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Prenatal hyperandrogenic environment induced autistic-like behavior in rat offspring

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HIGHLIGHTS

- Letrozole at 1 μg/kg/day produced a hyperandrogenic environment for the fetus.
- Prenatal hyperandrogenism exposure induced less ultrasonic vocalizations in rat pups.
- Prenatal hyperandrogenism exposure caused impaired social interaction in female rats.
- Heterosexual interaction was negatively correlated with maternal TSTO in female rats.

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ABSTRACT

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by persistent impairment in social communication and social interaction. Recent studies revealed that environmental factors, especially the intrauterine developmental environment, played important roles in the development of ASD. It is hypothesized that maternal hyperandrogenism during pregnancy may increase the susceptibility of the fetus to ASD. In the present study, pregnant rats were treated with a low dose of letrozole (1 μg/kg/day) in an attempt to produce a hyperandrogenic intrauterine environment for the developing fetus. Results showed that rat pups prenatally exposed to hyperandrogenic intrauterine environment emitted less number of ultrasonic vocalizations when isolated from their dams and littermates. Additionally, the female rats in the treatment group spent less time in social interaction in adolescence and exhibited impaired heterosexual interaction in adult. Moreover, the duration of social interaction and heterosexual interaction of the female offspring were negatively correlated with maternal serum testosterone levels during pregnancy. These results suggest that prenatal exposure to hyperandrogenic intrauterine environment could induce autistic-like behavior in female rats and maternal hyperandrogenism during pregnancy should be considered as a potential risk factor for the etiology of ASD.

1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by persistent impairment in social communication and social interaction, plus restricted and repetitive patterns of behavior, with the symptoms manifested in the early postnatal period [1]. The prevalence of ASD has increased considerably over the past decade with no specific neurophysiological or genetic marker identified yet [2–5]. Results of recent studies suggest that instead of a single factor causative effect, the combined effects and interplay between genetic heritability and environmental risk factors may be more important in the etiology of ASD [6,7].

The hypothesis that dysregulation of sex-steroid hormones may be involved in the onset and development of ASD has been supported by several lines of evidence [8–10]. It has been suggested that children born from hyperandrogenic women may express more autistic traits and might be under a higher risks for autism [11]. In one of our previous studies, we have also shown that mothers of autistic children had higher levels of testosterone in plasma [12]. It is well established that sex hormones play important roles in fetal brain development [13–15]. Since during pregnancy the maternal–fetal unit can be considered as a

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highly integrated endocrine co-system [16], it is possible that the intrauterine environment may greatly impact the susceptibility of the fetus to neurodevelopmental disorders, such as ASD.

The successful development of an animal model of ASD may greatly facilitate the mechanistic study of the disorder. In mammals, androgens are converted into estrogens by aromatase [17]. Letrozole is a potent aromatase inhibitor that attenuates estrogen biosynthesis and causes androgen to be accumulated in the body [18]. In the present study, pregnant rats were treated with a low dose of letrozole (1 μg/kg/day) for 3 consecutive weeks in an attempt to produce a hyperandrogenic intrauterine environment for the developing fetus. The objective of this study was to assess the effect of maternal hyperandrogenism during pregnancy on the behavior of their offspring in rats. Findings in this study may provide useful information on the etiology and/or prevention of ASD.

2. Methods

2.1. Animals

Adult Sprague–Dawley rats used in this study were obtained from the Department of Experimental Animal Sciences, Peking University Health Science Center. The animals were housed at 24 ± 1 °C and a 12 h light/dark cycle (light on at 7:00 AM) with food and water ad libitum. The study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, USA, and all procedures were approved by the Animal Use Committee of Peking University Health Science Center.

In order to produce timed mating, virgin female rats (weighing 220–240 g) were individually housed in cages and mated overnight with adult males. Detection of the vaginal plug was taken as evidence of mating and the day in which the vaginal plug was found was counted as gestation day 0 (G0). Pregnant females were randomly assigned into two groups as described below.

2.2. Experimental groups

Letrozole (Femara, 2.5 mg tablet, Novartis Pharma Stein AG, Switzerland) was dissolved in 20% ethanol in sesame oil, as described by Moradi-Azani et al. [19], at a concentration of 1 μg/ml. Pregnant rats in the treatment group received subcutaneous injection of letrozole at a dose of 1 μg/kg once daily for 3 weeks (from G0 to G20), while control rats received only the vehicle with the same volume at the same time.

