of sex hormones on hypercapnia-induced c-Fos immunoreactivity in the LC noradrenergic neurons of female and male rats. The same procedures employed in the protocol above were used in this experiment; however, the experiments were conducted in OVX and OVX + E2 females and ORX, ORX + E2, and ORX + T males.

RESULTS

Uterine and seminal vesicle weights

E2 and T treatments caused an increase in uterine and seminal vesicle weights, respectively. Hypercapnia did not change either uterine or seminal vesicle weights (data not show). Thus, in the tables, animals exposed to room air and animals exposed to CO_2 were grouped, and only differences in hormonal status were analyzed. OVX rats had a smaller uterus compared to intact females ($P < 0.0001$), whereas the OVX + E2 group displayed a larger uterus in comparison to intact ($P < 0.05$) and OVX rats ($P < 0.0001$) (Table 1). Table 2 shows that the ORX and ORX + E2 groups presented smaller seminal vesicles compared to intact male rats ($P < 0.0001$). ORX + T rats, in turn, displayed seminal vesicles of greater weights compared to intact ($P < 0.05$), ORX and ORX + E2 rats ($P < 0.0001$).

Plasma hormone concentrations

Table 3 shows plasma T and E2 concentrations in male rats. Since hypercapnia did not change the hormone

Table 1. Uterus weight of diestrous, OVX, and OVX + E2 rats

Uterus weight (mg/100 g)		
Diestrus ($n = 10$)	OVX ($n = 12$)	OVX + E2 ($n = 12$)
154 ± 10	77 ± 4*	194 ± 8**,***

Results are reported as mean ± SEM.
* Significant difference compared to diestrus ($P < 0.0001$).
** Significant difference compared to diestrus ($P < 0.05$).
*** Significant difference compared to the OVX group ($P < 0.0001$).

Table 2. Seminal vesicle weight of intact, ORX, ORX + E2, and ORX + T rats

Seminal vesicle weight (mg/100 g)			
Intact (n = 12)	ORX (n = 12)	ORX + E2 (n = 12)	ORX + T (n = 10)
350 ± 27	117 ± 17*	114 ± 11*	469 ± 36**,***

Results are reported as mean ± SEM.
* Significant difference compared to the intact group ($P < 0.0001$).
** Significant difference compared to the intact group ($P < 0.05$).
*** Significant difference compared to the ORX and ORX + E2 groups ($P < 0.0001$).

Table 3. Plasma testosterone and estradiol levels in intact, ORX, ORX + T, and ORX + E2 male rats

	Testosterone (ng/mL)	Estradiol (pg/mL)
Intact	0.9 ± 0.2 (n = 11)	75.5 ± 4.4**** (n = 12)
ORX	0.01 ± 0.003* (n = 12)	53.1 ± 4.8 (n = 11)
ORX + E2	0.01 ± 0.003* (n = 12)	139 ± 10.2*** (n = 10)
ORX + T	1.8 ± 0.2*,** (n = 8)	73.4 ± 8.6**** (n = 10)

Results are reported as mean ± SEM. Statistic analyses between animals subjected to the same hormonal treatment.
* Significant difference compared to the intact group ($P < 0.05$).
** Significant difference compared to the ORX and ORX + E2 groups ($P < 0.001$).
*** Significant difference compared to the intact, ORX and ORX + T groups ($P < 0.001$).
**** Significant difference compared to the ORX group ($P < 0.05$).

Table 4. Plasma estradiol levels in cycling females on diestrus and OVX and OVX + E2 rats

	Estradiol (pg/mL)
Diestrus	96.1 ± 5.8 (n = 12)
OVX	64.2 ± 7.6* (n = 10)
OVX + E2	139.1 ± 7.2*,** (n = 12)

Results are reported as mean ± SEM.
* Significant difference compared to the diestrous group ($P < 0.05$).
** Significant difference compared to the OVX group ($P < 0.001$).

