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RESEARCH ARTICLE

Estradiol Valerate and Remifemin ameliorate ovariectomy-induced decrease in a serotonin dorsal raphe–preoptic hypothalamus pathway in rats

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ABSTRACT

Perimenopausal syndromes begin as ovarian function ceases and the most common symptoms are hot flushes. Data indicate that the projections of serotonin to hypothalamus may be involved in the mechanism of hot flushes. Therefore, the aim of this study is to investigate the potential role of the serotonin dorsal raphe–preoptic hypothalamus pathway for hot flushes in an animal model of menopause. We determined the changes in serotonin expression in the dorsal raphe (DR) and preoptic anterior hypothalamus (POAH) in ovariectomized rats. We also explored the therapeutical effects of estradiol valerate and Remifemin in this model. Eighty female Sprague-Dawley rats were randomly assigned to sham-operated (SHAM) group, ovariectomy (OVX) group with vehicle, ovariectomy with estradiol valerate treatment (OVX + E) group and ovariectomy with Remifemin (OVX + ICR) group. Serotonin expression was evaluated in the DR and POAH using immunofluorescence and quantified in the DR using an enzyme-linked immunosorbent assay (ELISA). Apoptosis was analyzed in the DR by TUNEL assay. The number of serotonin immunoreactive neurons and the level of serotonin expression in the DR decreased significantly following OVX compared to the SHAM group. No TUNEL-positive cells were detected in the DR in any group. In addition, following OVX, the number of serotonin-positive fibers decreased significantly in the ventromedial preoptic nucleus (VMPO), especially in the ventrolateral preoptic nucleus (VLPO). Treatment with either estradiol or Remifemin for 4 weeks countered the OVX-induced decreases in serotonin levels in both the DR and the hypothalamus, with levels in the treated rats similar to those in the SHAM group. A fluorescently labeled retrograde tracer was injected into the VLPO at the 4-week time point. A significantly lower percentage of serotonin with CTB double-labeled neurons in CTB-labeled neurons was demonstrated after ovariectomy, and both estradiol and Remifemin countered this OVX-induced decrease. We conclude that serotonin pathway is changed after ovariectomy, including the serotonin synthesis in DR and serotonin fibers in PO/AH, both E and Remifemin have an equivalent therapeutic effect on it.

1. Introduction

The perimenopausal syndrome begins as ovarian function ceases and includes vasomotor, neuropsychiatric, urinary, and reproductive system dysfunction. The most common symptoms are hot flushes during the day and sweating at night (Hui et al., 2012). Women with hot flushes have an increased core temperature and a reduced thermoneutral zone (Shen and Stearns, 2009). The decrease in the reproductive hormones during menopause has been shown to cause thermoregulatory dysfunction. We have previously

Abbreviations: ANOVA, analysis of variance; CTB, cholera toxin B subunit; DR, dorsal raphe; ERα/ERβ, estrogen receptor-α/β; LPO, lateral preoptic area; MPA, medial preoptic area; OVX, ovariectomized rats; OVX + E, ovariectomized rats receiving estradiol valerate; OVX + ICR, ovariectomized rats receiving Remifemin; PBS, phosphate-buffered saline; POAH, preoptic anterior hypothalamus; SHAM, sham-operated controls; VLPO, ventrolateral preoptic nucleus; VMPO, ventromedial preoptic nucleus.

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shown that excitation of thermoregulatory neurons in the hypothalamus decreased and the temperature sensitive range narrowed in an ovariectomized (OVX) rat model (Freedman et al., 2011). Moreover, OVX rats exhibit abnormal thermoregulation characterized by increases in their day and night central body temperatures (Ma et al., 2011a).

Ample evidence has shown that serotonin is involved in thermoregulatory processes. Notably, serotonin reuptake inhibitors can alleviate hot flushes (Loprinzi et al., 2009). In addition, OVX rats have thermoregulatory disorders and an increased number of serotonin receptors in the thermoregulatory center associated with heat dissipation and production (Ma et al., 2011a). Neurotoxic drugs can affect serotonin and its receptors, which in turn damage thermoregulatory function (Lizarraga et al., 2014). Furthermore, stimulation of the central cholinergic system promotes heat dissipation in running rats that is related to decreased serotonin content in the preoptic area (Rodrigues et al., 2009). Animal studies have revealed that 17β-estradiol is a neuromodulator of the central serotonin system and can modulate serotonin pathways by affecting serotonin synthesis, concentration, the number of presynaptic and postsynaptic binding sites, and reuptake (Rossmanith and Ruebberdt, 2009; Cummings et al., 2011; Brenchat et al., 2012). Thus, 17β-estradiol modulation of the serotonin system is considered a potential mechanism of thermoregulation abnormalities induced by perimenopausal hormone fluctuations.

