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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

Bioscience Company). Expression levels of the target genes (Oxt, Avp, Oxtr, Avpr1a) and the endogenous control gene (Beta-actin) was estimated by real-time PCR using TaqMan® Gene Expression Assays (Assay ID: Oxt — Rn00564446_g1, Oxtr — Rn00563503_m1, Avp — Rn00690189_g1, Avpr1a — Rn00583910_m1, Beta-actin — Rn00667869_m1) on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) under standard amplification conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. For each sample, duplicate reactions were performed in a volume of 20 μl. Data were transformed using the ΔΔCT method with Beta-actin as the reference gene, and further normalized to the control samples in the vehicle group for comparison.

### 2.9. Statistics

Statistical analyses were performed with Statistical Package for the Social Science version 19.0 (SPSS Inc., Chicago, Illinois) and GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). Data were presented as mean ± SEM and comparisons between groups were performed using Student's *t* test within the same-sex animals, unless otherwise specified. The Pearson correlation analysis was used to assess the association between maternal testosterone levels and their offspring's behavior. For all tests, a value of $p < 0.05$ (two-tailed) was considered statistically significant.

## 3. Results

### 3.1. Serum testosterone and estradiol levels in pregnant rats

Blood samples of the pregnant rats were collected on G21 before the Cesarean sections. Serum testosterone levels of the pregnant rats were higher in the treatment group (mean ± SEM: 40.07 ± 1.462 ng/dl) as compared to the vehicle group (32.96 ± 1.455 ng/dl, $p < 0.05$). No significant difference was found on serum estradiol levels between the two groups (Fig. 1).

### 3.2. Ultrasonic vocalizations of isolated rat pups

The isolation-induced ultrasonic vocalizations (USVs) of rat pups were tested on PND7. The total number of USV emitted by the rat pups was less in the letrozole treatment group (274.2 ± 59.94) as compared to the vehicle group (431.0 ± 39.16, $p < 0.05$). For the three different types of USVs, only the "Frequency-modulated" vocalizations showed statistical difference between groups (Letrozole: 201.5 ± 53.03 vs. Vehicle: 340.8 ± 37.08, $p < 0.05$) (Fig. 2).

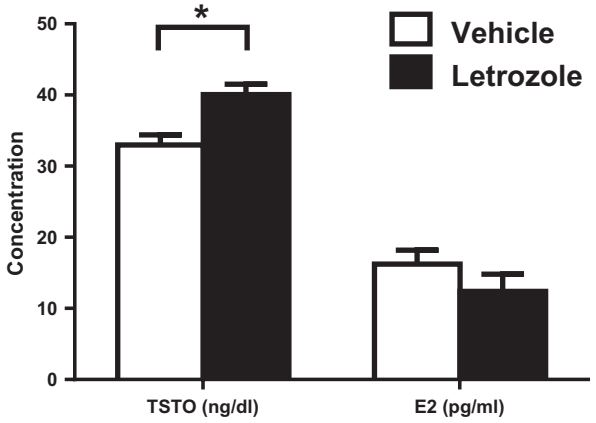


**Fig. 1.** Higher levels of serum testosterone were observed in pregnant rats following letrozole treatment. Pregnant rats in the treatment group (n = 3) had higher levels of serum testosterone as compared to the vehicle group (n = 4), while there was no significant difference in serum estradiol levels between the two groups. TSTO: testosterone; E2: estradiol. *$p < 0.05$.

### 3.3. Three-chamber sociability test

The sociability of the offspring was tested on PND45–55 (adolescence) and PND85–95 (adult), respectively, with the same-sex animals used as the model rats. During the adolescence period, the female offspring in the treatment group spent less time in social interaction (214.4 ± 42.89 s) as compared to the vehicle group (339.6 ± 13.44 s, $p < 0.01$), while no significant difference between groups was found for the male offspring (Fig. 3A). There was no difference on social interaction time between the two groups for the adult offspring in both sexes (Fig. 3B).

The heterosexual social interactions of the adult female offspring were also tested on PND100–110 when they were in estrous phases. As can be expected, the female offspring in control group showed greater preference in heterosexual social interactions towards the male model rats (349.2 ± 15.14 s vs. 148.2 ± 9.817 s, $p < 0.001$). Interestingly, the female offspring lost their interest towards male model rats following treatment with letrozole (349.2 ± 15.14 s vs. 246.6 ± 38.74 s, $p < 0.01$) (Fig. 4).

### 3.4. Relationships between maternal serum testosterone levels and their offspring's behavior

The Pearson correlation analysis was used to assess the relationship between serum testosterone levels in pregnant rats and their offspring's behavior. Maternal serum testosterone levels showed significant negative correlations with their female offspring's social behavior in adolescence ($r = -0.501$, $p < 0.01$) (Fig. 5A) but not in adult (Fig. 5B). Moreover, the significant negative correlations were also found between maternal testosterone levels and their female offspring's heterosexual social interactions ($r = -0.498$, $p < 0.01$) (Fig. 5C). In contrast, none of these correlations were significant when male offspring were tested (data not shown).

### 3.5. Expression of OXT, AVP and their receptors

For the female offspring (decapitated in estrous phases), the mRNA levels of OXT and AVP were estimated in the PVN and SON of the hypothalamus. In addition, the mRNA levels of OXT and AVP receptor genes (OXTR and AVPR1a) were estimated in amygdala. No significant difference between the control and letrozole treatment groups was found in the expressions of these genes (Fig. 6A). In addition, the mRNA levels of OXT and AVP showed significant correlation with each other in the PVN ($p < 0.05$) (Fig. 6B) and SON ($p < 0.001$) (Fig. 6C), respectively.