levels, data from controls and hypercapnic rats were grouped for hormonal analyses. The ORX + T group presented higher plasma T levels compared to ORX, ORX + E2 ($P < 0.001$) and intact rats ($P < 0.05$). Plasma T levels decreased in ORX and ORX + E2 groups compared to the intact group ($P < 0.05$). E2 levels were higher in ORX + E2 rats in comparison to the other groups ($P < 0.001$). Orchidectomy reduced plasma E2 levels ($P < 0.05$), and there were no differences between intact and ORX + T groups. Table 4 shows plasma E2 levels in diestrous, OVX and OVX + E2 female rats. Hypercapnia did not change the hormone levels (data not show). Thus, in the tables, animals exposed to room air and animals exposed to CO_2 were grouped, and only differences in hormonal status were analyzed. The OVX + E2 group showed higher plasma E2 levels than diestrous ($P < 0.05$) and OVX rats ($P < 0.001$). In the OVX group, ovariectomy reduced E2 levels in comparison to the diestrous group ($P < 0.05$).

c-Fos immunoreactivity in gonad-intact and castrated rats during normocapnia

There was no difference in the number of c-Fos-positive neurons during normocapnia between the gonad-intact animals and castrated rats treated with oil (for males: intact = 3.9 ± 1.0 vs ORX with oil = 2.3 ± 0.7, $P < 0.05$; for females: intact = 3.6 ± 0.9 and OVX with oil = 3.3 ± 0.6; $P < 0.05$), confirming that surgical intervention and hormonal injections did not cause differential c-Fos activation in the LC.

Effect of hypercapnia on c-Fos immunoreactivity in the LC noradrenergic neurons of female and male rats

Fig. 1 shows representative photomicrographs of c-Fos immunoreactivity in the LC noradrenergic neurons of diestrous female (Fig. 1A) and intact male rats (Fig. 1B) during normocapnia and hypercapnia. During normocapnic condition, the number of c-Fos/TH-ir neurons was similar in female (3.6 ± 0.9 neurons/section; n = 4) and male rats (3.9 ± 1.1 neurons/section; n = 5) (Fig. 1C). Hypercapnia increased the number of c-Fos/TH-ir neurons in females (7.5 ± 3.7 neurons/section; $P < 0.05$; n = 4) and males (9.7 ± 2.3 neurons/section; $P < 0.05$; n = 6) compared to room air (Fig. 1C). Additionally, the double staining was similar in female and male rats exposed to hypercapnia (Fig. 1C).

Effect of sex hormones on hypercapnia-induced c-Fos immunoreactivity in the LC noradrenergic neurons of female and male rats

Fig. 2 shows representative photomicrographs of c-Fos immunoreactivity in the LC noradrenergic neurons of OVX (Fig. 2A) and OVX + E2 rats (Fig. 2B) during normocapnia and hypercapnia. Hypercapnia increased the number of c-Fos/TH-ir neurons in OVX (7.5 ± 1.9 neurons/section; $P < 0.05$; n = 4) and in OVX + E2 (4 ± 0.8 neurons/section; $P < 0.05$; n = 4) compared to the room air condition (3.3 ± 0.6 and 2 ± 0.1 neurons/section for OVX and OVX + E2, respectively; n = 4 per group) (Fig. 2C). Nevertheless, the double labeling in the OVX + E2 group was less pronounced in normocapnia compared to the OVX group during the same condition (Fig. 2C; $P < 0.05$). Fig. 3 shows representative photomicrographs of c-Fos immunoreactivity in the LC noradrenergic neurons in ORX (Fig. 3A), ORX + E2 (Fig. 3B), and ORX + T (Fig. 3C) during normocapnia and hypercapnia. Hypercapnia increased the number of c-Fos/TH-ir neurons in ORX (7.6 ± 4 neurons/section; $P < 0.05$; n = 5), ORX + E2 (7.1 ± 1.8 neurons/section; $P < 0.05$; n = 5), and ORX + T rats (8.1 ± 3.2 neurons/section; $P < 0.05$; n = 5) compared to normocapnia (2.3 ± 0.7, n = 4; 2.1 ± 0.6, n = 3; 2.4 ± 1.7, n = 4, neurons/section for ORX, ORX + E2, and ORX + T, respectively) (Fig. 3C). However, castration and E2 or T replacement did not change the response of LC neurons to hypercapnia, as compared with control male rats.

