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The advancement of the onset of vaginal opening in female rats subjected to chronic testosterone treatment occurs independently of hypothalamic Kiss1 and RFRP expression

Takeshi Iwasa [1], Toshiya Matsuzaki [1], Altankhuu Tungalagsuvd [1], Munkhsaikhan Munkhzaya [1], Akira Kuwahara [1], Toshiyuki Yasui [2], Minoru Irahara [1]

1 Department of Obstetrics and Gynecology, Institute of Biomedical Sciences, Tokushima University Graduate School, Kuramoto-Cho, Tokushima, Japan
2 Department of Reproductive and Menopausal Medicine, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan

Correspondence to: Takeshi Iwasa
Department of Obstetrics and Gynecology, Institute of Biomedical Sciences
Tokushima University Graduate School
3-18-15 Kuramoto-Cho, Tokushima 770-8503, Japan.
TEL: +81 88 633 7177; E-MAIL: iwasa.takeshi@tokushima-u.ac.jp

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Abstract

OBJECTIVE: The neonatal and/or prepubertal androgen milieu affects sexual maturation. In rodents, neonatal chronic testosterone treatment, which is used as a model of polycystic ovary syndrome (PCOS), results in the onset of vaginal opening occurring earlier in the pubertal period.
DESIGN: In the present study, the changes in hypothalamic Kiss1 (a gonadotropin-releasing hormone (GnRH)-stimulating factor) and RF-amide related peptide (RFRP; a GnRH inhibitory factor) mRNA expression induced by testosterone treatment were examined in order to clarify whether these factors are involved in the testosterone-induced acceleration of sexual maturation.
RESULTS: The onset of vaginal opening occurred earlier and uterine weight was increased in female rats subjected to chronic (from postnatal day 23 to day 31) testosterone treatment. Contrary to our expectations, the rats' hypothalamic Kiss1 and Kiss1 receptor mRNA levels were not changed, and their serum luteinizing hormone (LH) levels were decreased. Although hypothalamic RFRP mRNA expression was decreased in the testosterone-treated rats, this change was not reflected in their serum LH levels.
CONCLUSIONS: These results indicate that the advancement of sexual maturation observed in chronic testosterone-treated rats might be caused by a peripheral, rather than a central, mechanism.

INTRODUCTION

The neonatal and/or prepubertal androgen milieu affects sexual maturation and disturbs reproductive function in adulthood (Walters *et al.* 2012). Some studies have indicated that in rodents chronic neonatal testosterone treatment results in vaginal opening occurring earlier in the pubertal period and the development of an irregular estrous cycle; i.e., constant vaginal estrous, in

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adulthood (Weisz & Lloyd 1965; McDonald *et al.* 1972; Nass *et al.* 1984; Hutter & Bibson 1988). In addition, two of these studies found that the luteinizing hormone (LH) surge was diminished or absent in rats that were administered testosterone in the immature period (Nass *et al.* 1984; Hutter & Bibson 1988). As such animals exhibit similar ovarian and/or metabolic characteristics to human polycystic ovary syndrome (PCOS) patients, they have also been used as PCOS animal models (Beloosesky *et al.* 2004; Walters *et al.* 2012).

It has been reported that hypothalamic kisspeptin, which is encoded by the Kiss1 gene, and hypothalamic RF-amide related peptide-3 (RFRP-3), which is encoded by the RFRP gene, and their receptors, Kiss1r and G-protein-coupled receptor 147 (GPR147), play pivotal roles in sexual maturation and the onset of puberty in many species (Wahab *et al.* 2015). Kisspeptin and RFRP act as positive and negative regulators, respectively, of gonadotropin-releasing hormone (GnRH) at the central level (Iwasa *et al.* 2014a). Therefore, increased kisspeptin activity and decreased RFRP activity around the peripubertal period are considered to stimulate GnRH release and induce sexual maturation. As mentioned above, neonatal and/or peripubertal testosterone treatment accelerates vaginal opening, which is the most important indicator of the onset of puberty. However, the mechanisms by which such alterations in sexual maturation are induced by testosterone have not been clarified. Thus, in the present study, we examined the changes in the hypothalamic mRNA levels of Kiss1, RFRP, and their receptors induced by testosterone treatment in order to clarify whether these factors are involved in the effects of testosterone on sexual maturation.

