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EFFECTS OF GONADAL STEROIDS AND OF ESTROGEN RECEPTOR AGONISTS ON THE EXPRESSION OF ESTROGEN RECEPTOR ALPHA IN THE MEDIAL PREOPTIC NUCLEUS OF FEMALE RATS

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Abstract—The medial preoptic nucleus (MPN) is a sexually dimorphic cell group of the medial preoptic area that plays a central role in the integration of olfactory and hormonal stimuli that modulate sexually differentiated behaviors. The influence of sex steroids in these behaviors is mediated through activation of estrogen receptors (ERs), which are highly expressed in this nucleus. Little is known about the effects of progesterone (P) or the selective activation of each ER subtype on the expression of estrogen receptor alpha (ERα) in the MPN of female rats. We have addressed this subject in the current investigation by estimating, using stereological tools, the total number of MPN neurons that express ERα in rats at each phase of the estrous cycle and in ovariectomized rats treated with estradiol benzoate (EB), P or the ERα- and estrogen receptor beta (ERβ)-specific agonists. Results show that the total number of ERα-immunoreactive neurons does not change over the estrous cycle, except at proestrus when the number is reduced. A similar effect was observed after the administration of EB, but not of P. Results also show that the estradiol-induced down-regulation of the ERα is mediated by activation of both ER subtypes, and that ERβ activation leads to a reduction in the total number of ERα-immunoreactive neurons that is twice that resulting from ERα activation. Present data suggest that ERα activation triggers a sort of negative feedback mechanism in MPN neurons that reduces its own expression, which might be of importance for the regulation of estradiol-dependent physiological and behavioral responses.

Key words: medial preoptic nucleus, estrous cycle, estrogen receptor alpha, estrogen receptor agonists, immunohistochemistry, stereology.

INTRODUCTION

The medial preoptic nucleus (MPN) is the largest cell group of the medial preoptic area, a complex region of the central nervous system that is critically involved in homeostatic mechanisms and autonomic adjustments (reviewed in Madeira and Lieberman, 1995; Blaustein and Erskine, 2002). In female rodents, activation of the MPN facilitates proceptive behaviors and inhibits the display of the receptive component of feminine sexual behavior, the lordosis reflex (Hoshina et al., 1994; Micevych et al., 2003; Guarraci et al., 2004). These actions are achieved through the bidirectional connections it establishes with main components of the limbic system, including the bed nucleus of the *stria terminalis* (BNST) and the hypothalamic ventromedial and arcuate nuclei (Berk and Finkelstein, 1981; Simerly and Swanson, 1986, 1988; Micevych and Christensen, 2012), and with the ventral tegmental area (Sakuma, 1994; Takeo and Sakuma, 1995). The MPN is also the brain region that contains the highest concentration of cells expressing receptors for gonadal steroids (Simerly et al., 1990; Shughrue et al., 1997a, 1998). These receptors mediate the influence of sex steroids on the functional activity of MPN neurons and on the structural and neurochemical organization of the MPN, both during development and adulthood (Swanson, 1976; Gorski et al., 1980; Döhler, 1991; Madeira and Lieberman, 1995).

Estradiol acts through membrane and two nuclear estrogen receptors (ERs), estrogen receptor alpha (ERα) (Gorski et al., 1968; Jensen et al., 1968; Greene et al., 1986) and estrogen receptor beta (ERβ) (Kuiper et al., 1996). In the MPN, ERα and ERβ, although more densely concentrated in the medial division (Yuri and Kawata, 1991; Shughrue et al., 1997b; Madeira et al., 2000), are expressed along its rostrocaudal extension. There is ample evidence from the literature that ERα is essential for the modulation of the physiological mechanisms that mediate the proceptive and receptive components of female sexual behavior (for a review see Blaustein and Mani, 2007; Blaustein, 2009). It has also been demonstrated that, in the preoptic area in general and in the MPN in particular, ERα mRNA and protein levels vary across the estrous cycle, being highest at metestrus and lowest at proestrus (Shughrue et al., 1992; Zhou et al., 1995). They also increase after ovariectomy, and this effect is reversed, in a dose-dependent manner, by estradiol administration (Lauber et al., 1991;

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Abbreviations: ANOVA, analysis of variance; BNST, bed nucleus of the stria terminalis; DPN, diaryl-propionitrile; EB, estradiol benzoate; ER, estrogen receptor; ERα, estrogen receptor alpha; ERα-ir, estrogen receptor alpha-immunoreactive; ERβ, estrogen receptor beta; LHRH, luteinizing hormone-releasing hormone; MPN, medial preoptic nucleus; OVX, ovariectomized; P, progesterone; PB, phosphate buffer; PPT, propyl-pyrazole triol.

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Shughrue et al., 1992). Contrariwise, there are no available data on the effects of ERβ activation on the functions ascribed to the MPN or on the expression of ERα by MPN neurons. It is known that ERα and ERβ have different transcriptional activity and their activation leads to modifications in the proportion of ERα/ERβ co-expression and, therefore, in the cell response to estradiol (Patisaul et al., 1999; Safe and Kim, 2008; Arnal et al., 2012).