### 3.6. Relationships between gene expressions and behavior in the female offspring

As revealed by the Pearson correlation analysis, the female offspring's level of social interactions showed a positive relationship with the mRNA level of AVP in the PVN ($p < 0.05$) (Fig. 7A). There were also negative correlations between the level of female offspring's social interaction and their AVPR1a expression level in the amygdala ($p < 0.05$) (Fig. 7B). No other correlations between levels of gene expression and behavior in the female offspring were observed (Table 1).

## 4. Discussion

As a neurodevelopmental disorder, ASD has received more attention around the world because of its increasing prevalence over the past decade [2–4]. Recent studies revealed that environmental factors, especially the intrauterine developmental environment, played important

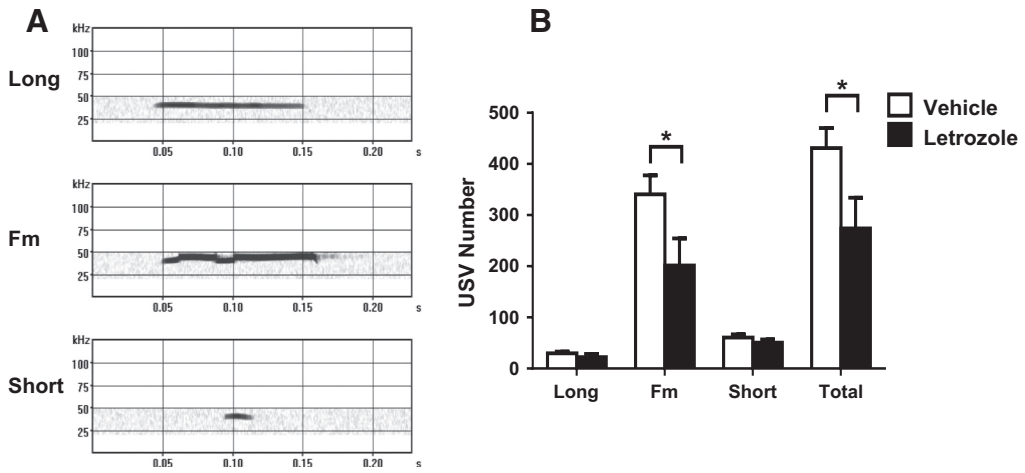


**Fig. 2.** Effect of letrozole treatment on the ultrasonic vocalization of isolated rat pups. An illustration of the three types of USVs (A). Rat pups in the treatment group (n = 20) emitted less "F m" and total number of USVs than those in the vehicle group (n = 38) (B). F m: Frequency-modulated; USV: ultrasonic vocalization. $^{*}p < 0.05$.

roles in the development of ASD [11,30,31]. Clinical evidence also suggested that dysregulation of brain development in ASD may begin at prenatal developmental stages [32]. The development of related animal models may provide important information for the prevention and treatment of this disorder. In the present study, we focused on the abnormal intrauterine environment of the fetus and demonstrated that exposure to low dose letrozole prenatally could produce behavioral changes in rats, especially in the females.

It is well established that sex hormones play important and diverse roles in the development of the central nerve system (CNS) [13,15,33]. Androgens and estrogens regulate the expression level of many genes including OXT and AVP, which have been found to be very important modulators of social behaviors in mammals [34–37]. As a potent aromatase inhibitor, letrozole is widely used for the treatment of hormonally-responsive breast cancer in postmenopausal women [18]. Nowadays, the off-label uses of letrozole as an ovulation-inducing agent in some countries draw attentions of many researchers since little information is available about the potential developmental hazard posed by the agent [38,39]. In the present study, we used letrozole at a relatively low dose to produce hyperandrogenemia in pregnant rats. The dose was chosen based on our preliminary study, which demonstrated that the 1 μg/kg dose significantly elevated serum testosterone level in pregnant rats with relatively minute embryonic toxicity. Letrozole was administrated to the pregnant rats for the whole duration of gestation (from G0 to G20) to create a hyperandrogenic intrauterine environment for the developing fetus. Serum testosterone levels in the pregnant rats increased by 21.6% in the treatment group, which is within the physiological range.

Ultrasonic vocalizations emitted by the rats provide substantial information on their physical and psychological state [40–42]. Rat pups typically exhibit vocalizations around 40 kHz range in response to distress situations, such as separation from their mothers and littermates [42,43]. These vocalizations can be classified into "Long", "Short" and "Frequency-modulated" types [22]. It has been suggested that the frequency-modulated vocalizations may better reflect the pup's emotional state since the largest change occurs in this type of USV in response to isolation and reunion with the dams [22,44]. It is also believed that frequency-modulated vocalizations can be detected more easily by the dams than the other two types [44]. In the present study, the rat pups' isolation-induced USVs were examined on PND7 and we found that pups in the letrozole treatment group emitted less USV after being isolated from their mothers and littermates. Moreover, when the three types of vocalizations were analyzed separately, only the frequency-modulated vocalizations were significantly reduced in letrozole group. It seemed that the pups in the treatment group cared less about their surrounding conditions, including the olfactory and tactile stimuli from their littermates or the licking and care from the dams. Our data suggest that rat pups grow up in a prenatal environment

