Prenatal testosterone supplementation alters puberty onset, aggressive behavior, and partner preference in adult male rats

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Abstract The objective of this study was to investigate whether prenatal exposure to testosterone (T) could change the body weight (BW), anogenital distance (AGD), anogenital distance index (AGDI), puberty onset, social behavior, fertility, sexual behavior, sexual preference, and T level of male rats in adulthood. To test this hypothesis, pregnant rats received either 1 mg/animal of T propionate diluted in 0.1 ml peanut oil or 0.1 ml peanut oil, as control, on the 17th, 18th and 19th gestational days. No alterations in BW, AGD, AGDI, fertility, and sexual behavior were observed (p > 0.05). Delayed onset of puberty (p < 0.0001), increased aggressive behavior (p > 0.05), altered pattern of sexual preference (p < 0.05), and reduced T plasma level (p < 0.05) were observed for adult male rats exposed prenatally to T. In conclusion, the results showed that prenatal exposure to T was able to alter important aspects of sexual and social behavior although these animals were efficient at producing descendants. In this sense more studies should be carried to evaluated the real impact of this hormonal alteration on critical period of sexual differentiation on humans, because pregnant women exposed to hyperandrogenemia and then potentially exposing their unborn children to elevated androgen levels in the uterus can undergo alteration of normal levels of T during the sexual differentiation period, and, as a consequence, affect the reproductive and behavior patterns of their children in adulthood.

Keywords Aggressive behavior · Male rats · Prenatal testosterone · Puberty onset · Sexual behavior · Sexual differentiation

Introduction

Studies have shown that prenatal exposure of female rodents to exogenous androgens results in physiological and behavioral masculinization: male-like genitalia, increased anogenital distance, delayed puberty, early constant estrus, delayed anovulatory syndrome, and male-like changes in brain nuclei [1–5]. There is some evidence that prenatal androgenization may be involved in human diseases [6]. In this sense, women with polycystic ovary syndrome (PCOS) may maintain elevated androgen levels during pregnancy [7], thus potentially exposing their unborn children to elevated androgen levels in uterus [8].

In humans, the developmental effects of prenatal androgens because of medical conditions remain an active area of investigation [6, 8].

Exposure to testicular steroids, for example testosterone (T), early in life masculinizes the developing brain, leading to permanent changes in behavior in a wide variety of animal models [9]. According to the aromatization hypothesis, T is converted by aromatase into 17-beta estradiol, which then acts on estrogen receptors to feminize the brain [10]. Traditionally, aromatization is...
believed to be the mechanism by which the rodent brain becomes masculinized and defeminized [11]. Prenatally, for male rats, a T surge is observed that starts on GD 16 and lasts through GD 20 [12] and between 0 and 2 h after birth [13]. This surge has been shown to be important in the differentiation of sexual behavior [14]. Although T is necessary for normal development of male sexual behavior, exogenous perinatal T treatment of an intact male results in disruption of normal male sexual behavior [15] and social behavior, for example increased aggressiveness [16].

For ethical and methodological reasons, studies evaluating the effect on adult life of excess hormones during pregnancy are unviable, especially for humans. Because rats and humans have more than 90% of their genes in common [17] and to provide a useful model for mechanistic studies that are difficult to perform on humans, in this study we used animal models to determine whether prenatal exposure to T could change the body weight (BW), anogenital distance (AGD), anogenital distance index (AGDI), puberty onset, social behavior, fertility assessment, sexual behavior, sexual preference, and T level of male rats in adulthood. Delayed onset of puberty, increased aggressive behavior, disruption of the pattern of sexual preference, and reduced plasma levels of testosterone were observed for the T-supplemented animals.

**Methods**

**Animals**

After acclimatization under standard conditions (temperature at 25 ± 1°C, humidity 55 ± 5%, and light from 06:00 a.m. to 6:00 p.m.), sixteen virgin adult female Wistar rats (up to 90 days old, 230 ± 10 g) were mated with one fertile untreated male of the same age. Vaginal smears were then inspected daily, and the first day of pregnancy was defined as the morning on which spermatozoa were found. Pregnant females were randomly assigned into two groups, according to treatment, as described below.

The animals used in this study were maintained in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and approved by Institute of Biosciences/UNESP-Botucatu Ethical Committee for Animal Research (Protocol number 029/06).

**Experimental groups**

Sixteen pregnant dams were injected subcutaneously with either 1 mg/animal of testosterone propionate (TP) (Sigma–Aldrich, St Louis, MO, USA—testosterone group) diluted in 0.1 ml peanut oil or with 0.1 ml peanut oil on gestational day (GD) 17–19 [18]. The dose used was on a per rat basis, without correction for body weight, in order to replicate methods used extensively by other investigators [5, 19, 20]. Additionally, a 1-mg dose was used because higher doses induce adverse effects including loss of litters, delayed delivery, decrease in pup weight, and extensive mortality in F1 females after weaning, because of reproductive tract malformations [5]. The pups were born naturally and left undisturbed with their mothers until weaning, always 8 newborns/dam (4 females and 4 males to ensure the presence of both sexes in the litters). On postnatal day 2 (PND 2), pups were counted, weighed, and sexed, and anogenital distance was measured to confirm the sex. In this study we evaluated male rats only; female rats were sacrificed 1 day after weaning (pup PND 23) by CO₂ asphyxiation followed by decapitation. The pups were weaned on PND 22 and housed with same-sex litter mates until 90 days of age, when behavioral tests were conducted. These males are referred to as testosterone and control groups, respectively. They were identified and housed in collective polypropylene cages (32 × 40 × 18 cm³), each with wood shavings bedding, 4 animals/cage. To reduce “litter effects”, for each set of experiments in adult life, one male sibling was chosen from each litter [21].

