Neuroscience Letters 534 (2013) 64–68

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

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

Atsushi Fukushima[a], Miyako Furuta[a], Fukuko Kimura[b], Tatsuo Akema[a], Toshiya Funabashi[a,b,*]

[a] Department of Physiology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8511, Japan
[b] Department of Physiology, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan

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.

ARTICLE INFO

Article history:
Received 28 July 2012
Received in revised form 16 October 2012
Accepted 16 November 2012

Keywords:
Neonate
Testosterone
Estrous cycle
BST
GnRH
Fos
Transsexual
Female-to-male
Menstrual cycle

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

* Corresponding author at: Department of Physiology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8511, Japan.
Tel.: +81 44 977 8111; fax: +81 44 977 8111.
E-mail address: toshiya@marianna-u.ac.jp (T. Funabashi).

0304-3940/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neulet.2012.11.065

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 $p < 0.05$.

3. Results

3.1. Effect of neonatal testosterone treatment on the surge in LH

In the first experiment, rats injected with sesame oil ($n = 8$) or 1 μg TP ($n = 13$) for 3 consecutive days during the neonatal period did not exhibit any disruption of estrous cyclicity when they became adults. Though 37.5% of rats injected with 5 μg TP ($n = 16$) for 3 consecutive days during the neonatal period demonstrated a regular estrous cycle (designated as the 5 μg TP-R group, $n = 6$), 62.5% of rats exhibited constant estrus (designated as the 5 μg TP-CE group, $n = 10$).

Neonatal treatment had a significant effect on estrogen-induced LH secretion in repeated ANOVA for treatment between subjects and time within subjects (Fig. 1, upper left panel; $F_{3,33} = 6.80$, $p < 0.002$). Post hoc comparison revealed that LH secretion was significantly decreased in the 1 μg TP ($n = 13$, $p < 0.05$), 5 μg TP-R ($n = 6$, $p < 0.05$), and 5 μg TP-CE groups ($n = 10$, $p < 0.0005$) compared to control rats treated with sesame oil ($n = 8$). The peak value for LH secretion was significantly different among the groups (one-way ANOVA, Fig. 1, upper right panel; $F_{3,33} = 7.33$, $p < 0.001$). Post hoc comparison revealed that the peak values for LH secretion were significantly lower in the 1 μg TP ($p < 0.05$), 5 μg TP-R ($p < 0.005$), and 5 μg TP-CE groups ($p < 0.0002$) compared to control rats. These results indicate that the LH secretory response to estrogen was impaired, even in rats with a regular estrous cycle.

LH secretion induced by estrogen plus progesterone also varied significantly among the groups (Fig. 1, lower left panel; repeated ANOVA $F_{3,33} = 4.78$, $p < 0.01$). Post hoc comparison revealed that LH secretion was significantly decreased in the 5 μg TP-CE group ($p < 0.001$), but not in the 1 μg TP ($p > 0.2$) or 5 μg TP-R groups ($p > 0.08$), compared to control rats. The peak values for LH secretion were significantly different among the groups (Fig. 1, lower right panel; $F_{3,33} = 4.46$, $p < 0.01$). Post hoc comparison revealed that the secretion of LH induced by estrogen plus progesterone treatment was significantly decreased in the 5 μg TP-CE group ($p < 0.002$), but not in the 1 μg TP ($p > 0.2$) or 5 μg TP-R ($p > 0.09$) group, compared to control rats, suggesting that the LH secretory response to estrogen plus progesterone was in accordance with estrous cyclicity.