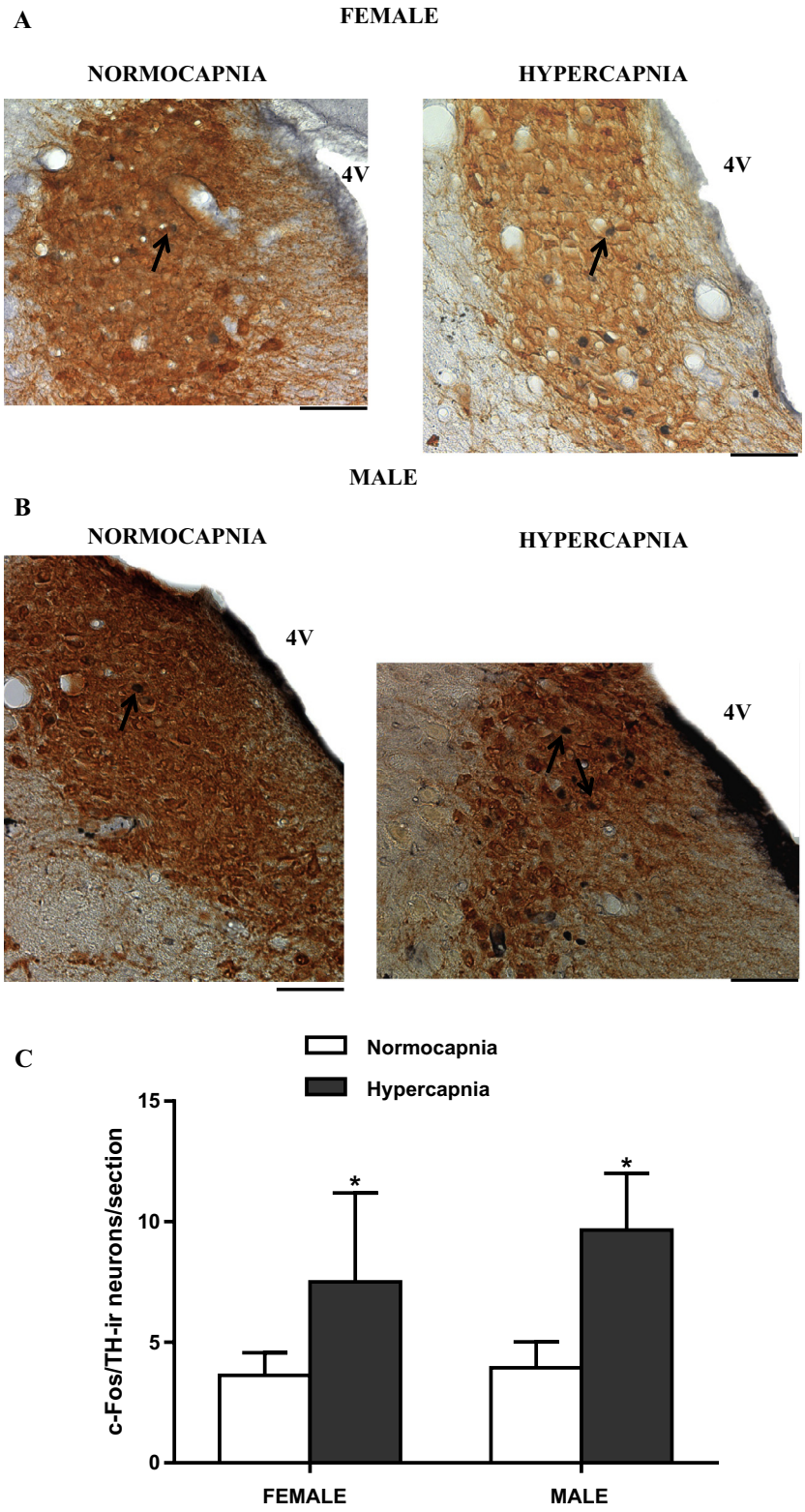

Fig. 1. (A, B) Photomicrographs of LC coronal sections double-labeled to c-Fos and tyrosine hydroxylase (TH) in cycling female rats on diestrus (A) and gonad-intact male rats (B) during normocapnia and hypercapnia (7% CO_2). Arrows indicate examples of double-labeled neurons (c-Fos and TH). 4V: fourth ventricle. Scale bar = 50 μm. (C) Mean ± SEM number of c-Fos/TH-immunoreactive (ir) neurons/section in the LC of female and male rats after normocapnia or hypercapnia. * Indicates a difference between normocapnia and hypercapnia ($P < 0.05$).

