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The impact of neonatal exposure to 17alpha-ethynylestradiol on the development of kisspeptin neurons in female rats

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ABSTRACT

Neonatal exposure to 17alpha-ethynylestradiol (EE) at relatively low doses leads to delayed effects characterized by the early onset of age-related anovulation. Kisspeptin neurons in the anteroventral periventricular nucleus (AVPV), located at the anterior hypothalamus, are proposed to play key roles in appearance of these delayed effects after maturation. To understand the initial changes, we investigated Kiss1 mRNA expression in the anterior and posterior hypothalamus before weaning in female rats that received neonatal exposure to EE at various doses (0.002–2000 μg/kg). The level of Kiss1 mRNA in the anterior hypothalamus was decreased from 0.002 μg/kg which did not induce delayed effects. In the posterior hypothalamus, Kiss1 mRNA expression did not differ among the groups except 2000 μg/kg group. These results suggest that neonatal exposure to EE affects the development of kisspeptin neurons and kisspeptin neurons in the AVPV are highly susceptible to neonatal EE treatment.
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1. Introduction

Exposure to chemicals with estrogenic activity during the critical time for brain sex differentiation (late embryonic to early postnatal stages in rodents) is known to cause irreversible reproductive deficits [1,2]. At a high dose, defeminization effects characterized by masculinized sexual behavior, lower gonadotropin levels during puberty, malformation of the reproductive tract, and cessation of cyclic ovulation occurs during the pre- or peri-pubertal periods [3,4]. In cases of low dose exposure, increased carcinogenic risk and impaired reproductive function can be apparent later in life in rodents as well as in humans, even though normal development occurs through maturation [5–7]. These are regarded as delayed effects, as distinguished from defeminization which occurs earlier. For chemical risk assessment, delayed effects have become a serious issue as they might be overlooked in existing reproductive toxicity or developmental toxicity studies in accordance with current authorized guidelines which only require limited observation periods.

We have previously investigated estrous cyclicity in rats that received a single injection of 17α-ethynylestradiol (EE) at dose levels of 0.02–200 μg/kg during the neonatal period [8]. In this study, although the vaginal opening was not affected, early onset of age-related anovulation was induced in a dose-dependent fashion after sexual maturation at 0.2 μg/kg of EE or more, and was considered a delayed effect. Although estrous cyclicity was regarded as a very useful indicator of delayed toxic effects on the female reproductive tract, which clearly demonstrated age- and dose-dependent effects, it takes a protracted time to detect the effects caused by neonatal exposure to estrogenic compounds [8]. Thus, toxicologic indicators applicable to early detection of delayed adverse effects are required for risk assessment of offspring toxicity.

From these view point, we have examined the changes occurring prior to abnormal estrous cycle, to find early indicators for subsequent delayed effects. As a result, we found that decreased expression of Kiss1 mRNA (encoding kisspeptin) in the anteroventral periventricular nucleus (AVPV) as well as concurrent depression of LH surges preceded the onset of abnormal estrous cycling [9]. In the arcuate nucleus (ARC), however, Kiss1 expression was not changed [9]. The AVPV is involved in control of the female estrous cycle [10], and kisspeptin neurons in the AVPV are presumed to be key players in the onset of these delayed effects. Significant reductions in Kiss1 mRNA expression in the hypothala-

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mus (including both the AVPV and ARC) were observed in rats that received neonatal injections of EE at postnatal day (PND) 14, even at the low dose of 0.02 μg/kg which was not sufficient to induce delayed effects [11]. Therefore, it is possible that neonatal exposure to EE might have an impact on kisspeptin neurons from very low dose before weaning. Given that the expression pattern of Kiss1 during the postnatal period has been reported to differ between the AVPV and ARC [12], region-specific analysis of Kiss1 during the developmental period would provide valuable insight into this process.

In this study, to clarify the initial changes leading to the delayed effects induced by neonatal exposure to EE, we investigated Kiss1 mRNA expression in the anterior and posterior hypothalamus before weaning, and examined the potential of Kiss1 as an early indicator for toxicological evaluation of delayed effects.