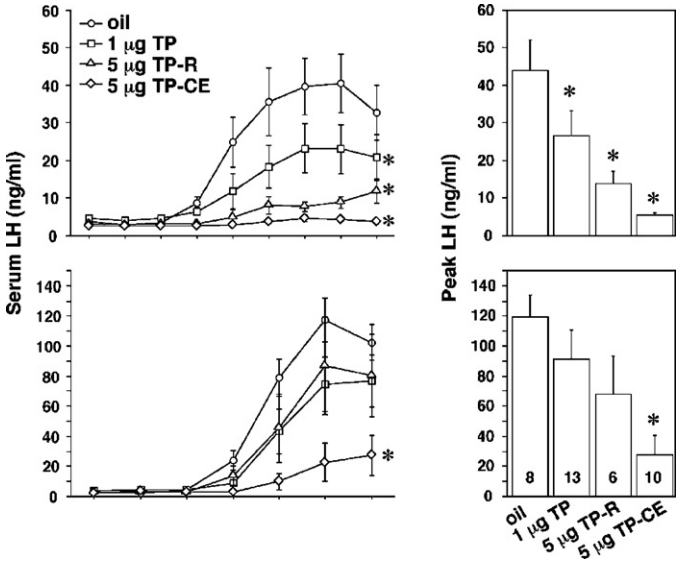

Fig. 1. Effects of neonatal testosterone treatment on LH secretion induced by estrogen (upper panels) or estrogen plus progesterone (lower panels). The right panels show the mean peak values. Data are mean ±SEM. Numbers in columns refer to the number of rats. Oil, oil-injected (control) rats; 1 μg TP, 1 μg testosterone propionate (TP)-injected rats; 5 μg TP-R, 5 mg TP-injected rats demonstrating a regular estrous cycle; 5 μg TP-CE, 5 μg TP-injected rats demonstrating constant estrus. *$p < 0.05$ vs. the oil-injected (control) group.

3.2. Effect of neonatal testosterone treatment on Fos expression in GnRH neurons

Estrogen plus progesterone treatment induced Fos expression in GnRH neurons (Fig. 2a) as reported previously [17,21]. The number of GnRH-ir cells in control rats injected with sesame oil, the 1 μg TP group, 5 μg TP-R group, and 5 μg TP-CE group was 138.5 ± 5.8 ($n = 8$), 148.1 ± 7.0 ($n = 13$), 156.8 ± 13.3 ($n = 6$), and 131.6 ± 8.5 ($n = 10$), respectively. The differences were not significant (one-way ANOVA, $F_{3,33} = 1.49$, $p > 0.2$). However, the percentage of GnRH-ir cells expressing Fos-ir was significantly decreased ($F_{3,33} = 5.63$, $p < 0.004$) in the 1 μg TP ($p < 0.003$), 5 μg TP-R ($p < 0.03$), and 5 μg TP-CE groups ($p < 0.0004$, Fig. 2b).

3.3. Effect of neonatal testosterone treatment on the development of CRH neurons

In the second experiment, rats injected with sesame oil ($n = 12$) or 1 μg TP ($n = 10$) for 3 consecutive days during the neonatal