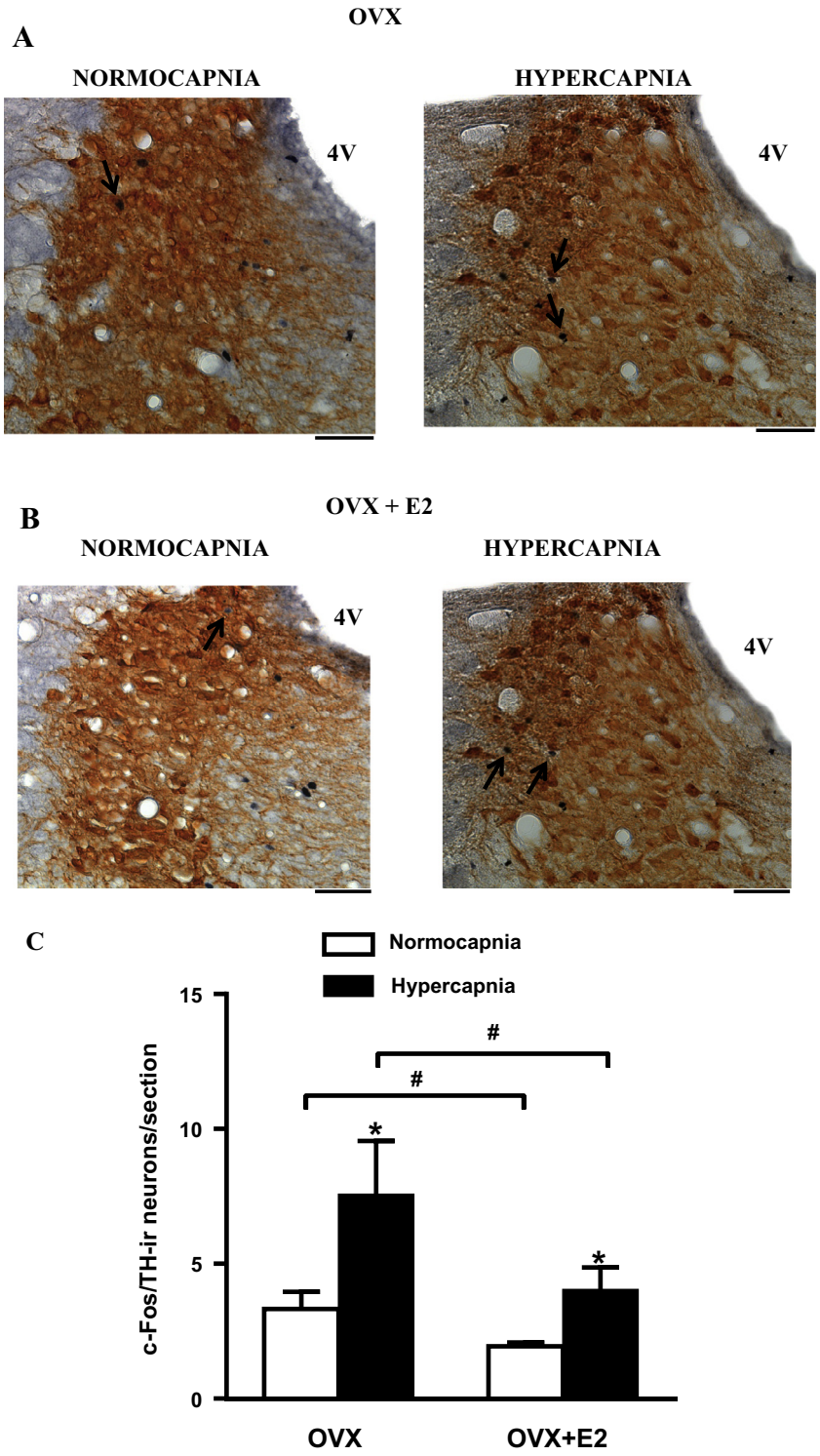

Fig. 2. (A, B) Photomicrographs of LC coronal sections double-labeled to c-Fos and tyrosine hydroxylase (TH) in ovariectomized rats treated with oil (vehicle; OVX; A) or estradiol (OVX + E2; B) during normocapnia and hypercapnia (7% CO_2). Arrows indicate examples of double-labeled neurons (c-Fos and TH). 4V: fourth ventricle. Scale bar = 50 μm. (C) Mean ± SEM number of c-Fos/TH-immunoreactive (ir) neurons/section in the LC of OVX and OVX + E2 rats after normocapnia or hypercapnia. * Indicates a difference between normocapnia and hypercapnia ($P < 0.05$). # Indicates a difference between OVX and OVX + E2 groups ($P < 0.05$).