MATERIALS & METHODS

Pregnant Sprague-Dawley rats were purchased (Charles River Japan, Inc., Tokyo, Japan) and housed individually. The animal rooms were maintained under controlled lighting (14 h light, 10 h dark cycle) and temperature (24 °C) conditions. All animal experiments were conducted in accordance with the ethical standards of the animal care and use committee of the University of Tokushima.

Pregnant rats were fed ad libitum during pregnancy. The day of delivery was defined as postnatal day 0. On postnatal day 2, female pups were selected and randomized, and litter size was adjusted to 13–14 per dam. On postnatal day 21, the pups were weaned and housed at 4 per cage. On postnatal day 23, the pups were randomly divided into control and testosterone-treated groups. The pups in the testosterone-treated group were implanted with a silastic tube (inner diameter: 3 mm; length: 10 mm) filled with crystalline T, and those in the control group were implanted with empty tubes. The crystalline T implantation method was described in a previous study (Urban *et al.* 1993) and was matched to the body weight of the rats used in this study. On day 31 (8 days after the tube implantation), all of the rats were weighed, and vaginal opening was checked. Then, brain and blood samples were collected, and rats' uterine weight was measured after they had been decapitated under sevoflurane anesthesia. The rats' serum LH levels were measured using radioimmunoassay kits, as described previously (Iwasa *et al.* 2014a; 2015a,b). In addition, hypothalamic explants were dissected, total RNA was isolated from them, and cDNA was synthesized, as described previously (Iwasa *et al.* 2015a,b). Real-time polymerase chain reaction (PCR) analysis was performed using the StepOnePlus™ real-time PCR system (PE Applied Biosystems, Foster City, CA, USA) and SYBR green in order to quantify the relative mRNA expression levels of Kiss1, Kiss1r, RFRP, GPR147, and GnRH. All expression levels were normalized to the mRNA expression level of GAPDH. The primer sequences and PCR conditions were described previously (Iwasa *et al.* 2014a–e; 2015a,b).

All data are presented as mean ± SE values. The Student's *t* test or Mann-Whitney U test was used for comparisons of serum or hypothalamic factors, and the chi-square test was used for comparisons of the frequency of vaginal opening. Statistical significance was defined as $p<0.05$.

RESULTS

The body weights of the testosterone-treated rats were significantly higher than those of the control rats on postnatal day 31 (Figure 1A). The frequency of vaginal opening was 100% in the testosterone-treated rats on postnatal day 31, whereas none of the control rats had undergone vaginal opening by this point (Figure 1B). The uterine weights of the testosterone-treated rats were significantly heavier than those of the control rats (Figure 1C). The serum LH levels of the testosterone-treated rats were significantly lower than those of the control rats (Figure 1D).

There were no significant differences between the hypothalamic Kiss1, Kiss1r, GPR147, or GnRH mRNA levels of the testosterone-treated and control rats. However, the hypothalamic RFRP mRNA levels of the testosterone-treated rats were significantly higher than those of the control rats (Figure 2).

DISCUSSION

In the present study, the onset of vaginal opening occurred earlier in testosterone-treated rats than in the control rats, which agreed with the findings of previous studies (Weisz and Lloyd, 1965; McDonald *et al.* 1972; Nass *et al.* 1984; Hutter and Bibson, 1988). As the neuroendocrine mechanisms by which testosterone induces the advancement of sexual maturation have not been examined previously, we investigated them by focus-