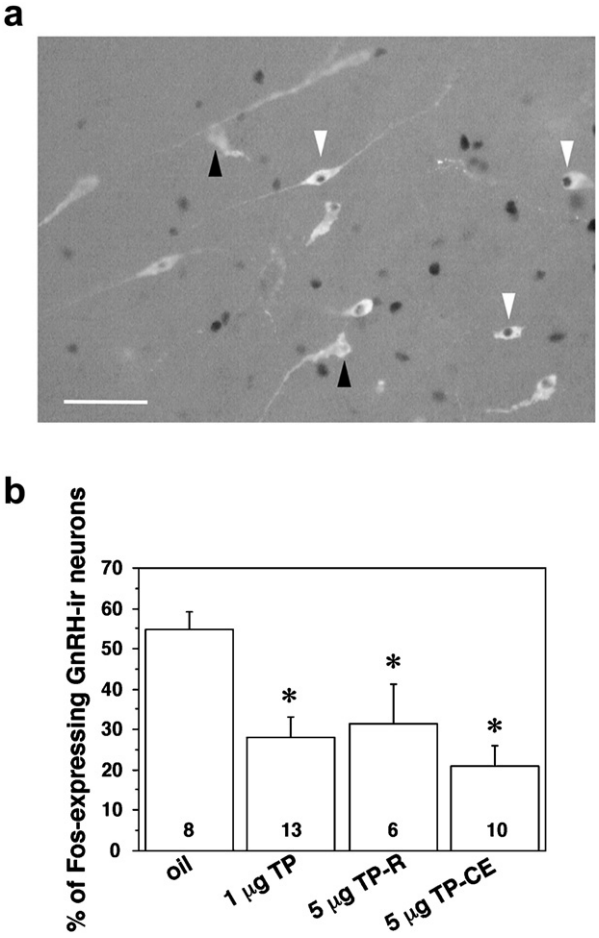

Fig. 2. Representative photograph of cells that are both Fos-immunoreactive (blue-black dots) and GnRH-immunoreactive (white cytoplasm). White and black arrowheads indicate double (for Fos and GnRH) and single (for GnRH only) cells. Scale bar = 50 μm. (b) Effects of neonatal testosterone treatment on Fos immunoreactivity in GnRH-immunoreactive neurons. Columns and vertical lines indicate means and SEM, respectively. Numbers in parentheses refer to the number of rats. Oil, oil-injected (control) rats; 1 μg TP, 1 μg testosterone propionate (TP)-injected rats; 5 μg TP-R, 5 μg TP-injected rats demonstrating a regular estrous cycle; 5 μg TP-CE, 5 μg TP-injected rats demonstrating constant estrus. *$p < 0.05$ vs. oil-injected (control) group.

period did not exhibit any disruption in estrous cyclicity when they became adults, similar to the first experiment. Though 40.0% of rats injected with 5 μg TP ($n = 10$) for 3 consecutive days during the neonatal period demonstrated a regular estrous cycle ($n = 4$, 5 μg TP-R group), 60.0% of rats exhibited constant estrus ($n = 6$, 5 μg TP-CE group). These results were similar to those of the first experiment.

In the BST, the number of CRH-ir cells (Fig. 3a) varied significantly among the groups (one-way ANOVA, Fig. 3b; $F_{3,28} = 6.21$, $p < 0.002$). Post hoc comparison revealed that the number of CRH-ir cells was significantly lower in the 5 μg TP-R group ($p < 0.05$) and 5 μg TP-CE group ($p < 0.005$) compared to control rats. Although the differences were not significant, the number of CRH-ir cells in the 1 μg TP group tended to be less than that of the control group. In the preoptic area, no significant difference in the number of CRH-ir cells was found among the groups ($F_{3,27} = 1.81$, $p > 0.1$), which suggests that the sexually dimorphic development of CRH neurons in the preoptic area was not affected by neonatal testosterone treatment.