DISCUSSION

In the present study, we determined the effects of sex steroids on the activation of LC noradrenergic neurons in female and male rats using c-Fos expression after exposure to 7% CO_2. We analyzed cycling female rats on the day of diestrus, as well as in models of OVX and OVX + E2 rats. Gonad-intact adult males were

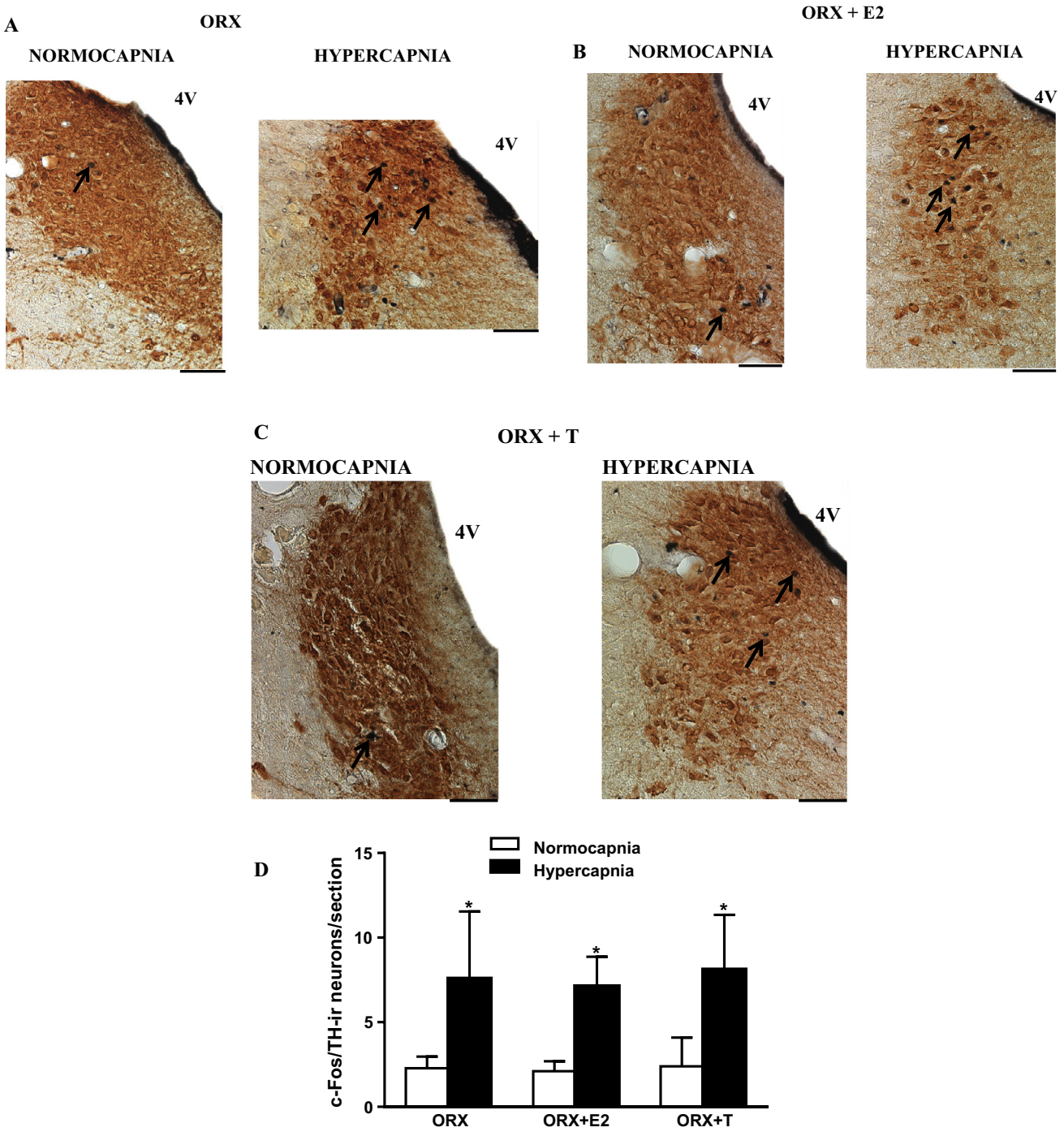

Fig. 3. (A–C) Photomicrographs of LC coronal sections double-labeled to c-Fos and tyrosine hydroxylase (TH) in orchidectomized rats treated with oil (vehicle; ORX; A), estradiol (ORX + E2; B) or testosterone (ORX + T; C) during normocapnia and hypercapnia (7% CO_2). Arrows indicate examples of double-labeled neurons (c-Fos and TH). 4V: fourth ventricle. Scale bar = 50 μm. (D) Mean ± SEM number of c-Fos/TH-immunoreactive (ir) neurons/section in the LC of ORX, ORX + E2 and ORX + T groups after normocapnia or hypercapnia. * Indicates a difference between normocapnia and hypercapnia ($P < 0.05$).