2.3. Cesarean section from decapitated, unanesthetized dams

Pregnant rats in both groups were decapitated on G21 and blood samples were collected for biochemical analysis. As described by Vaillancourt et al. [20], the abdominal incision was made immediately after decapitation and pups were quickly delivered from the isolated uterus. The operation was taken on a heating pad (37 °C) to maintain the pups' body temperature. Within 0.5 h after birth, all pups were fostered to a dam which had given birth within the past 24 h. Litter size was restricted to 10 at maximum (5 males, 5 females). Pups were weaned at postnatal day 21(PND21) and siblings of the same sex were then housed together under standard conditions.

2.4. Measurements of serum testosterone and estradiol concentrations

Testosterone and estradiol levels in the serum samples of the pregnant rats were determined by radioimmunoassay (RIA) (North Institute of Biological Technology, Beijing, China). The sensitivities of assay for testosterone and estradiol were 20 pg/ml and 5 pg/ml, respectively, with no or very little cross-reactivity with other steroid hormones. The *r*-values of standard curves were greater than 0.99 for both assays. Analyses were conducted blindly in respect to which group the samples belonged to. Two pregnant rats were excluded from this study because of their abnormal hormone levels as revealed by the RIA tests.

2.5. Measurement of isolation-induced ultrasonic vocalizations

Isolation-induced ultrasonic vocalizations (USVs) were tested on PND7 between 13:00 and 16:00. Briefly, the dam was removed from the home cage and the pups were then gently transported in random order to the test chamber in a separate room on a heating pad (37 °C). The vocalizations were recorded for 5 min for each pup.

The emitted USV was collected by a condenser microphone (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) suspended approximately 25 cm above the base of the chamber, with the amplifier (AUSG-116H, Avisoft Bioacoustics, Berlin, Germany) set at a sampling rate of 250 kHz. The recorded files were transferred to Avisoft SASLab Pro (Version 4.52) for fast Fourier transform (512 FFT-length, 100% frame size, Hamming window, 50% time window overlap), with a 125 kHz low-pass filter [21]. The classification algorithm of USV was set according to Li et al. [22]: "Long" USVs were defined as waveforms that were greater than 50 ms long, with a frequency deviation of less than 3 kHz; "Short" USVs were any waveforms that were less than 50 ms long; "Frequency-modulated" USVs were greater than 50 ms long and had a frequency deviation of greater than 3 kHz.

2.6. Three-chamber sociability test

The test was designed based on previous studies [23,24] with slight modifications. The apparatus consisted of three Plexiglas chambers (40 cm × 34 cm × 24 cm) with the side chambers each connected to the middle chamber by a corridor (10 cm × 10 cm × 15 cm). At the beginning of the test, the rat was placed into the middle chamber and allowed the exploration of the three chambers for 5 min. Then a model rat, locked in a small cage, was placed in one of the side chambers, and an empty cage of the same size and design was placed in the other side chamber. The testing rat was allowed to freely explore the apparatus and interact with the model rat for 10 min. The duration of time spent by the testing rat in the chamber containing the model rat was interpreted as social interaction time. The model rat was about the same age as the testing rat but it was never met before. All behavioral tests were carried out during the dark period of the light cycle under dim red illumination.

2.7. Detection of the estrous cycle phases of female rats

The estrous cycle phases of the adult female rats were determined based on the cell types observed in the vaginal smear [25]. Heterosexual social interactions were tested when the female rats were in estrous phases.

2.8. Real-time quantitative PCR

Brain tissues for quantitative PCR were obtained by using micropunch technique [26]. Briefly, rats were euthanized by decapitation and the brains were quickly removed and cut into 450 μm coronal sections. As described by previous studies [27,28], bilateral micropunches were taken from the following regions: the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus and the amygdala. All coordinates were based on the rat brain atlas [29].

Total RNA was extracted from the brain tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Trace DNA contamination was removed by DNase digestion (Promega, Madison, WI) and cDNA was synthesized from 1 μg DNase-treated total RNA using PrimeScript RT-PCR kit (TaKaRa, Dalian, China) on a Carefree Fast Gradient PCR Cycler (Coyote