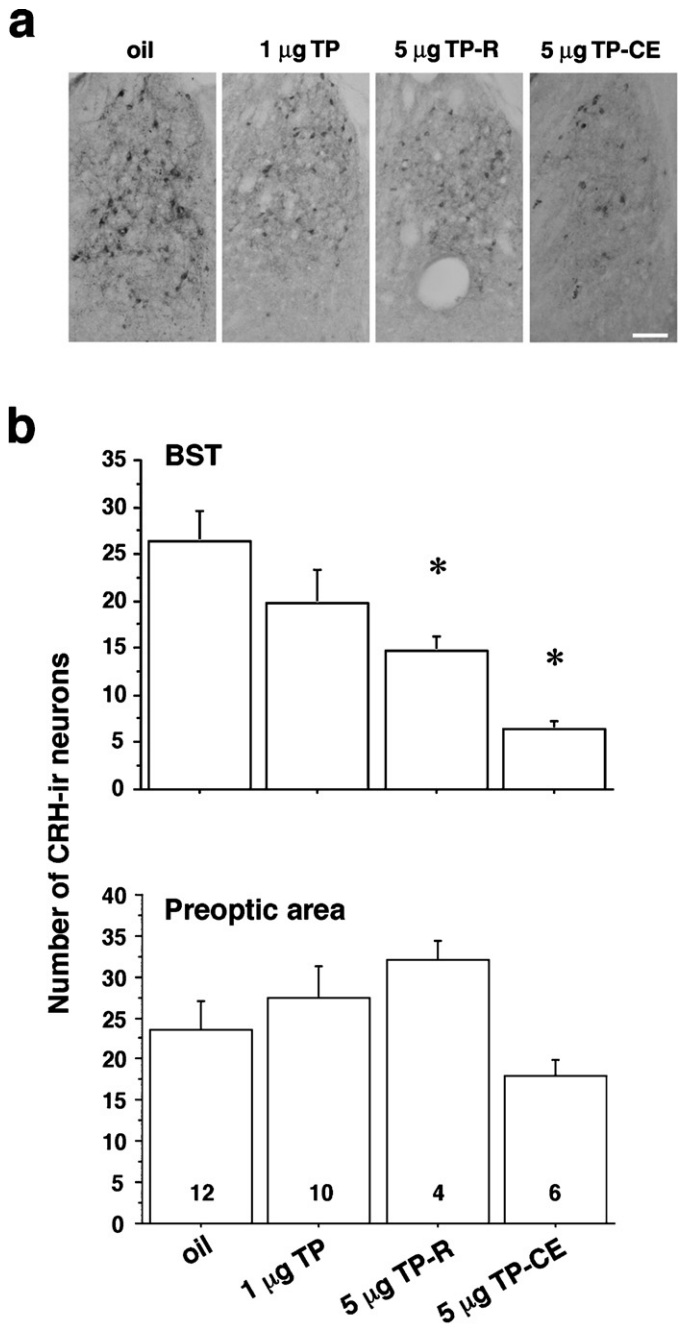

Fig. 3. (a) Representative photographs of CRH-immunoreactive cells in the BST. Scale bar = 100 μm. (b) Effects of neonatal testosterone treatment on the expression of CRH-immunoreactive neurons in the BST (upper panel) and preoptic area (lower panel). Columns and vertical lines indicate means and SEM, respectively. Numbers in parentheses refer to the number of rats. Oil, oil-injected (control) rats; 1 μg TP, 1 μg testosterone propionate (TP)-injected rats; 5 μg TP-R, 5 μg TP-injected rats demonstrating a regular estrous cycle; 5 μg TP-CE, 5 μg TP-injected rats demonstrating constant estrus. *$p < 0.05$ vs. oil-injected (control) group.

4. Discussion

The results of the present study indicate that the sex-based differences in the number of CRH neurons in the BSTLD, but not the preoptic area, are regulated by exposure to testosterone during the critical period of sexual differentiation of the brain. In addition, sexual differentiation of the brain and reproductive system is not homogeneous, but the fragility of differentiation may be selective to brain areas and functions. For example, some rats treated with 5 μg testosterone during the neonatal period exhibited a regular estrous cycle but a decreased number of CRH-ir cells in the BSTLD. Rats treated with 1 μg testosterone during the neonatal period had a decreased LH secretion response to estrogen without any changes in the maintenance of estrous cyclicity. Importantly, sex-based differences in the number of CRH neurons in the preoptic area were not affected by neonatal testosterone treatment.

The overall effects of neonatal testosterone exposure on reproductive functions are in accordance with the pioneering study in which constant estrus was called “delayed anovulatory syndrome” [11]. Testosterone treatment during the neonatal period results in sterility [12] at a frequency close to that of constant estrus found in the present study. Both the LH response and Fos expression in GnRH neurons are impaired by neonatal testosterone treatment. This result is also in accordance with a previous study [36]. Therefore, no claims exist regarding the effects of testosterone during the neonatal period on the sexual differentiation of the control system for LH secretion in rats [22,35]. Thus, we added new information regarding the CRH system in the BST. Knowing whether castration in male rats during the critical period increases the CRH neurons in the BST is important. Although no direct information is available at the moment, a previous report that the volume of the BSTLD is increased by the castration of male rats [5,13] suggests that the number of CRH neurons in the BSTLD is also increased by castration. Furthermore, whether testosterone or estrogen converted from testosterone is critical should be determined in future studies.

The mechanism of different effects of 5 μg TP on the estrous cyclicity is puzzling. We speculate that sensitivity to steroid hormones is altered by exposure to testosterone during the critical period, as the number of CRH in the BSTLD was decreased and the positive feedback effects of estrogen on LH secretion attenuated in the 5 μg TP-CE group compared to the 5 μg TP-R group. In addition, the number of CRH neurons in the preoptic area of the 5 μg TP-CE group seemed to be decreased compared to the 5 μg TP-R group, suggesting a robust effect of testosterone even though the difference was not significant.

LH secretion has been reported to be affected by the BST [2,19], and stress mediated by CRH neurons influences reproductive function in females [6]. These results lead us to think that CRH in the BST is involved in the regulation of gonadotropin secretion in a direct manner. However, CRH neurons mediate the inhibitory effects of stressors on LH secretion, but do not directly involve CRH projections to GnRH neurons [15]. Furthermore, CRH neurons in the BST

do not express estrogen receptor α [33]. Restraint stress does not induce Fos in the CRH neurons in the BSTLD. Therefore, how CRH neurons in the BST are involved in the stress-mediated suppression of gonadotropin needs to be determined.