Although the changes in ERα mRNA and protein levels that occur in the MPN over the estrous cycle or as a result of the exogenous administration of estradiol are relatively well characterized, in this study we have re-visited this topic and extended the investigation to assess the influence of progesterone (P) in the regulation of ERα expression in the MPN. To do so, we have used stereological methods to estimate the total number of ERα-immunoreactive (ERα-ir) neurons in the MPN of rats at each phase of the estrous cycle and in ovariectomized (OVX) rats treated with estradiol benzoate (EB) and/or P. There are also no data on the role played by the activation of each ER subtype or the effects of the ERα/ERβ interaction in the regulation of ERα expression in the MPN. To shed light on this subject, we have also estimated the total number of ERα-ir neurons in the MPN of rats that, after ovariectomy, were treated with the specific agonist of the ERα, propyl-pyrazole triol (PPT), the specific agonist of the ERβ, diaryl-propionitrile (DPN) or a combination of both.

EXPERIMENTAL PROCEDURES

Animals

Female Wistar rats obtained from the Institute for Molecular and Cell Biology (Porto, Portugal) were kept in a 12-h light–dark cycle (lights on at 07:00 am), at ambient temperature of 23 °C and free access to food and water. Starting at 8 weeks of age, estrous cycles were monitored daily by vaginal smear cytology between 2:00 and 4:00 pm. Only females with regular 4- to 5-day estrous cycles were included in the experiments. At 10 weeks of age, 35 rats were randomly selected and bilaterally OVX under deep anesthesia by sequential administration of promethazine (10 mg/kg body weight, sc), xylazine (2.6 mg/kg body weight, im) and ketamine (50 mg/kg body weight, im). The remaining rats (n = 20) were kept intact and were killed at 12 weeks of age, at each phase of the estrous cycle, between 2:00 and 4:00 pm (n = 5 per estrous cycle stage). All animal experimentation was conducted according to the European Communities Council Directives of 22 September 2010 (2010/63/EU) and Portuguese Act no. 113/13.

Treatments

After recovery for 12 days, OVX rats were allocated to one of seven groups (n = 5 per group). Rats in each group received two subcutaneous injections, 24 h apart, of the following solutions: (1) 100 μl sesame oil (Oil group); (2) 10 μg EB (EB group); (3) 10 μg EB followed by 500 μg P 44 h after the last EB injection (EB + P group); (4) 500 μg P (P group); (5) 500 μg propyl-pyrazole triol (PPT group); (6) 500 μg diaryl-propionitrile (DPN group) or (7) a solution containing 500 μg PPT and 500 μg DPN (PPT + DPN group). EB and P were purchased from Sigma–Aldrich Company Ltd. (Madrid, Spain), and PPT and DPN from Tocris BioScience (Bristol, UK). All solutions were prepared in 0.1 ml of sesame oil and injected subcutaneously. Rats were sacrificed 48 h after the last injection, except in the EB + P group in which rats were sacrificed 4 h after the injection of P. The doses of hormones and ER agonists and the schedule of administration used in this study are recognized as effective in inducing changes in sexual receptivity in OVX rats and in the expression of ER and P receptors in several regions of the brain, including the preoptic area and hypothalamus (Boling and Blandau, 1939; Pfaff and Sakuma, 1979; McEwen and Alves, 1999; Pfaus et al., 2006; Mazzucco et al., 2008; Sá et al., 2013).

Tissue preparation

Rats were anesthetized with 3 ml/kg body weight of a solution containing sodium pentobarbital (10 mg/ml) and killed by intracardiac perfusion of a fixative solution containing 4% paraformaldehyde in phosphate buffer (PB), pH 7.0. Brains were removed from the skulls, immersed in the same fixative solution for 1 h and kept overnight in a solution of 10% sucrose in PB at 4 °C. Uteri were excised, freed from connective tissue and fat, and weighed in order to confirm the stage of the estrous cycle in intact rats and the efficacy of the treatments in OVX rats (Fig. 1). Brains were transected in the coronal plane through the anterior border of the optic chiasm and the posterior limit of the mammillary bodies, mounted on a Vibratome and sectioned at 40 μm, in the coronal plane, along the rostrocaudal extent of the preoptic area. Immunostaining was carried out on free-floating sections using a rabbit polyclonal antiserum directed at the C-terminus of the ERα (MC20, Santa Cruz Biotechnology, Germany). Sections were incubated for 72 h, at 4 °C, in the primary antibody diluted 1:1,000 in phosphate-buffered saline containing 0.5% Triton-X 100 (PBS + T). Then, they were incubated in biotinylated horse anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA), diluted 1:400, followed by avidin–biotin peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories) at a dilution of 1:800. Between each step, sections were thoroughly washed with PBS + T. Following a final wash, sections were incubated for 90 s in 0.05% diaminobenzidine (Sigma, St. Louis, MO, USA) to which 0.01% H_2O_2 was added. Stained sections were mounted on gelatin-coated slides, air-dried overnight at room temperature and counterstained with Giemsa for examination of MPN cytoarchitectural details (Fig. 2). They were then dehydrated in a series of ethanol solutions, cleared in xylol, and coverslipped using Histomount (National Diagnostics, Atlanta, GA, USA). To minimize the variation in staining between each run, immunohistochemical procedures were carried out in parallel with at least two rats per group. The rabbit