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

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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,

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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.

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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%