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Neonatal exposure to SERMs disrupts neuroendocrine development and postnatal reproductive function through alteration of hypothalamic kisspeptin neurons in female rats

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ABSTRACT

Selective estrogen receptor modulators (SERMs) are a class of therapeutic chemicals which present tissue-specific estrogen receptor modulating activity. Neonatal exposure to SERMs has been reported to adversely affect central nervous system development, however, mechanism and involvement of hypothalamic kisspeptin neurone in this impairment remains undetermined. To clarify this uncertainty, neonates from female Donryu rats were subcutaneously injected with raloxifene (RLX) at 0.1, 1, and 10 mg/kg or tamoxifen (TMX) at 10 mg/kg on postnatal day 0, and then hypothalamic KiSS1 mRNA expression and gonadotropin levels were investigated during young adulthood and estrous cycling was monitored until middle age. Treatment with RLX or TMX at 10 mg/kg significantly depressed luteinizing hormone surge levels and KiSS1 mRNA expression in the anteroventral periventricular nucleus (AVPV), the control center of estrous cyclicity. The 10 mg/kg TMX group also showed decreased levels of follicle-stimulating hormone and KiSS1 mRNA expression in the arcuate nucleus (ARC). Early cessation of normal estrous cycling was observed in the 10 mg/kg RLX group, while the estrous cycle in the 10 mg/kg TMX group had ceased by the start of the analysis. The same dose of tamoxifen or raloxifene had either weak-estrogenic or anti-estrogenic activity on the uterus, respectively; however, treatment in adulthood with both SERMs did not affect KiSS1 mRNA expression in either the AVPV or ARC in the present study. These results indicate that neonatal exposure to SERMs could disrupt neuroendocrine development and postnatal reproductive function through the alteration of kisspeptin neurons.

1. Introduction

Exposure to estrogenic chemicals during critical periods of development is widely known to cause irreversible damage to the programming of the hypothalamus-pituitary-gonadal (HPG) axis in a wide range of species. This leads to persistent impairment of reproductive function later in life (Dickerson et al., 2011; Gore et al., 2011). High-dose exposure completely disrupts sexual differentiation in neonatal brains, causes masculinized sexual

Abbreviations: ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; BW, body weight; CNS, central nervous system; CL, corpora lutea; EB, estradiol benzoate; EE, 17α-ethynylestradiol; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; HE, hematoxylin and eosin; HPG, hypothalamus-pituitary-gonadal; IHC, immunohistochemistry; ISH, in situ hybridization; LH, luteinizing hormone; OVX, ovariectomy; PND, postnatal day; P4, progesterone; SERMs, selective estrogen receptor modulators; VO, vaginal opening; 3V, third ventricle.

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behavior, malformation of the reproductive tract, and reduced fertility in adults- effects commonly recognized as "masculinization" or "defeminization" (Christensen and Gorski, 1978; Katsuda et al., 2000; McLachlan et al., 1982). In contrast, lower-dose exposure is reported to induce other effects such as early onset of age-matched abnormal estrus cycling, advanced reproductive senescence and increased uterine carcinogenic risk in young adult or aging rats (Gore et al., 2011; Yoshida et al., 2002, 2011); these are known as "delayed effects" or "delayed reproductive dysfunction" (Yoshida et al., 2011; Takahashi et al., 2013; Shirota et al., 2012; Ohta et al., 2012). In previous studies, we investigated the delayed effects caused by neonatal exposure to single, relatively low doses of 17α-ethynylestradiol (EE) in rats (Takahashi et al., 2013, 2014; Ichimura et al., 2015a,b). These studies revealed that attenuation of the luteinizing hormone (LH) surge and reduction of KiSS1 mRNA levels in the anteroventral periventricular nucleus (AVPV) occurred prior to the early onset of abnormal estrous cycling (Ichimura et al., 2015a). In the rat brain, kisspeptin neurons are mainly located in the AVPV and arcuate nucleus (ARC), and the AVPV is known to trigger secretion of the GnRH/LH surge and subsequent ovulation (Kauffman et al., 2007; Maeda et al., 2007; Dungan et al., 2006). These studies indicated that KiSS1 depression in the AVPV and a reduced LH surge might be early key events in late-onset reproductive dysfunction, a delayed effect (Ichimura et al., 2015a).