investigated, as well as ORX, ORX + E2 and ORX + T rats. Plasma levels of sex hormones and the trophic effects of E2 and T on the weights of the uterus and the seminal vesicle, respectively, validated the efficacy of the regimen of hormonal treatments used. The present findings demonstrate that the response of LC to hypercapnia does not differ between males and diestrous females. In males, neither lack of gonadal hormones caused by orchidectomy, nor replacement with T or E2, influenced the LC response to hypercapnia. In females, although ovariectomy was also ineffective in altering the activation of the LC, treatment with high physiological levels of E2 in OVX + E2 rats reduced LC neuron activation, without altering their CO_2

responsiveness. These results reveal a complex role of E2 in the LC in females.

c-Fos is a protein expressed by the *c-fos* gene in response to various stimuli, and was introduced as a tool for determining activity changes neuronal activity (Hoffman and Lyo, 2002). In the present study, ovariectomy or orchidectomy did not induce c-fos expression in the LC, as demonstrated by the lack of difference between gonad-intact and castrated rats during normocapnia, which strengthens the specificity of the hypercapnic stimulus. Hypercapnia increased c-Fos expression in LC noradrenergic neurons in all groups evaluated, consistent with a previous report by Haxhiu et al. (1996) exposing male rats to 15% CO_2. Studies *in vitro* demonstrated that the firing rate of LC neurons increases in response to hypercapnia (Oyamada et al., 1998; Stunden et al., 2001; Filosa et al., 2002) and approximately 80% of LC neurons are considered to be chemosensitive (Oyamada et al., 1998; Filosa et al., 2002). Additionally, an increase (~44%) in firing frequency of LC neurons in response to hypercapnic acidosis was reported in brain slice preparations (Stunden et al., 2001). Likewise, Johnson et al. (2008), using Prp57 transgenic mice that express green fluorescent protein (GFP) in the LC, reported that the LC exhibits chemosensitivity in culture after pharmacological blockade of fast excitatory and inhibitory synaptic transmissions, and that more than 85% of GFP-positive LC neurons are stimulated by elevated CO_2/H^+. However, as far as we are aware, no previous study evaluated whether CO_2–induced LC activation is modulated by sex hormones or is sex-dependent.

Orchidectomy, followed or not by T or E2 treatment, did not change c-Fos expression during hypercapnia in male rats. Similarly, a previous study also showed that orchidectomy did not change c-Fos expression in the LC after restraint stress (Chen and Herbert, 1995). Since aromatase catalyzes the conversion of androgens into estrogens in the central nervous system of both sexes (Roselli et al., 1987; Naftolin et al., 1996), we evaluated whether E2 affects LC noradrenergic neuron activation in males during hypercapnia. However, E2 treatment in males did not change LC activation during room air and CO_2 challenge, which differs from what we observed in females. This differential response may be related to the sexual dimorphism of the LC, since the LC contains more and larger neurons in females than in males (Guillamóm et al., 1988; Bangasser et al., 2011). In addition, males possess more ERβ than females in the LC (Pendergast et al., 2008), and ERβ antagonizes the function of ERα in some brain areas like the hippocampus (Bean et al., 2014). Thus, one alternative possibility is that E2 does not affect the LC response in males due to a local inhibition of ERα by ERβ.