2. Materials and methods

2.1. Animals and chemicals

A total of 26 pregnant Wistar Hannover GALAS rats were obtained from CLEA Japan, Inc. (Tokyo, Japan) at gestational day 14 ($n = 9$) and gestational day 15 ($n = 17$). The rats 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. After delivery, 24 litters were used for the experiment, excluding 2 dams in which the timing of delivery was too delayed for dosing. The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

EE was purchased from Sigma (CAS No. 57-63-6; St. Louis, MO, USA) with purity > 98%. EE was stirred into a small amount of sesame oil overnight then used after dilution. EE was selected because of its rapid excretion and lower binding affinity for α-fetoprotein in neonatal blood. We previously confirmed that EE injected into neonatal rats was distributed to the brain and mostly excreted within 24 h, indicating that exposure time to EE is limited to several hours on PND0-1 [8].

2.2. Experimental design

To lessen the genetic difference between litters, pups born on the same day were collected and randomized within 24 h after birth. Then, 8 pups per dam (with a female predominance) were allocated to foster dams. Dams were assigned to 6 groups (4 dams/group), and all pups received a single subcutaneous injection of EE. The doses of EE were set based on our previous study [8] as follows: doses which did not induce delayed effects (no-effect level, 0.002 and 0.02 μg/kg body weight), doses which did induce delayed effects (delayed effect level, 0.2 and 20 μg/kg body weight) and a dose that leads to defeminization (2000 μg/kg body weight). The pups of the control group were injected with sesame oil (5 mL/kg body weight) as a vehicle.

On PND12, 14 and 21, 5 female pups per group were autopsied after measurement of body weight. The age at autopsy was determined based on the timing of follicle-stimulating hormone (FSH) secretion and Kiss1 mRNA expression during postnatal stages [11,13,14]. The animals were decapitated, and blood samples were collected for hormone assays. At PND14 and 21, the pituitary, ovaries, uterus, vagina and mammary glands were removed and fixed in 10% neutral buffered formalin. The weights of the ovaries and uteri were measured after fixation. These tissues were routinely processed and sectioned for hematoxylin and eosin (HE) staining. The intact uterine horns were cut into cross-section at 3 mm intervals. To elucidate the development of uterine glands, the number of uterine glands located away from the endometrium was counted, and the number of uterine glands per section per animal was calculated by dividing by the number of sections analyzed.

The brains were removed from the skulls, and the hypothalami were 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 immediately removed upon decapitation at PND14 and 21, and frozen in liquid nitrogen, then stored at −80 °C until processing for RNA analysis. The hypothalami from male rats of the control group were also collected at the same time for reference.

2.3. Hormone assays

Serum samples obtained after decapitation were stored at −80 °C until ready for assessment. Serum concentrations of FSH and luteinizing hormone (LH) were determined using double-antibody radioimmunoassays and ^{125}I-labeled radio-ligands. National Digestive and Kidney Disease (NIDDK) radioimmunoassay kits were used (NIAMDD, NIH, Bethesda, MD, USA) as described previously [15].

2.4. Real-time RT-PCR for Kiss1

Total RNA was isolated from the anterior and posterior hypothalamus using ISOGEN (NIPPON GENE Co., Ltd., Tokyo), and reverse transcription reactions were performed using 2 μg of total RNA with High Capacity Reverse Transcription kits (Applied Biosystems Japan Ltd., Tokyo, Japan). Following the manufacturer's instructions, real-time PCR was performed with an ABI Prism 7900HT (Applied Biosystems Japan Ltd.). Taqman® Gene Expression Assays (Applied Biosystems Japan Ltd.) were used to measure mRNA levels of Kiss1 metastasis-suppressor (Kiss1, Rn00710914_ml). The expression level of Kiss1 gene was calculated using the relative standard curve method and normalized against endogenous GAPDH (TaqMan Rodent GAPDH Control Reagent, Applied Biosystems Japan Ltd.). The expression level in the anterior hypothalamus of the control group at PND14 was expressed as 1, and relative levels were calculated for the other groups.

2.5. Statistical analysis

Following Bartlett's test, variance in data for body and organ weights, the number of uterine glands, hormone assays and real-time RT-PCR were compared to the control group by one-way analysis of variance or the Kruskal–Wallis test. When statistically significant differences were detected, Dunnett's multiple comparison test was employed for comparison between the control group and the treatment groups. The mRNA expression levels in males were compared using Student's *t*-test following a test for equal variance.

3. Results

3.1. Mortality and body growth

One animal in each of the control and 2000 μg/kg group died before PND7. No other deaths or abnormal clinical signs were found

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