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Original article

The role of estrogen receptor subtypes for induction of delayed effects on the estrous cycle and female reproductive organs in rats☆

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ABSTRACT

It has been reported that neonatal exposure to estrogens at relatively low doses can induce early onset anovulation as a delayed effect in female rats. Dysfunction of kisspeptin neurons in the anteroventral periventricular nucleus (AVPV) was proposed to be a trigger for this effect. To determine the roles of estrogen receptor (ER) subtypes in the induction of delayed effects, we conducted a series of experiments using Donryu rats to examine whether neonatal injection of an ERα agonist (PPT), an ERβ agonist (DPN) or an ERα antagonist (ICI) could induce delayed effects. Also, involvement of the kisspeptin neurons in the AVPV for induction of delayed effect by PPT and DPN was investigated. We observed that neonatal exposure to PPT, DPN and ICI induced the early onset of abnormal estrous cyclicity after sexual maturation, suggesting that the compounds capable of inducing delayed effects are not limited to ERα agonists. On the other hand, the data suggested the possibility that DPN and ICI functioned partially as ERα agonists in the neonatal brain. Regardless of the agents used, there is a possibility that dysfunction of kisspeptin neurons in the AVPV might contribute to induction of early onset anovulation.

1. Introduction

A critical period for sexual differentiation of the rodent brain extends from the prenatal to the early postnatal period [1]. While the female rodent brain develops in the relative absence of sex steroid hormones, the brain of neonatal males is exposed to higher levels of testosterone produced at fetal testis, leading to masculinization of the reproductive tract and the brain [1]. In addition, estradiol converted from testosterone is also necessary for normal sexual differentiation of the male brain. The feedback circuits of the hypothalamus-pituitary-gonadal (HPG) axis are coordinated at this time. Therefore, the neonatal period is particularly sensitive to disruption by hormones or hormone-like compounds, resulting in irreversible reproductive deficits [2,3]. The derived effects differ, and they are dependent on the dose. Administration of high dose testosterone or estradiol benzoate to females during the critical period can induce a male-like brain phenotype and reproductive physiology [4,5]. In contrast, low dose exposure to estrogenic compound such as diethylstilbestrol has been reported to increase carcinogenic risk and impaired reproductive function later in life as delayed effects [6,7].

We have been investigating delayed effects on the female rat's reproductive tract following a single injection of low dose 17α-ethynylestradiol (EE) during the neonatal period. In our research, delayed effects consisted of early onset abnormal estrus cycling (anovulation in brief) after sexual maturation. This occurred in a dose-dependent fashion at doses ≥0.2 μg/kg, although the vaginal opening was not affected [8]. Additionally, we found that decreased expression of *Kiss1* mRNA in the anteroventral periventricular nucleus (AVPV) and depression of the luteinizing

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hormone (LH) surge occurred prior to the onset of abnormal cycling, indicating that kisspeptin neurons in the AVPV that control ovulation play a key role in the induction of abnormal estrus cycling [9].

Classically, the effects of estrogens are mediated through two subtypes of nuclear receptor, estrogen receptor (ER) α and ERβ [10]. There have been a small number of studies investigating the roles of ER subtype in the induction of abnormal cycling [11–13]. From the results of studies using a selective agonist for ERα and ERβ, such as propyl pyrazole triol (PPT) and diarylpropionitrile (DPN), respectively, it has been concluded that ERα plays a major role in regulating the estrous cycle in the rat brain because DPN produces a less severe outcome than PPT on estrous cyclicity [11–13]. However, a study using another ERβ agonist (ZK 281738) showed strong induction of persistent estrous [14], and neonatal exposure to ICI 182,780 (ICI), an ERα antagonist, has been reported to induce an abnormal cycle after sexual maturation [15,16]. Therefore, the mechanistic roles of ER subtypes in the induction of delayed effects remain incompletely characterized.

From a risk assessment perspective, delayed effects have become a serious issue as they are overlooked by existing toxicity studies due to the limited period of observation that is used. Characterization of agents with delayed effects will improve our understanding of such molecules. To determine the roles of ER subtypes for induction of delayed effects, we investigated the long-term effects of neonatal exposure to PPT, DPN and ICI on the female reproductive tract, such as vaginal opening, estrous cyclicity and histology. Also, to validate the *in vivo* estrogenic activity of PPT and DPN at the dose using in the observation of estrous cyclicity, we performed uterotrophic assays. In addition, the levels of *Kiss1* mRNA and serum LH at the period prior to onset of abnormal cycling were examined to confirm the involvement of kisspeptin neurons in the AVPV for delayed effects induced by PPT and DPN.