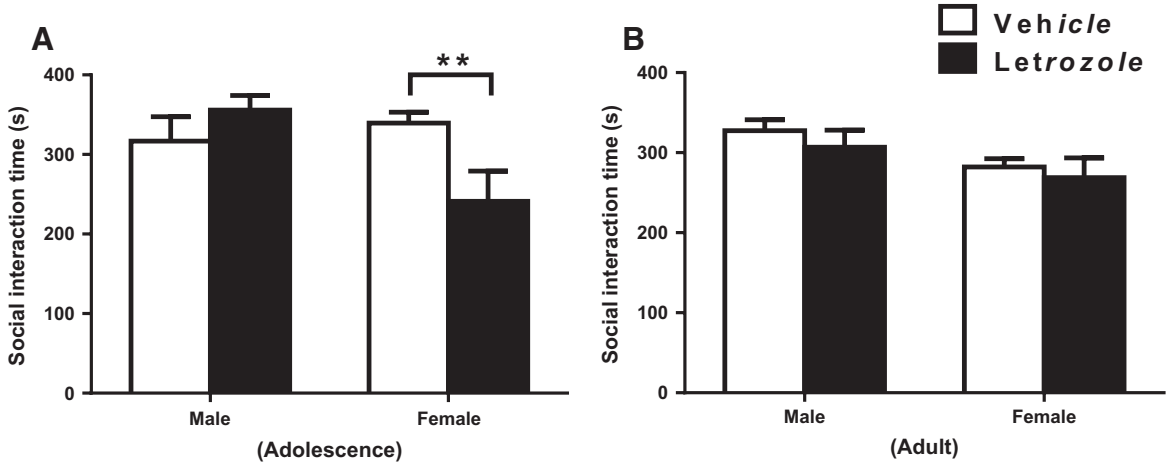


**Fig. 3.** Effect of letrozole treatment on social interactions in male and female offspring. The adolescence female offspring in the treatment group (n = 9) spent less time in social interaction compared to those in the vehicle group (n = 18) (A). In contrast, no difference between the two groups was seen in male offspring and the adult female offspring (A and B). $^{**}p < 0.01$.

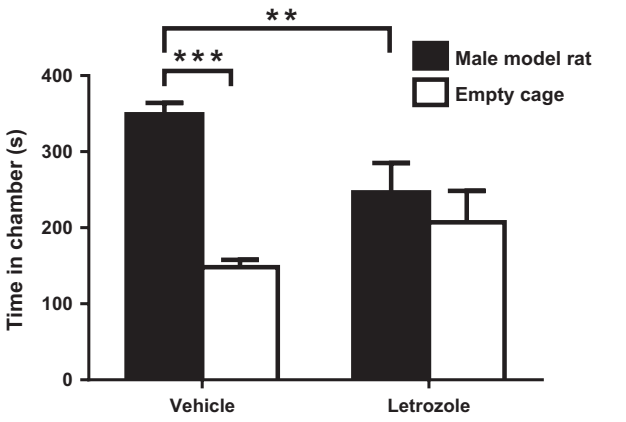


**Fig. 4.** Effect of letrozole treatment on heterosexual social interactions in the female offspring. Females in the vehicle group exhibited greater preference to the heterosexual interactions, manifested as spending much more time in the chamber containing male model rats than in the chamber containing the empty cages. Females in the letrozole treatment group (n = 9) lost their interest towards male model rats compared with rats in control group (n = 19). $^{**}p < 0.01$, $^{***}p < 0.001$.

with a high level of testosterone that may develop a behavior pattern with less sensitivity to their dams and littermates during isolation. This seems similar to the situation in ASD children, showing less attachment with their mothers and siblings [45].

The results from clinical observation suggest that there are sex differences in the etiology and presentation of ASD [46,47]. Social interaction impairment is one of the most important symptoms of ASD [1]. The results of three-chamber sociability test indicated that prenatal exposure to letrozole induced different patterns of social behavior changes for the male and female rats. In contrast to male rats which showed no response to letrozole exposure, female rats in the treatment group showed decreased sociability in adolescence. The greater impact of prenatal letrozole exposure on the female offspring seen in the present study is consistent with the results of a previous clinical study, where daughters of patients with polycystic ovary syndrome (PCOS) were found to be at higher risks for pervasive developmental disorders [11]. These results indicated that females are more susceptible to the hyperandrogenic intrauterine environment. During the prenatal development of the male fetus, the androgen in the chemical environment is derived from both maternal and fetal sources [48]. It is believed that higher maternal testosterone levels may reduce testicular androgen production prenatally in the male fetus to minimize the androgen elevation [11]. However, such counter-regulatory mechanism is not available in the female fetus. Thus the maternal hyperandrogenic effect would be more obvious and would have a greater impact on the development of female offspring [11]. This hypothesis can be used to interpret our data which demonstrated that a significant correlation between maternal testosterone levels and the change in their offspring's social behavior was only found in adolescence female offspring, but not in the male offspring.

It seemed that the effect of prenatal exposure to letrozole on the female offspring's sociability would not last a whole lifetime, since the abnormal behaviors observed in the three-chamber test manifested during adolescence disappeared in adulthood. We are not sure whether this is because of the compounding effect of the sex hormones in the adult rats, since the sex hormones produced by the adult females themselves could also modulate the expressions of many genes in the brain, thus influencing their social interactions [13,34,49].

