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Testosterone exposure during the critical period decreases corticotropin-releasing hormone-immunoreactive neurons in the bed nucleus of the stria terminalis of female rats

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HIGHLIGHTS

- We previously described female rats have more CRH neurons than male rats.
- We hypothesized that testosterone exposure during the critical period decreased CRH neurons.
- Testosterone exposure resulted defeminize female reproductive system.
- CRH neurons in the BSTLD but not in the preoptic area changed by testosterone.
- Testosterone results the sexual differentiation of CRH neurons in the BSTLD.

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ABSTRACT

We previously described sex differences in the number of corticotropin-releasing hormone-immunoreactive (CRH-ir) neurons in the dorsolateral division of the bed nucleus of the stria terminalis (BSTLD). Female rats were found to have more CRH neurons than male rats. We hypothesized that testosterone exposure during the critical period of sexual differentiation of the brain decreased the number of CRH-ir neurons in the hypothalamus, including the BSTLD and preoptic area. In the present study we confirm that testosterone exposure during the neonatal period results in changes to a variety of typical aspects of the female reproductive system, including estrous cyclicity as shown by virginal smear, the positive feedback effects of estrogen alone or combined with progesterone, luteinizing hormone secretions, and estrogen and progesterone-induced Fos expression in gonadotropin-releasing hormone neurons. The number of CRH-ir neurons in the preoptic area did not change, whereas CRH-ir neurons in the BSTLD significantly decreased in estrogen-primed ovariectomized rats exposed to testosterone during the neonatal period. These results suggest that the sexual differentiation of CRH neurons in the BSTLD is a result of testosterone exposure during the critical period and the BSTLD is more fragile than the preoptic area during sexual differentiation. Furthermore, sex differences in CRH in the preoptic area may not be caused by testosterone during this period.

1. Introduction

The structure of the bed nucleus of the stria terminalis (BST) has been shown to exhibit sexual dimorphism [5,13,16,30]. We previously reported a sex-based difference in the number of corticotropin-releasing hormone-immunoreactive (CRH-ir) neurons in the dorsolateral division of the BST (BSTLD) [10] as well as in the preoptic area [24]. The exact function of the BSTLD is not well known, but fibers connect the BSTLD to various brain regions [7,8] involved in many different types of motivational behaviors [4,32,34] and gonadotropin secretions [2,19]. Recently, we reported that the BSTLD is involved in modulating pain responses [14]. In addition to a sex-based difference in the number of adult CRH-ir neurons, the development of this sexually dimorphic nucleus may be susceptible to changes in environmental factors as exposure to endocrine disruptors is known to affect BST development [10].

Sexual differentiation of the number of CRH-ir neurons in the BSTLD may be caused by exposure to testosterone during the critical period of sexual differentiation of the brain; the principal nucleus of the BST [13,16] and the hypothalamus [23] exhibit sex-based structural differences caused by testosterone. That is, sexual differentiation of the brain occurs during a critical period

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just before and after birth, when testosterone exposure permanently organizes the brain [29]. Some hypothalamic areas are well documented as being sexually differentiated due to testosterone and/or testosterone converted to estrogen [26,37]. In the case of the brain structure controlling gonadotropin, testosterone converted to estradiol is thought to increase GABA tone, causing masculinization of astrocytes, which may contribute to the lack of positive feedback by estradiol and a luteinizing hormone (LH) surge in males [29]. Loss of the capacity of estrogen to induce a LH surge is indicative of exposure to testosterone during the critical period.

In the present study, we examined the effects of testosterone exposure during the critical period of sexual differentiation in female rats, particularly in relation to reproductive functions that exhibit testosterone-induced differences, such as the secretion of LH and the occurrence of regular estrous cyclicity [23].

2. Materials and methods

2.1. Animals

Female Wistar rats (weight 250.0 g; Charles River, Yokohama, Japan) were maintained under controlled temperature (24–26 °C) and lighting conditions (light on 05:00–19:00 h) with food (Oriental Yeast co., Ltd., Tokyo, Japan) and water available ad libitum. The day of birth was defined as the first day. Three-day-old female rats were injected subcutaneously with 1 μg (n = 23) or 5 μg (n = 26) testosterone propionate (TP) dissolved in 25 μl sesame oil once daily for 3 consecutive days. Control rats were injected with sesame oil (n = 20). Two series of experiments were carried out when the animals became adults. Daily vaginal smears were taken from the age of 6 weeks, and rats exhibiting three or more consecutive 4-day estrous cycles were defined as normal with regard to estrous cycle. Constant estrus was defined in rats exhibiting an estrous smear for 10 or more consecutive days. In the first experiment 2 weeks after ovariectomy, estrogen priming was performed under isoflurane anesthesia around noon, and the day was defined as Day 1. A silicone tube (inner diameter = 1.5 mm, outer diameter = 2.5 mm, length = 25 mm) containing 20% 17β-estradiol (E_2; Sigma Chemical Co.) dissolved in cholesterol was implanted subcutaneously. An intra-atrial cannula was implanted through the jugular vein on Day 4. At noon on Day 6, the rats were injected with 1.5 mg progesterone dissolved in 200 μl sesame oil.