OVX rats displayed higher c-Fos expression in the LC than OVX + E2 rats during normocapnia, suggesting that circulating E2 reduces overall neuronal activation in the LC. E2 also reduced c-Fos expression in the central nervous system in OVX rats submitted to restraint stress (Dayas et al., 2000; Ueyama et al., 2006). In the present study, the number of c-fos labeled neurons increased from 3.3 to 7.5 (2.27 times) in OVX-rats and from 2 to 4 in OVX + E2 rats (2 times), indicating that the number of LC c-Fos labeled neurons increased similarly in both groups. Therefore, our data demonstrate that E2 reduces LC neuron activation without altering their CO_2 responsiveness. Corroborating our results, Szawka et al. (2009) demonstrated an inhibitory effect of this hormone in the LC of female rats, because E2 reduced the firing rates of LC neurons in slices from OVX rats. In addition, previous studies have also reported an E2 inhibition of c-Fos expression in the LC of OVX rats in response to immobilization stress (Ueyama et al., 2006) and magnetic fields (Cason et al., 2010). Interestingly, our findings suggest that the E2 modulation of the LC response to hypercapnia depends on its circulating levels or regimen of administration. The low physiological levels of E2 found in cycling rats on the day of diestrus seem to exert no influence on the activity of LC neurons, because there was no noticeable difference in the expression of c-Fos between diestrous and OVX rats. On the other hand, OVX + E2 rats displayed lower levels of c-Fos expression than those in OVX and diestrous rats. The E2 treatment regimen used promoted slightly, but significantly, higher estrogen bioactivity and circulating hormonal levels compared to diestrous rats, as determined by the uterine weight and plasma E2 levels. It seems, therefore, that E2 concentrations higher than basal levels of the rat estrous cycle are required for suppressing LC activation. Such high levels are likely to occur during the preovulatory period in cycling animals and at late pregnancy. Thus, further studies are required to determine whether the LC activation is reduced during these physiological conditions of high E2.

Additionally, E2 is known to regulate the activity of TH and the DβH genes, promoting an increase in TH and DBH transcription in the LC of female rats (Liaw et al., 1992; Serova et al., 2002). Thus, E2 plays a complex modulatory role in LC neurons, probably increasing noradrenaline synthesis and storage inside synaptic vesicles, but inhibiting its release. During the ovarian cycle in mammals, the E2 surge prior to ovulation is followed by an increase in progesterone levels. Unlike E2, progesterone has an excitatory effect on the LC (Szawka et al., 2009) and promotes the release of a large amount of noradrenaline in the hypothalamus (Szawka et al., 2013). Given the diffuse nature of the central noradrenergic system (Kvetnansky et al., 2009), progesterone probably also increases noradrenaline release in diverse areas of the central nervous system (Szabadi, 2013), including those related to respiratory control. Most of the central effects of progesterone are expected to require an estrogen primer, because E2 up-regulates expression of progesterone receptors in the brain (MacLusky and McEwen, 1978; Thornton et al., 1986). Thus, it remains to be investigated whether progesterone plays a role in the LC response to hypercapnia in females and, more specifically, in conditions of E2-induced suppression of LC neuronal activity.

E2 is an important hormone for respiratory control since reductions in this hormone levels after menopause are associated with the appearance of respiratory disorders, such as obstructive sleep apnea, which is

attenuated by E2 replacement therapy (Shahar et al., 2003; Wesström et al., 2005). Considering that LC is the major noradrenergic nucleus in the central nervous system (Dahlström and Fuxe, 1964; Moore and Bloom, 1979), sending projections to diverse areas such as the amygdala, hippocampus, rostral ventrolateral medulla, raphe, facial nucleus and the hypothalamus (Szabadi, 2013), we believe that the inhibitory action of E2 upon the LC neurons during normocapnia without altered CO_2 responsiveness may have important physiological effects.

CONCLUSION

In conclusion, our data show that orchidectomy, T or E2 treatment does not affect c-Fos expression in the LC, indicating that LC activation is not influenced by gonadal hormones in male rats, unlike in females, where high levels of E2 reduce the activity of LC neurons during normocapnia, suggesting an inhibitory role for E2 in this nucleus despite not altering their CO_2 responsiveness. This study offers some new insights into an important chemosensitivity area of neurons, offering special attention to differences between females vs. males.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: D.C., L.H.G., R.E.S. Performed the experiments: D.C., D.A.M., M.P.B., C.M.L., R.A.J. Analyzed the data: D.C., M.P.B., C.M.L. Contributed reagents/materials/analysis tools: L.H.G., K.C.B., R.E.S., J.A.F. Wrote the paper: D.C., L.H.G., R.E.S. Revised the article critically for important intellectual content: L.H.G., K.C.B., R.E.S., JAF.

Acknowledgments—This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – Grant: 2012/19966-0), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Débora de Carvalho was the recipient of a FAPESP PHD scholarship (2008/57748-9).

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(Accepted 12 November 2015)
(Available online 19 November 2015)