The heterosexual behavior in adult female rats changes greatly during the phases of the estrous cycle [50]. In the present study, the estrous cycles of the females were determined by cytological observation on vaginal smears [25], and the heterosexual social behavior was tested only in their estrous phases. Females from the letrozole treatment group exhibited a significant impairment in the heterosexual

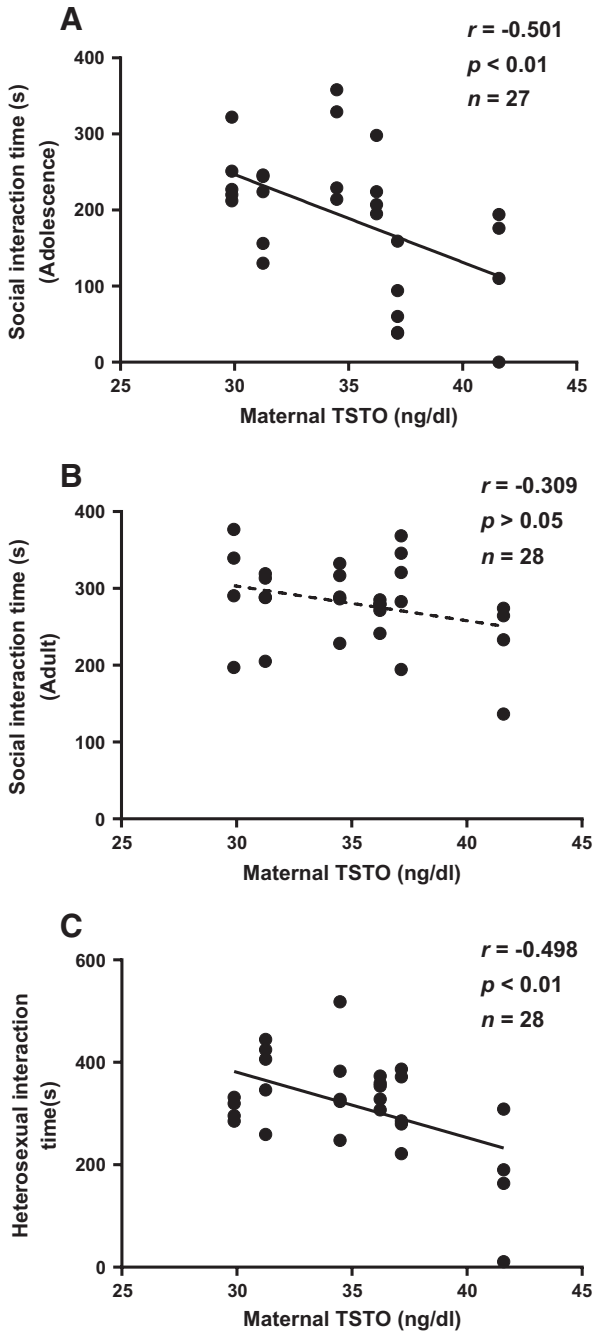


**Fig. 5.** Scatter-plot between maternal serum testosterone levels and their female offspring's social and heterosexual interactions. The testosterone levels in pregnant rats showed negative correlations with their female offspring's social behavior in adolescence (A) but not in adult (B). Higher levels of maternal serum testosterone were also associated with lower heterosexual interactions in adult female offspring (C). TSTO: testosterone.

interactions, even the natural preference to sexual interaction was not found. Furthermore, the heterosexual interactions in adult females were negatively correlated with their maternal serum testosterone levels. In a previous study, adult male rats were also found to be impaired in sexual behavior after prenatal exposure to 1 mg/kg/day letrozole on G21 and G22 [51]. These results suggest that drugs or situations that disrupt aromatase activity and cause imbalance in sex hormones during pregnancy could have long-standing impact on their offspring's brain development and sexual behavior. It further confirmed the presumption that females are more susceptible to the hyperandrogenic intrauterine environment since no such effect on