Selective estrogen receptor modulators (SERMs) are a class of chemicals which present tissue-specific estrogenic or anti-estrogenic activity, and are utilized for the treatment of various estrogen related diseases such as osteoporosis, postmenopausal symptoms and breast cancer (Musa et al., 2007; Pinkerton and Thomas, 2014). The most classic and clinically identified SERM is tamoxifen, which has been widely used for the treatment of breast cancer. Raloxifene, a second generation SERM, is known to have selective estrogenic activity in osseous tissue and slight anti-estrogenic activity in endometrial tissue. Thus, it has been prescribed for the treatment of osteoporosis with the advantage of less frequent adverse effects such as the increased risk of uterine cancer. Although the unique bilateral effects of SERMs on hormone-dependent tissues have been well studied, little evidence pertaining to the effects on the central nervous system (CNS), especially the effects of neonatal exposure to SERMs on the development of the CNS, has been reported. Previously, Pinilla et al. (2001a) reported that neonatal exposure to raloxifene during postnatal day (PND) 1–5 in female rats causes early cessation of the estrous cycle, decreased gonadotropin secretion and infertility at later ages (PND 90). The same group also compared the neonatal effects of raloxifene and tamoxifen in female rats. They reported that exposure to either SERM caused the same delayed adverse effects such as vaginal acyclicity, ovarian atrophy and sterility in adulthood (Pinilla et al., 2002). These results indicate the potential risk of SERMs in the disruption of CNS development and postnatal reproductive systems in neonatal rats. However, the precise mechanism of these delayed adverse effects caused by SERMs remains undetermined. Kisspeptin neurons are potential candidates for SERM targets in the developing CNS, as neonatal exposure to estrogens could disrupt neuronal function through the estrogen receptor (ER), which was investigated in the previous studies by EE. However, the ER modulating effects of SERMs in neonatal kisspeptin neurons and developing neonatal brains have not been elucidated.

To clarify these uncertainties, in the present study we investigated the effects of neonatal exposure to raloxifene or tamoxifen on the development of kisspeptin neurons using known sensitive indicators of neuroendocrine activity such as KiSS1 expression in the AVPV, the LH surge, and estrous cycling (Ichimura et al., 2015a,b; Homma et al., 2009). Depressed KiSS1 expression in the AVPV resulting in a decreased LH surge and early cessation of normal cycling are also known to be good indicators of delayed adverse effects on unexpected neonatal exposure to estrogens (Ichimura et al., 2015a,b; Takahashi et al., 2013; Shirota et al., 2012; Usuda et al., 2014). For further elucidation, we also studied the modulating (estrogenic or anti-estrogenic) activity of SERM on the uterus and hypothalamus in a modified uterotropic assay using adult rats.

2. Materials and methods

2.1. Animals

Pregnant Crj:Donryu rats maintained in house were used for Experiment 1 (n = 28); young adult Crj:Donryu rats (7 weeks of age) were used for Experiment 2 (n = 30) and Experiment 3 (n = 35). This strain was adopted for the present study because of their clear 4-day estrous cycle and abundant background data obtained from our previous studies (Ichimura et al., 2015a; Yoshida et al., 2011). The dams and their offspring were housed individually in polycarbonate cages with wood chip bedding and maintained in an air-conditioned animal room (temperature, 24 ± 1 °C; relative humidity, 55 ± 5%; 12-h light/dark cycle) with a basal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) and tap water available *ad libitum*. The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences (Japan).

2.2. Chemicals

EE (CAS No. 57-63-6; purity > 98%), raloxifene (CAS No.84449-90-1; purity > 99%), tamoxifen (CAS No.54965-24-1; purity > 99%) and ICI 182,780 (CAS No.129453-61-8; purity > 98%) were all purchased from Sigma (St. Louis, MO, USA). These chemicals were stirred into small amounts of sesame oil overnight, then used after dilution in sesame oil (Wako Pure Chemical, Osaka).

2.3. Experiment 1

All neonates were randomized within 24 h after birth, and 10 neonates were allocated to each dam with a female predominance. After randomization, dams were assigned to 5 groups (5–6 dams/group). Neonates in each group received a single subcutaneous injection of sesame oil (control group), raloxifene 0.1, 1 or 10 mg/kg (RLX0.1, 1 or 10 groups, respectively) or tamoxifen 10 mg/kg (TMX10 group) within 24 h after birth. The doses of EE, raloxifene and tamoxifen were selected as being capable of inducing early-onset abnormal estrous cycling based on previous reports (Takahashi et al., 2013; Pinilla et al., 2001a, 2002). On PND 21, the rats were weaned, and 26 female rats per group were housed 2–4 per cage. Starting on PND 23, we checked for vaginal opening (VO) every day; the day of VO and body weight on the day of VO were recorded. After checking for VO, the estrous cycle was monitored by vaginal smear for 5 consecutive days every week, and observations of clinical signs, body weight, and mortality were made throughout the experiment.

At 10 weeks of age, 10 out of 26 rats in each group were used for the investigation of hypothalamic mRNA expression and gonadotropins. The rats in each group received an ovariectomy (OVX) under isoflurane anesthesia. One week after OVX, all animals were subcutaneously treated with 2 μg of estradiol benzoate (EB) at 9:00 for 3 consecutive days and 500 μg of progesterone (P4) at 11:00 on the last day of EB treatment for artificial LH surge priming. Animals were decapitated (n = 5/group) or transcardially perfused with 4% paraformaldehyde (PFA, Nacalai Tesque, Inc., Kyoto, Japan) under deep anesthesia with pentobarbital sodium (Kyoritsu Seiyaku Corporation, Tokyo, Japan) (n = 5/group) between 16:00