The BSTLD is homologous to the human central BST [25], which may be involved in gender identity [38]. The human brain exhibits sexual dimorphism in its structure [31], and testosterone levels in human male newborns are higher than in female newborns, suggesting an effect of testosterone on sexual differentiation [3]. In addition, male-to-female transsexuals have a neuron number [38] and central BST size [20] that correspond to the respective norms for biological females, whereas female-to-male transsexuals exhibit a BST size that corresponds to the norm for biological males [20]. These data suggest that sexual differentiation of the brain and genitals go in opposite directions in transsexuals.

References

[1] D. Albe-Fessard, F. Stutinsky, S. Libouban, Atlas stéréotaxique du diencéphale du rat blanc, Editions du Centre National de la Recherche Scientifique, Paris, 1966.

[2] C. Beltramino, S. Taleisnik, Dual action of electrochemical stimulation of the bed nucleus of the stria terminalis on the release of LH, Neuroendocrinology 30 (1980) 238–242.

[3] P. Corbier, L. Dehennin, M. Castanier, A. Mebazaa, D.A. Edwards, J. Roffi, Sex differences in serum luteinizing hormone and testosterone in the human neonate during the first few hours after birth, Journal of Clinical Endocrinology and Metabolism 71 (1990) 1344–1348.

[4] J. G. de Vries, Sex differences in vasopressin and oxytocin innervation of the brain, Progress in Brain Research 170 (2008) 17–27.

[5] A. del Abril, S. Segovia, A. Guillamón, The bed nucleus of the stria terminalis in the rat: regional sex differences controlled by gonadal steroids early after birth, Developmental Brain Research 32 (1987) 295–300.

[6] H. Dobson, S. Ghuman, S. Prabhakar, R. Smith, A conceptual model of the influence of stress on female reproduction, Reproduction 125 (2003) 151–163.

[7] -W. H. Dong, D. G. Petrovich, G. A. Watts, W. L. Swanson, Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain, Journal of Comparative Neurology 436 (2001) 430–455.

[8] W. H.-. Dong, W. L. Swanson, Projections from the rhomboid nucleus of the bed nuclei of the stria terminalis: implications for cerebral hemisphere regulation of ingestive behaviors, Journal of Comparative Neurology 463 (2003) 434–472.

[9] T. Funabashi, K. Jinnai, F. Kimura, Bicuculline infusion advances the timing of Fos expression in LHRH neurons in the preoptic area of proestrous rats, NeuroReport 8 (1997) 771–774.

[10] T. Funabashi, M. Kawaguchi, M. Furuta, A. Fukushima, F. Kimura, Exposure to bisphenol A during gestation and lactation causes loss of sex difference in corticotropin-releasing hormone-immunoreactive neurons in the bed nucleus of the stria terminalis of rats, Psychoneuroendocrinology 29 (2004) 475–485.

[11] A. R. Gorski, Influence of age on the response to paranatal administration of a low dose of androgen, Endocrinology 82 (1968) 1001–1004.

[12] A. R. Gorski, A. C. Barraclough, Effects of low dosages of androgen on the differentiation of hypothalamic regulatory control of ovulation in the rat, Endocrinology 73 (1963) 210–216.

[13] A. Guillamón, S. Segovia, A. del Abril, Early effects of gonadal steroids on the neuron number in the medial posterior region and the lateral division of the bed nucleus of the stria terminalis in the rat, Developmental Brain Research 44 (1988) 281–290.

[14] H. Hagiwara, M. Ishida, J. Arita, D. Mitsushima, T. Takahashi, F. Kimura, T. Funabashi, The cAMP response element-binding protein in the bed nucleus of the stria terminalis modulates the formalin-induced pain behavior in the female rat, European Journal of Neuroscience 30 (2009) 2379–2386.

[15] J.D. Hahn, T. Kalamatianos, C.W. Coen, Studies on the neuroanatomical basis for stress-induced oestrogen-potentiated suppression of reproductive function: evidence against direct corticotropin-releasing hormone projections to the vicinity of luteinizing hormone-releasing hormone cell bodies in female rats, Journal of Neuroendocrinology 15 (2003) 732–742.

[16] L. M. Hines, S. Allen, A. R. Gorski, Sex differences in subregions of the medial nucleus of the amygdala and the bed nucleus of the stria terminalis of the rat, Brain Research 579 (1992) 321–326.