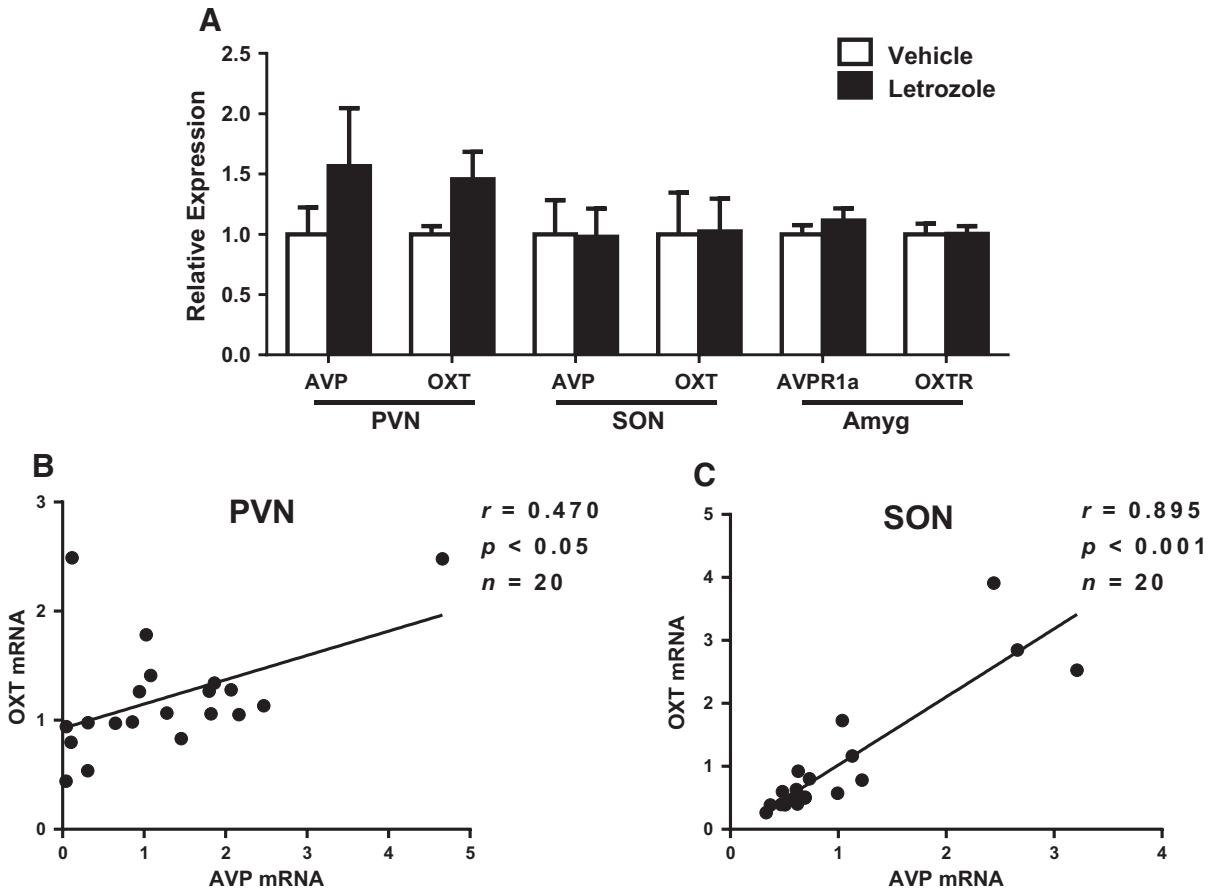


**Fig. 6.** The mRNA levels of OXT, AVP and their receptors in the brain nuclei in female offspring. Relative abundance of mRNAs in females in the letrozole treatment group (n = 9) was represented by fold increase over the mean expression level in control females (n = 11). None of the differences in expression levels of these genes have achieved statistically significant level between the two groups (A). Positive correlations were found between OXT and AVP mRNA levels in the PVN (B) and SON (C) of the hypothalamus. OXT: oxytocin; AVP: Arg-vasopressin; OXTR: oxytocin receptor; AVPR1a: Arg-vasopressin receptor 1a; PVN: paraventricular nucleus; SON: supraoptic nucleus; Amyg: amygdala.

heterosexual interactions was found in the males at this low dose of 1 μg/kg/day letrozole in the present study.

One recent study consisting of 415 women with autism spectrum conditions (ASC) and 415 normally developed controls revealed that women with ASC were at higher risks for gender dysphoria, transsexualism and differences in sexual preference [52]. This is somewhat in accordance with our heterosexual interaction results observed in the rats in that female rats with some autistic-like traits also exhibited a significant impairment in heterosexual interactions in adults. In another clinical study, women with ASD were also found to have more masculine traits whereas men with ASD displayed several feminized characteristics [53]. All these results suggest that ASD may constitute a gender defiant disorder, and it reminds us that the endocrine-disrupting chemicals (EDCs), which are abundant in our living environment, need to be evaluated as potential risk factors for the development of ASD and other neurodevelopmental disorders.

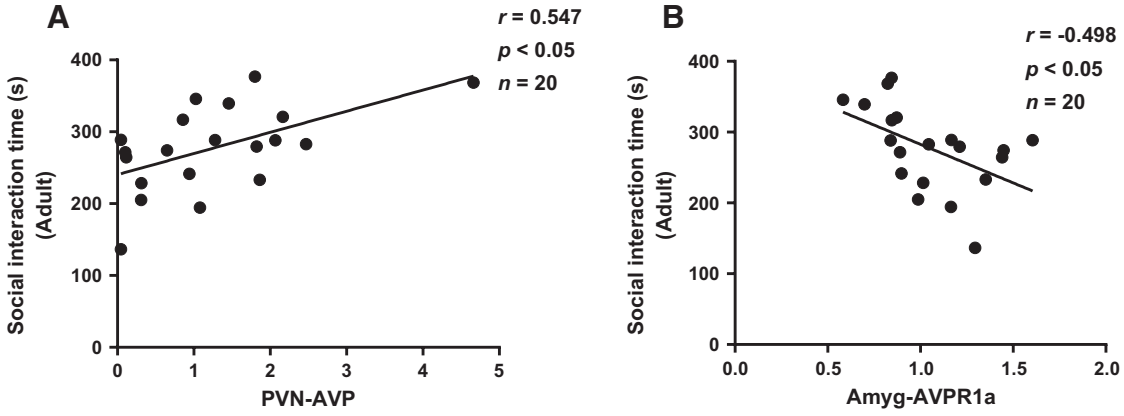


**Fig. 7.** Scatter-plot showing correlation between AVP or AVPR1a mRNA levels and duration of the female adult offspring's social interactions. Increased social interaction duration in the adult females was associated with higher mRNA levels of AVP in the PVN (A) and lower mRNA levels of AVPR1a in the amygdala (B). AVP: Arg-vasopressin; AVPR1a: Arg-vasopressin receptor 1a; PVN: paraventricular nucleus; Amyg: amygdala.