The dorsal raphe nucleus (DR) located in the midbrain and rostral pons is the largest serotonin-containing nucleus in the brain (Lee and Lee, 2014) and accounts for more than half of the serotonergic neurons in the central nervous system (Yoon and Lee, 2013). The DR is divided into medial, lateral, and caudal components. The medial component can be further subdivided into the mediodorsal and medioventral areas (Jacobs and Azmitia, 1992). Among the seven classes and 15 subtypes of serotonin receptors, 5-HT1A, 5-HT2A, and 5-HT7 receptors are mainly distributed in the preoptic anterior hypothalamus (POAH), which is considered the central integrator of thermoregulation (Henderson and Bethea, 2008; Hiroi and Neumaier, 2009; Naumenko et al., 2011; Voronova et al., 2011; Brenchat et al., 2012; Kondaurova et al., 2012; Vidal et al., 2013; Al-Safi et al., 2014). The POAH contains many nuclei, such as the medial preoptic area (MPA), lateral preoptic area (LPO), ventrolateral preoptic nucleus (VLPO), and ventromedial preoptic nucleus (VMPO), which are closely related to thermoregulation (Hui et al., 2012). Based on the current literature, we hypothesize that the thermoregulation mechanism of the central serotonin system is as follows: DR serotonergic neurons project to the POAH, transport serotonin to thermosensitive nuclei in this area, and exert thermoregulatory functions by acting on different subtypes of serotonin receptors. As a neuromodulator of the central serotonergic system, perimenopausal estrogen fluctuations can thus cause hot flushes by acting on this pathway.

Remifemin, a perennial herb medicine and an isopropanolic formulation of *Cimicifuga racemosa* (black cohosh), is applied widely for the relief of menopausal symptoms, including vasomotor symptoms, anxiety, and depression (Nadaoka et al., 2012a,b; Ross, 2014). The extract of black cohosh (Remifemin) is extremely complex, mainly containing triterpene glycosides and a phenolic substance. Unlike estradiol, Remifemin has no phytoestrogen and estrogenic effects on the mammary gland and uterus (Viereck et al., 2005; Wuttke et al., 2014). The mechanism by which black cohosh acts has not yet been determined, but evidence suggests that it might act as a partial agonist of the serotonin receptor (Burdette et al., 2003). Studies have confirmed that Remifemin can reduce hot flush frequency and subcutaneous temperature in OVX rats (Kapur et al., 2010). Moreover, in our previous study, the numbers of 5-HT1A and 5-HT2A receptors in the POAH changed after OVX, but treatment with estradiol valerate or Remifemin significantly inhibited these changes (Ma et al., 2011b).

In the current study, we determined the changes in serotonin in the DR and POAH after ovariectomy and further investigated whether these two regions were connected via retrograde tracing. We also examined the effects of estradiol and Remifemin in these nuclei in our model. Our results provide morphological evidence for a central effect of estradiol valerate and Remifemin in the DR and hypothalamus, and thus provide a theoretical basis for the mechanism of perimenopausal hot flushes.

2. Materials and methods

2.1. Ethics statement

All experimental procedures and protocols were approved by the Biomedical Ethics Committee of Peking University (the approval number: LA2012-81) and were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The operation was performed under 1% sodium pentobarbital intraperitoneal injection anesthesia, and all efforts were made to minimize suffering.

2.2. Reagents and instruments

Goat anti-rat serotonin antibody (1:500, ab66047) and Cy2-conjugated donkey anti-goat IgG (1:200, ab6948) were purchased from Abcam (Cambridge, UK). Cholera toxin B subunit (CTB)-conjugated Alexa Fluor 594 (C34777) was obtained from Invitrogen (Carlsbad, CA, USA). The TUNEL assay (In Situ Cell Death Detection Kit, POD) was purchased from Roche (Cat. No. 11684817910, Germany). DNase I solution was purchased from Beijing Atom Science and Technology Co., Ltd. (Cat. No. SL2076). The rat 5-HT ELISA Kit was purchased from Beijing Peak Albert Biotechnology Co., Ltd. (Cat. No. DRE20128I). The manufacturers of *Cimicifuga racemosa* (Remifemin® tablets) and estradiol valerate (Bujiale®, 1 mg/tablet) are Schaper & Brümmer GmbH & Co., KG (batch number 063471; Salzgitter, Germany) and the Guandong branch of Bayer Healthcare Co., Ltd. (batch number 026A 11), respectively. The active ingredients in each Remifemin tablet (0.018–0.026 mL liquid extract) correspond to, on average, 2.5 mg dry extract or 20 mg crude drug (extraction agent: 40% [v/v] isopropanol) (Genant et al., 2007). A Leica 1900 microtome and an Olympus BX51 microscope were also used in this study (Leica, Wetzlar, Germany; Olympus, Tokyo, Japan).

2.3. OVX model and experimental groups

Eighty 3-month-old, healthy female Sprague Dawley rats (weight: 250 ± 10 g) (purchased from the Laboratory Animal Science Department of the Peking University Health Science Center) were used in this study. Before surgery, all rats were housed in the laboratory and given 2 weeks to adapt to their surroundings. The laboratory was maintained at a constant temperature of 25 °C and relative humidity of 50% on a 12-h light/dark cycle. All rats were allowed free access to water and soy-free pellets, which were used to eliminate the effects of phytoestrogens throughout the experiment (Rachoń et al., 2008).

Eighty rats were randomly divided into four groups: a sham-operated control group that received vehicle (SHAM, $n = 20$) and OVX rats that received vehicle (OVX, $n = 20$), estradiol valerate (OVX + E, $n = 20$), and Remifemin (OVX + ICR, $n = 20$). In the OVX groups, rats were anesthetized with an intraperitoneal injection of 1% sodium pentobarbital (80 mL/kg), an incision was made at the midline of the abdomen, and both ovaries were removed. In the SHAM group, rats were anesthetized and their ovaries were