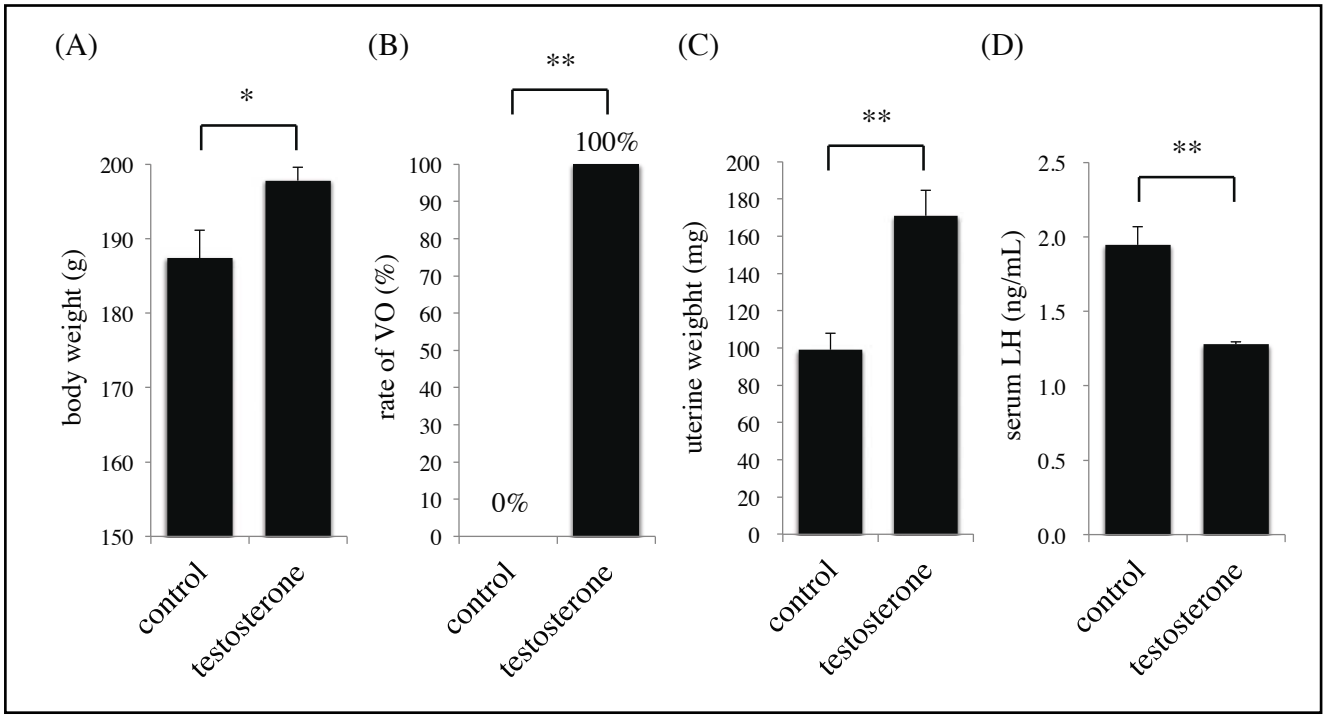

Fig. 1. The body weights (A), vaginal opening frequencies (VO) (B), uterine weights (C), and serum LH levels (D) of the control (n=8) and chronically testosterone-treated rats (n=8) at postnatal day 31. All data, except for the frequency of VO, are expressed as mean ± SE values. * $p<0.05$, ** $p<0.01$.