**Table 1**
Correlation analysis between levels of gene expression and social interactions in the female offspring.

| | | PVN | | SON | | Amyg | |
|---|---|---|---|---|---|---|---|
| | | OXT | AVP | OXT | AVP | OXTR | AVPR1a |
| Social interactions | *r* | 0.411 | **0.547*** | 0.045 | −0.007 | −0.343 | **−0.498*** |
| | *p* | 0.072 | **0.013** | 0.851 | 0.977 | 0.138 | **0.025** |
| Heterosexual interactions | *r* | −0.174 | 0.149 | 0.329 | 0.406 | 0.243 | −0.316 |
| | *p* | 0.463 | 0.530 | 0.157 | 0.075 | 0.303 | 0.174 |

Abbreviations: OXT: oxytocin; AVP: Arg-vasopressin; OXTR: oxytocin receptor; AVPR1a: Arg-vasopressin receptor 1a; PVN: paraventricular nucleus; SON: supraoptic nucleus; and Amyg: amygdala. n = 20.
* $p < 0.05$.

OXT and AVP are two "twin" neuropeptides which are thought to arise from a gene-duplication event through evolution [35,54]. They are highly conserved in structure and are both synthesized in the PVN and SON of the hypothalamus [35]. Plasma levels or changes of OXT and AVP are found to be correlated with each other in the basal physiological or dehydration status [12,55,56]. In the present study, the expression levels of OXT and AVP in the brain nuclei also showed significant correlations. All these results point to a presumption that the synthesis and release of the two neuropeptides may in some way be related. It has been suggested in several studies that the expressions of the two neuropeptides are both regulated by sex hormones [34–36]. One of the potential reasons to interpret why there is no significant difference on OXT or AVP expression levels between groups could be that letrozole was administered prenatally while gene expressions were determined in adulthood, when the females themselves could produce their own sex hormones.

The contribution of OXT and AVP to mammalian social behavior has been studied extensively over the past few decades [35,57,58]. Although no significant difference was found in this study on social interactions in adult females between groups, the gene expression levels of AVP in the PVN did show positive correlation with the duration of their social interactions. This is consistent with previous reports which demonstrated that AVP was important for social behavior in rodents [34,59]. One of the interesting findings in our study was that the expression levels of AVPR1a in the amygdala were negatively correlated with the duration of adult female's social interactions. This result seemed to be contrary to other studies [34,60]. The mechanism underlying this discrepancy remains to be established, and it is not clear whether this is a compensatory decrease of AVPR1a since its ligand (AVP) is more abundant in high sociability females.

Another major concern is that the OXT expression levels did not show significant relationships with the social behaviors as was presumed. One of the reasons for this may be that the expression of OXT is tightly associated with estrogen hormones [34–36], which fluctuate naturally during the estrous cycle in adult females. Although all the female rats were decapitated in the estrous phases, the natural fluctuation of the estradiol level may still be an interference factor which could distort the correlations between OXT and social behavior. In contrast to the homosexual social interactions, the duration of the heterosexual interactions showed no significant correlation with the level of gene expression of these neuropeptides, suggesting that heterosexual interactions are much more complex which are far beyond the major interest of this study.

There are several limitations ought to be mentioned: The restricted and repetitive patterns of behavior are one of the symptoms of ASD. However, the levels of such behavior were not evaluated in this study. Additionally, although letrozole was used at a low dose of 1 μg/kg/day, which corresponds to 2.5% of the recommended daily human dose (2.5 mg) in a person weighing 60 kg (41.7 μg/kg/day), it may still have some side effects on the development of the fetus rather than just inhibit the aromatase activity to produce the hyperandrogenic intrauterine environment. The litters used in this study were relatively small, and the number and sex ratio of the fetus in each pregnant rat could not be controlled due to technical reasons. All these factors may cause some confounding effect on the interpretation of the results.

## 5. Conclusions

Prenatal hyperandrogen exposure could induce less ultrasonic vocalizations in rat pups and impaired behavior pattern of interaction in female rats. Moreover, the duration of social and heterosexual interaction of the female offspring was negatively correlated with maternal serum testosterone levels during pregnancy. These results indicate that maternal hyperandrogenism during pregnancy may serve as a potential risk factor for the development of ASD, hence be useful for the understanding and prevention of this ever expending neurodevelopmental disorder.