Approximately 200 μl of blood was collected under free-moving conditions at 1-h intervals from 12:00 h to 20:00 h on Day 5 and from 12:00 h to 18:00 h on Day 6. The blood was replaced by an equal volume of heparinized saline (2 IU/ml) after each blood sampling. After blood sampling on Day 6, the rats were intravenously injected with an overdose of pentobarbital sodium (100 mg/kg) and perfused through the cardiac ventricle with 4% paraformaldehyde in phosphate buffer (pH 7.5) at 4 °C.

In the second experiment, intact female rats (3 days old) were treated with TP during the neonatal period as described above, and daily vaginal smears were taken when they became adults. The rats were ovariectomized under isoflurane anesthesia. Two weeks after ovariectomy, estrogen priming was performed under isoflurane anesthesia around noon and the day defined as Day 1. The silicone tube containing 20% 17β-estradiol as indicated in the first experiment was implanted subcutaneously. Colchicine dissolved in sterile saline (30 μg/μl) at a dose of 250 μg/kg body weight was injected into the lateral ventricle under pentobarbital sodium (32.5 mg/kg) anesthesia through an acutely inserted cannula according to the atlas of Albe-Fessard et al. [1] (stereotaxic co-ordinates: A = 7.4 mm, V = 7.5 mm, and L = 1.25 mm) on Day 4. The next day (Day 5), the rats were intraperitoneally injected with an overdose of pentobarbital sodium (100 mg/kg) and perfused through the cardiac ventricle with 4% paraformaldehyde in phosphate buffer (pH 7.5) at 4 °C.

Animals were housed and surgical procedures carried out according to the guidelines implemented by the Institutional Animal Care and Use Committee of the Yokohama City University School of Medicine.

2.2. LH assay

Serum concentrations of LH were measured by double antibody radioimmunoassay using materials supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). The reference standard was NIDDK rat LH-RP-3, but the amounts of LH are expressed in terms of NIH LH-S1. The minimal detectable amount of LH in three assays was 0.60 ng/ml. The intra-assay and inter-assay coefficients of variation (CVs) estimated at the mean LH level of 12.8 ng/ml were 5.7% and 6.3%, respectively.

2.3. Immunocytochemistry

Frozen coronal brain sections (30 μm) were cut using a cryostat. The sections were incubated overnight with rabbit polyclonal antibodies to cFos diluted 1:20,000 (first experiment, Oncogene) or rabbit polyclonal antibodies to CRH diluted 1:1000 (second experiment, DiaSorin Inc.). The next day, sections were incubated with biotinylated anti-rabbit IgG diluted 1:200 in PBS containing 1.5% NGS and 0.05% Triton X-100, and then incubated with streptavidin–biotin-peroxidase complex (Vectastain Elite ABC Kit). Bound peroxidase was visualized by incubating sections for 6 min (first experiment) or 10 min (second experiment) in 0.05% 3,3′-diaminobenzidine with H_2O_2. For double staining in the first experiment, sections were incubated overnight with mouse monoclonal antibody to GnRH diluted 1:8000 (LRH13) [27]. The sections were also incubated with biotinylated anti-mouse IgG diluted 1:200, then with Cy3-labeled streptavidin (Amersham Pharmacia Biotech). Finally, sections were mounted on glass slides, dehydrated in graded alcohol, cleared in xylene, and coverslipped with Permount.

2.4. Histological analysis

Cells were counted by an investigator who was blinded to experimental conditions and expectations. In the first experiment, GnRH-immunoreactive (ir) cells in which a clearly visible nucleus was surrounded by fluorescent cytoplasmic staining were counted in the preoptic area (approximately bregma 0.36 mm to −0.40 mm [28]) as described previously [9]. Cells expressing Fos immunoreactivity were observed in the same sections, and cells were defined as double-stained for GnRH and Fos when a blue-black nucleus (Fos-ir) was surrounded by fluorescent cytoplasm (GnRH-ir) at 200× magnification. In the second experiment, the number of CRH-ir cells was counted in the BSTLD and preoptic area as described previously [10]. The BSTLD (approximately bregma −0.26 mm to −0.40 mm [28]) includes the oval nucleus shown by Ju et al. [18] and the anterior lateral subnucleus shown by Moga et al. [25]. The preoptic area (approximately bregma 0.36 mm to −0.40 mm [28]) includes the anteroventral periventricular preoptic area and medial preoptic area where the sexually dimorphic population of CRH neurons is found [24].

The data were analyzed by analysis of variance (ANOVA) or repeated ANOVA followed by Fisher's protected LSD post hoc test. Significance was set at $p < 0.05$.