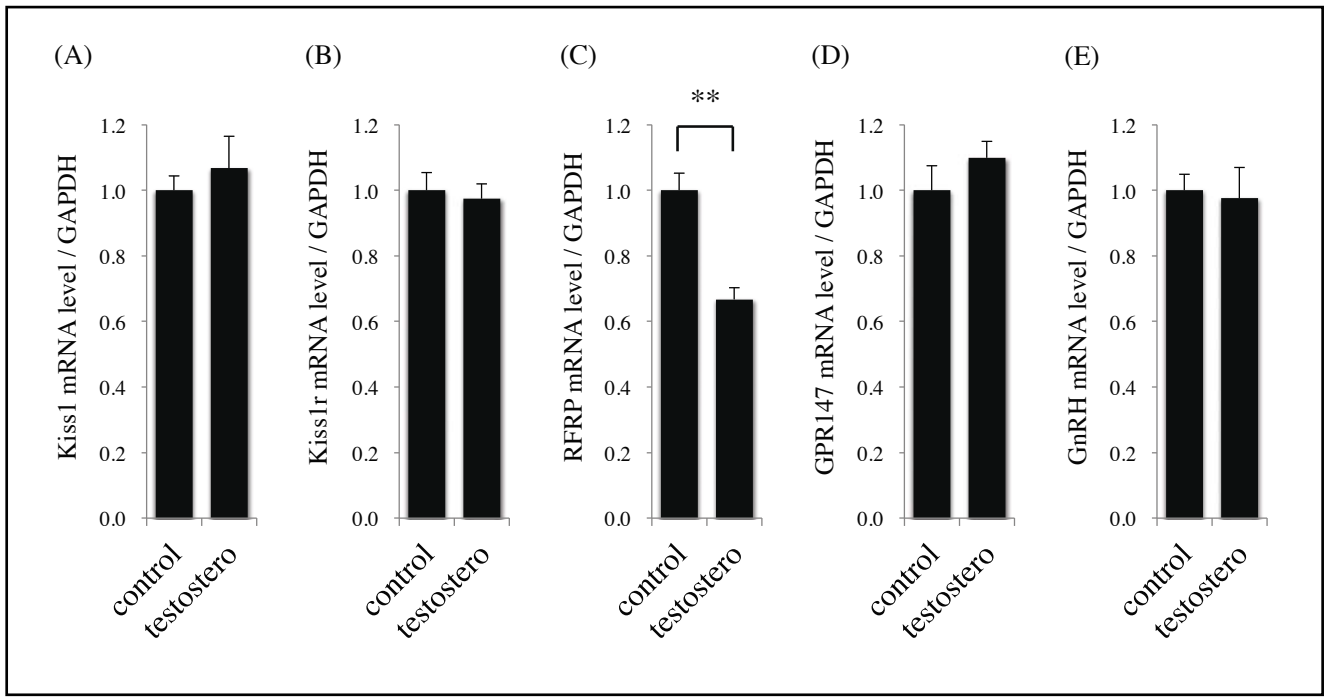

Fig. 2. Hypothalamic Kiss1 (A), Kiss1r (B), RFRP (C), GPR147 (D), and GnRH (E) mRNA levels of the control (n = 8) and chronically testosterone-treated rats (n=8) at postnatal day 31. Data are expressed as mean ± SE values. ** $p<0.01$.

ing on two substantial neuropeptides; i.e., kisspeptin and RFRP. It has been well established that kisspeptin stimulates GnRH release, increases the serum gonadotropin level, and plays pivotal roles in sexual maturation and the onset of puberty. It has been reported that kisspeptin activity is increased during the peripubertal period and that the administration of kisspeptin results in vaginal opening occurring earlier in experimental animals (Navarro and Tena-Sempere, 2012). In addition, kisspeptin receptor-deficient mice exhibit

delayed sexual maturation (Navarro and Tena-Sempere, 2012). On the other hand, RFRP inhibits GnRH release and decreases serum gonadotropin levels. It has been reported that RFRP activity is decreased in the early pre-pubertal period (Semaan and Kauffman, 2015), suggesting that such changes promote GnRH release and sexual maturation. Therefore, we speculated that hypothalamic Kiss1 and RFRP mRNA expression would be increased and decreased, respectively, and therefore, the serum gonadotropin level would be increased in testosterone-treated rats. However, contrary to our expectations, the hypothalamic Kiss1 expression levels of the testosterone-treated rats were similar to those of the control rats, and their serum gonadotropin levels were lower than those of the control rats. Although hypothalamic RFRP expression was decreased in the testosterone-treated rats, the causes and roles of these changes could not be clarified in this study. As the uterine weight of the testosterone-treated rats was increased in spite of the fact that they had decreased serum LH levels, the advancement of sexual maturation might have been induced by peripheral alterations. Further studies are needed to clarify the mechanisms underlying the effects of testosterone on sexual maturation. As noted above, chronically androgen-treated animals are used as PCOS models because they exhibit similar characteristics, e.g., insulin resistance and ovulatory disorders, to human PCOS patients (Beloosesky *et al.* 2004; Walters *et al.* 2012). However, while the serum LH levels of PCOS patients tend to be elevated, those of the testosterone-treated rats were decreased. Therefore, it is possible that the underlying mechanism of ovulatory disorders might differ slightly between animal models and PCOS patients.

In summary, we have shown that the onset of vaginal opening occurred earlier and uterine weight was increased in chronically testosterone-treated female rats. As the rats' hypothalamic Kiss1 mRNA expression levels were not changed and their serum LH levels were decreased, the advancement of sexual maturation might have been induced by a peripheral, rather than a central, mechanism.

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