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## References

[1] Association AP. Diagnostic and statistical manual of mental disorders. 5th ed. Arlington, VA: American Psychiatric Association; 2013.
[2] Fombonne E. Epidemiology of pervasive developmental disorders. Pediatr Res 2009; 65:591–8.
[3] Autism, Developmental Disabilities Monitoring Network Surveillance Year Principal I, Centers for Disease C, Prevention. Prevalence of autism spectrum disorders — autism and developmental disabilities monitoring network, 14 sites, United States, 2008. MMWR Surveill Summ 2012;61:1–19.
[4] Developmental Disabilities Monitoring Network Surveillance Year Principal I, Centers for Disease C, Prevention. Prevalence of autism spectrum disorder among children aged 8 years — autism and developmental disabilities monitoring network, 11 sites, United States, 2010. MMWR Surveill Summ 2014;63:1–21.
[5] Geschwind DH. Advances in autism. Annu Rev Med 2009;60:367–80.
[6] Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, Torigoe T, et al. Genetic heritability and shared environmental factors among twin pairs with autism. Arch Gen Psychiatry 2011;68:1095–102.
[7] Ronald A, Hoekstra RA. Autism spectrum disorders and autistic traits: a decade of new twin studies. Am J Med Genet B Neuropsychiatr Genet 2011;156B:255–74.
[8] Sarachana T, Xu M, Wu RC, Hu VW. Sex hormones in autism: androgens and estrogens differentially and reciprocally regulate RORA, a novel candidate gene for autism. PLoS One 2011;6:e17116.
[9] Ruta L, Ingudomnukul E, Taylor K, Chakrabarti B, Baron-Cohen S. Increased serum androstenedione in adults with autism spectrum conditions. Psychoneuroendocrinology 2011;36:1154–63.
[10] Chakrabarti B, Dudbridge F, Kent L, Wheelwright S, Hill-Cawthorne G, Allison C, et al. Genes related to sex steroids, neural growth, and social–emotional behavior are associated with autistic traits, empathy, and Asperger syndrome. Autism Res 2009; 2:157–77.
[11] Palomba S, Marotta R, Di Cello A, Russo T, Falbo A, Orio F, et al. Pervasive developmental disorders in children of hyperandrogenic women with polycystic ovary syndrome: a longitudinal case–control study. Clin Endocrinol (Oxf) 2012;77:898–904.
[12] Xu XJ, Shou XJ, Li J, Jia MX, Zhang JS, Guo Y, et al. Mothers of autistic children: lower plasma levels of oxytocin and Arg-vasopressin and a higher level of testosterone. PLoS One 2013;8:e74849.
[13] Keefe DL. Sex hormones and neural mechanisms. Arch Sex Behav 2002;31:401–3.
[14] Bonthuis PJ, Cox KH, Searcy BT, Kumar P, Tobet S, Rissman EF. Of mice and rats: key species variations in the sexual differentiation of brain and behavior. Front Neuroendocrinol 2010;31:341–58.
[15] Phoenix CH, Goy RW, Gerall AA, Young WC. Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. Endocrinology 1959;65:369–82.
[16] Bagasra O, Golkar Z, Garcia M, Rice LN, Pace DG. Role of perfumes in pathogenesis of autism. Med Hypotheses 2013;80:795–803.
[17] Cole PA, Robinson CH. Mechanism and inhibition of cytochrome P-450 aromatase. J Med Chem 1990;33:2933–42.
[18] Haynes BP, Dowsett M, Miller WR, Dixon JM, Bhatnagar AS. The pharmacology of letrozole. J Steroid Biochem Mol Biol 2003;87:35–45.