2. Materials and methods

2.1. Animals

Pregnant Crj:Donryu rats maintained in-house were used for Experiment (Exp.) 1 (n = 13), Exp. 2 (n = 27) and Exp. 3 (n = 15). Female Donryu rats at 6 week of age were used for uterotrophic assays. This strain possesses a regular 4-day cycle after puberty [17]. The rats were housed individually (Exp. 1–3) or 3 per cage (uterotrophic assay) in polycarbonate cages with woodchip bedding and maintained in an air-conditioned animal room (temperature: 24 ± 1◦C; relative humidity: 55 ± 5%; 12-h light/dark cycle: light on, 5:00–17:00; light off, 17:00–5:00) 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

Propyl pyrazole triol (PPT, CAS No. 263717-53-9, purity > 99%) and diarylpropionitrile (DPN, CAS No. 1428-67-7, purity > 99%) were purchased from Tocris Bioscience (Bristol, UK). ICI 182,780 (ICI, CAS No. 129453-61-8, purity > 98%) was obtained from Sigma (St. Louis, MO, USA).

The dose of PPT and DPN was chosen by reference to a previous report [13]. We used a dose of ICI that completely inhibited the EE-induced uterotrophic response based on our preliminary study. After dissolving in DMSO, PPT and DPN were diluted 10-fold in sesame oil. ICI was dissolved in ethanol and then stirred into 100 vol of sesame oil. The dosage volume was 5 mL/kg body weight and solutions were prepared just before dosage every time.

2.3. Experiment 1 (observation of estrous cyclicity at a medium dose of PPT and DPN)

Dams were assigned to 3 groups (4–5 dams/group) before delivery. All of the pups in each group received a single subcutaneous injection of vehicle (10%DMSO/sesame oil), 1000 μg/kg of PPT or DPN within 24 h of birth. Litters were culled randomly to preserve 10 pups, with a female predominance on postnatal day (PND) 4. On PND21, the offspring were weaned, and female rats were housed 3–4 per cage (control group, n = 24; PPT group, n = 27; DPN group, n = 29). After PND 26, we checked vaginal openings daily and measured body weight at the day that vaginal opening was confirmed. After that, all animals were assessed for estrous cyclicity by vaginal smear for 5 consecutive days every week from 7 to 21 weeks of age (except 8 weeks of age). The analysis of the cycle pattern was based on 5-day observation. Regular 4- or 5-day cycles were defined as normal cycles, and other patterns were judged to be abnormal. In particular, the animals showing proestrus and estrus continuously for 5 days were designated as persistent estrus. Observations regarding clinical signs, body weight, and mortality were made throughout the experimental period.

2.4. Experiment 2 (observation of estrous cyclicity at high dose of PPT and DPN)

Thirteen and fourteen dams normally delivered pups within two days. Pups born on the same day were randomized within 24 h of birth, and 8 pups per dam were cross-fostered with a female predominance to minimize litter effects. Dams were assigned to 4 groups (6–7 dams/group). Immediately after that, all pups received a single subcutaneous injection of vehicle (10%DMSO/sesame oil), 10,000 μg/kg of PPT or DPN. The pups in the fourth group were injected with 10,000 μg/kg of both PPT and DPN at separate sites on the back (PPT + DPN group). On PND 21, the offspring were weaned, and 3 female rats were housed per cage (control, n = 36; PPT group, n = 32; DPN group, n = 36; PPT + DPN group, n = 36). From PND 24, we checked vaginal openings every day, and measured body weight on the day that vaginal opening was confirmed. After that, all animals were observed for estrous cyclicity at least every other week from 7 to 25 weeks of age in the same manner as Exp. 1.

At 10 weeks of age, 8 randomly selected rats per group showing normal estrous cycles were autopsied on proestrus at 16:00–17:00 (near the peak time of LH surge in our laboratory). Five animals per group were decapitated, and blood samples were collected for hormone assays. Then, the brain, pituitary, ovaries, uteri, vagina and mammary glands were removed, and weights of the ovaries and uteri were recorded. We excluded 3 animals per group that underwent transcardial perfusion for blood and hypothalamic sampling and measurement of organ weights. Except for the brain, tissues were fixed in 10% neutral buffered formalin, and routinely processed and sectioned for hematoxylin and eosin (H&E) staining.

After removal of the brain from the skull, the hypothalamus was dissected out as described in a previous report [9]. A horizontal cut about 2 mm in depth was made with the following boundaries: 1 mm anteriorly from the optic chiasm, the posterior border of the mammillary bodies, and the hypothalamic fissures. Dissected hypothalami were macroscopically divided using the optic chiasm as a boundary into the anterior and posterior hypothalamus, each containing the AVPV and ARC. We had previously confirmed that the expression of *Kiss1* mRNA in the anterior and posterior hypothalamus was equivalent to that in the AVPV and ARC, respectively [9]. Hypothalamic samples were snap frozen in liquid nitrogen and then stored at −80 °C until processing for RNA analysis.