[17] E. G. Hoffman, S. W. Lee, V. B. Attardi, M. Yann, D. Fitzsimmons, Luteinizing hormone-releasing hormone neurons express c-fos antigen after steroid activation, Endocrinology 126 (1990) 1736–1741.

[18] G. Ju, L.W. Swanson, R.B. Simerly, Studies on the cellular architecture of the bed nuclei of the stria terminalis in the rat: II. Chemoarchitecture, Journal of Comparative Neurology 280 (1989) 603–621.

[19] F. Kimura, M. Kawakami, Reanalysis of the preoptic afferents and efferents involved in the surge of LH, FSH and prolactin release in the proestrous rat, Neuroendocrinology 27 (1978) 74–85.

[20] F.P.M. Kruijver, J.N. Zhou, C.W. Pool, M.A. Hofman, L.J.G. Gooren, D.F. Swaab, Male-to-female transsexuals have female neuron numbers in a limbic nucleus, Journal of Clinical Endocrinology and Metabolism 85 (2000) 2034–2041.

[21] S. W. Lee, S. M. Smith, E. G. Hoffman, Progesterone enhances the surge of luteinizing hormone by increasing the activation of luteinizing hormone-releasing hormone neuron, Endocrinology 127 (1990) 2604–2606.

[22] J. N. MacLusky, F. Naftolin, Sexual differentiation of the central nervous system, Science 211 (1981) 1294–1302.

[23] M. McCarthy, Estradiol and the developing brain, Physiological Reviews 88 (2008) 91–124.

[24] A.J. McDonald, F. Mascagni, M.A. Wilson, A sexually dimorphic population of CRF neurons in the medial preoptic area, NeuroReport 5 (1994) 653–656.

[25] M.M. Moga, C.B. Saper, T.S. Gray, Bed nucleus of the stria terminalis: cytoarchitecture, immunohistochemistry, and projection to the parabrachial nucleus in the rat, Journal of Comparative Neurology 283 (1989) 315–332.

[26] B.M. Nugent, M.M. McCarthy, Epigenetic underpinnings of developmental sex differences in the brain, Neuroendocrinology 93 (2011) 150–158.

[27] K. M. Park, K. Wakabayashi, Preparation of a monoclonal antibody to common amino acid sequence of LHRH and its application, Endocrinologia Japonica 33 (1986) 257–272.

[28] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press, Australia, 1986.

[29] J.M. Schwarz, M.M. McCarthy, Cellular mechanisms of estradiol-mediated masculinization of the brain, Journal of Steroid Biochemistry and Molecular Biology 109 (2008) 300–306.

[30] N. Stefanova, A. Bozhilova-Pastirova, W. Ovtscharoff, Sex and age differences of neurons expressing GABA-immunoreactivity in the rat bed nucleus of the stria terminalis, International Journal of Developmental Neuroscience 16 (1998) 443–448.

[31] F. D. Swaab, C. W. Chung, P. F. Kruijver, A. M., M. Hofman, A. T. Ishunina, Structural and functional sex differences in the human hypothalamus, Hormones and Behavior 50 (2001) 93–98.

[32] D. Toufexis, Region- and sex-specific modulation of anxiety behaviours in the rat, Journal of Neuroendocrinology 19 (2007) 461–473.

[33] G.A. Traslavina, C.R. Franci, Divergent roles of the CRH receptors in the control of gonadotropin secretion induced by acute restraint stress at proestrus, Endocrinology 153 (2012) 4838–4848.

[34] L. D. Walker, M. Davis, Role of the extended amygdala in short-duration versus sustained fear: a tribute to Dr. Lennart Heimer, Brain Structure and Function 213 (2008) 29–42.

[35] A. C. Wilson, C. D. Davies, The control of sexual differentiation of the reproductive system and brain, Reproduction 133 (2007) 331–359.

[36] I. R. Wood, S.J. Kim, D.L. Foster, Prenatal androgens defeminize activation of GnRH neurons in response to estradiol stimulation, Journal of Neuroendocrinology 8 (1996) 617–625.

[37] M.V. Wu, N.M. Shah, Control of masculinization of the brain and behavior, Current Opinion in Neurobiology 21 (2011) 116–123.

[38] J.-N. Zhou, M.A. Hofman, L.J.G. Gooren, D.F. Swaab, A sex difference in the human brain and its relation to transsexuality, Nature 378 (1995) 68–70.