[19] Moradi-Azani M, Ahmadiani A, Amini H. Increase in formalin-induced tonic pain by 5alpha-reductase and aromatase inhibition in female rats. Pharmacol Biochem Behav 2011;98:62–6.
[20] Vaillancourt C, Berger N, Boksa P. Effects of vaginal birth versus caesarean section birth with general anesthesia on blood gases and brain energy metabolism in neonatal rats. Exp Neurol 1999;160:142–50.
[21] Umeda T, Takashima N, Nakagawa R, Maekawa M, Ikegami S, Yoshikawa T, et al. Evaluation of Pax6 mutant rat as a model for autism. PLoS One 2010;5:e15500.
[22] Li M, He W, Heupel K. Administration of clozapine to a mother rat potentiates pup ultrasonic vocalization in response to separation and re-separation: contrast with haloperidol. Behav Brain Res 2011;222:385–9.
[23] Todeschin AS, Winkelmann-Duarte EC, Jacob MH, Aranda BC, Jacobs S, Fernandes MC, et al. Effects of neonatal handling on social memory, social interaction, and number of oxytocin and vasopressin neurons in rats. Horm Behav 2009;56:93–100.
[24] Bambini-Junior V, Rodrigues L, Behr GA, Moreira JC, Riesgo R, Gottfried C. Animal model of autism induced by prenatal exposure to valproate: behavioral changes and liver parameters. Brain Res 2011;1408:8–16.
[25] Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: some helpful considerations. Braz J Biol 2002;62:609–14.
[26] Palkovits M. Isolated removal of hypothalamic or other brain nuclei of the rat. Brain Res 1973;59:449–50.
[27] Nephew BC, Bridges RS, Lovelock DF, Byrnes EM. Enhanced maternal aggression and associated changes in neuropeptide gene expression in multiparous rats. Behav Neurosci 2009;123:949–57.
[28] Zheng J, Babygirija R, Bulbul M, Cerjak D, Ludwig K, Takahashi T. Hypothalamic oxytocin mediates adaptation mechanism against chronic stress in rats. Am J Physiol Gastrointest Liver Physiol 2010;299:G946–53.
[29] Paxions G, Watson C. The rat brain in stereotaxic coordinates. 3rd ed. San Francisco, CA: Academic Press; 1996.
[30] Auyeung B, Baron-Cohen S, Ashwin E, Knickmeyer R, Taylor K, Hackett G. Fetal testosterone and autistic traits. Br J Psychol 2009;100:1–22.
[31] Baron-Cohen S, Lombardo MV, Auyeung B, Ashwin E, Chakrabarti B, Knickmeyer R. Why are autism spectrum conditions more prevalent in males? PLoS Biol 2011;9:e1001081.
[32] Stoner R, Chow ML, Boyle MP, Sunkin SM, Mouton PR, Roy S, et al. Patches of disorganization in the neocortex of children with autism. N Engl J Med 2014;370:1209–19.
[33] Woolley CS. Effects of estrogen in the CNS. Curr Opin Neurobiol 1999;9:349–54.
[34] Murakami G, Hunter RG, Fontaine C, Ribeiro A, Pfaff D. Relationships among estrogen receptor, oxytocin and vasopressin gene expression and social interaction in male mice. Eur J Neurosci 2011;34:469–77.
[35] Harony H, Wagner S. The contribution of oxytocin and vasopressin to mammalian social behavior: potential role in autism spectrum disorder. Neurosignals 2010;18:82–97.
[36] Okabe S, Kitano K, Nagasawa M, Mogi K, Kikusui T. Testosterone inhibits facilitating effects of parenting experience on parental behavior and the oxytocin neural system in mice. Physiol Behav 2013;118:159–64.
[37] Swaab DF. Sexual differentiation of the brain and behavior. Best Pract Res Clin Endocrinol Metab 2007;21:431–44.
[38] Tiboni GM, Marotta F, Rossi C, Giampietro F. Effects of the aromatase inhibitor letrozole on in utero development in rats. Hum Reprod 2008;23:1719–23.
[39] Casper RF. Aromatase inhibitors in ovarian stimulation. J Steroid Biochem Mol Biol 2007;106:71–5.
[40] Branchi I, Santucci D, Alleva E. Ultrasonic vocalisation emitted by infant rodents: a tool for assessment of neurobehavioural development. Behav Brain Res 2001;125:49–56.
[41] Ehret G. Infant rodent ultrasounds — a gate to the understanding of sound communication. Behav Genet 2005;35:19–29.
[42] Portfors CV. Types and functions of ultrasonic vocalizations in laboratory rats and mice. J Am Assoc Lab Anim Sci 2007;46:28–34.
[43] Blumberg MS, Alberts JR. On the significance of similarities between ultrasonic vocalizations of infant and adult rats. Neurosci Biobehav Rev 1991;15:383–90.
[44] Brudzynski SM, Kehoe P, Callahan M. Sonographic structure of isolation-induced ultrasonic calls of rat pups. Dev Psychobiol 1999;34:195–204.
[45] Rutgers AH, Bakermans-Kranenburg MJ, van Ijzendoorn MH, van Berckelaer-Onnes IA. Autism and attachment: a meta-analytic review. J Child Psychol Psychiatry 2004;45:1123–34.
[46] Hartley SL, Sikora DM. Sex differences in autism spectrum disorder: an examination of developmental functioning, autistic symptoms, and coexisting behavior problems in toddlers. J Autism Dev Disord 2009;39:1715–22.
[47] Lamb JA, Barnby G, Bonora E, Sykes N, Bacchelli E, Blasi F, et al. Analysis of IMGSAC autism susceptibility loci: evidence for sex limited and parent of origin specific effects. J Med Genet 2005;42:132–7.
[48] Martin CR. Endocrine physiology. New York: Oxford University Press; 1985.
[49] Shepard KN, Michopoulos V, Toufexis DJ, Wilson ME. Genetic, epigenetic and environmental impact on sex differences in social behavior. Physiol Behav 2009;97:157–70.
[50] Adler NT, Bell D. Constant estrus in rats — vaginal reflexive and behavioral changes. Physiol Behav 1969;4:151–3.
[51] Gerardin DC, Pereira OC. Reproductive changes in male rats treated perinatally with an aromatase inhibitor. Pharmacol Biochem Behav 2002;71:301–5.
[52] Pohl A, Cassidy S, Auyeung B, Baron-Cohen S. Uncovering steroidopathy in women with autism: a latent class analysis. Mol Autism 2014;5:27.
[53] Bejerot S, Eriksson JM, Bonde S, Carlstrom K, Humble MB, Eriksson E. The extreme male brain revisited: gender coherence in adults with autism spectrum disorder. Br J Psychiatry 2012;201:116–23.
[54] Donaldson ZR, Young LJ. Oxytocin, vasopressin, and the neurogenetics of sociality. Science 2008;322:900–4.
[55] Ludwig M, Callahan MF, Neumann I, Landgraf R, Morris M. Systemic osmotic stimulation increases vasopressin and oxytocin release within the supraoptic nucleus. J Neuroendocrinol 1994;6:369–73.
[56] Landgraf R, Neumann I, Schwarzberg H. Central and peripheral release of vasopressin and oxytocin in the conscious rat after osmotic stimulation. Brain Res 1988;457:219–25.
[57] Meyer-Lindenberg A, Domes G, Kirsch P, Heinrichs M. Oxytocin and vasopressin in the human brain: social neuropeptides for translational medicine. Nat Rev Neurosci 2011;12:524–38.
[58] Ebstein RP, Knafo A, Mankuta D, Chew SH, Lai PS. The contributions of oxytocin and vasopressin pathway genes to human behavior. Horm Behav 2012;61:359–79.
[59] Lukas M, Neumann ID. Oxytocin and vasopressin in rodent behaviors related to social dysfunctions in autism spectrum disorders. Behav Brain Res 2013;251:85–94.
[60] Bielsky IF, Hu SB, Szegda KL, Westphal H, Young LJ. Profound impairment in social recognition and reduction in anxiety-like behavior in vasopressin V1a receptor knockout mice. Neuropsychopharmacology 2004;29:483–93.