On PND 90, for each characteristic, 8 males per group from different mothers (1 male/litter) were used for analysis of social behavior, fertility, sexual behavior, sexual partner preference assessment, and plasma testosterone quantification, because any of these procedures could interfere with the others.

Sexually experienced, gonadally intact adult Wistar male rats, 90 days old were used as stimulus animals for the behavioral tests.

**Body weight, anogenital distance, anogenital distance index, and puberty onset**

At birth and at 22 (weaning) days of age, males’ descend-ants from both groups were weighed and the anogenital distance (AGD) (distance from anal opening to genitals) was measured by use of a vernier-caliper. Anogenital distance Index (AGDI) was calculated by use of the formula (AGD/BW) × 100 [22]. To determine the day of puberty onset, the animals were inspected daily for balanopreputial separation.

**Aggressive behavior test: resident–intruder model**

The aggressive behavior test was designed to determine whether T supplementation could make these animals more aggressive.

The study utilized a model of aggression based on the resident–intruder model. This method enables observation
of the social interaction and offensive behavior of the resident, and defensive aspects of the behavior of the intruder, and reflects intraspecific aggression. Moreover, this model has the advantage of detecting mild aggressive behavior involving minimal injury to the animal. Resident animals were individually housed in cages; intruders were grouped and housed under the same environmental conditions [23].

The home territory that residents defended was their usual cage, which each animal had occupied since the beginning of the test. The cages were washed once a week during the study. A different intruder was used for each experimental animal. The same intruder was never used more than once [23].

Animals of the testosterone and control groups were each isolated in a cage for 4 weeks, and aggressive tendencies were evaluated once only. The session lasted 15 min (900 s). Latency and duration of each attack, and frequency of back attacks, lateral attacks, and bites were tabulated for each resident. A composite aggressive score (CAS) was calculated as follows: (number of attack-s) + 0.2 × (attack duration [s]) (adapted from Ref. [24]). If no attacks were made the composite aggression was scored as 0.0 and latency as 900 s. This procedure provides a mathematically derived and a more stable estimate of hormonal aggression than any other observation taken alone [24]. Each encounter was videotaped and was later scored for analysis. No animals were seriously injured, openly wounded, or mutilated during the duration of the study.

Fertility assessment

This experiment was designed to evaluate whether animals treated prenatally with T could produce descendants.

This assessment was conducted in accordance with an experimental procedure previously used in our laboratory [25, 26]. Each male rat of both groups was housed in a large cage with two regularly cycling females. Vaginal smears were examined daily to detect the presence of spermatozoa, indicating copulation. On gestation day 21, all mated females were killed in a CO2 gas chamber for exploratory laparotomy. The contents of the uterine horns were removed and litter size, gestational index, fertility index, fetal viability index, and rates of implantation and of pre and post-implantation losses were analyzed. Gestational index was calculated by use of the formula [(number of pregnant females with live fetuses)/(total number of pregnant females)] × 100. Fertility index was calculated by use of the formula [(number of pregnant females)/(number of mated females with successful copulation)] × 100. Fetal viability index was calculated by use of the formula [(number of live fetuses)/(number of points of implantation)] × 100, for each female individually. Post-implantation loss rate was calculated for each female individually, by use of the formula [(points of implantation – live fetuses)/(points of implantation)] × 100. Pre-implantation loss was also calculated for each mother, by use of the formula [(number of corpora lutea – points of implantation)/(number of corpora lutea)] × 100, and incidence of implantation was calculated use of the formula [(points of implantation)/(number of corpora lutea)] × 100 [27].

Male sexual behavior

This experiment was designed to determine whether the effects of T supplementation could change the male’s motivation to approach female rats.

T-treated male rats were allowed to mount female rats that were presenting natural estrus phase, detected by use of vaginal smears. Each male was placed in a Plexiglas cage and after 10 min of adaptation the estrus female was introduced. For 30 min, the following behavior was recorded: latency to first intromission (vaginal penetration, behavior that starts with a mount, but suddenly the male makes a deep thrust forward and stops pelvic thrusting, then vigorously withdraws and always licks his genitals); number of intromissions until the first ejaculation; latency to the first ejaculation (time from the initial intromission to ejaculation, starting with an intromission, but after vaginal penetration the male remains on the female for 1–3 s); post ejaculatory intromission latency; number of post ejaculatory intromissions; and total number of intromissions and ejaculations. In this study, the male rats used for sexual behavior evaluation were sexually experienced, because they had passed the fertility assessment test before being used for the sexual behavior test. For this reason, mounts with intromission were observed for all male rats of both experimental groups, so the latency to first mount and number of mounts without intromission were excluded from the results. If a male did not mount or intromit within 10 min, the evaluation was ended and the male was considered sexually inactive [28, 29]. For this reason males with sexual experience were used for evaluation of sexual behavior.

Sexual partner preference evaluation in adulthood

The evaluation of sexual partner preference was designed to determine whether T supplementation could change this behavior when the experimental animals were placed together with female and male rats. Test for partner preference (20 min duration) were adapted from Refs. [30, 31].

The sexual partner preference evaluation apparatus utilizes a semicircular arena (100 × 50 cm) with 2 cages
(25 × 15 cm) positioned on opposite sides, outside the arena, in which the stimulus animals, a sexually active male and a receptive female in estrus, were placed. The partition between the stimulus animals and the experimentally sexually experienced adult male rats (gonadally intact) consisted of a wire mesh enabling both animals to see, smell, and hear each other. The floor in front of the stimulus animals was demarcated into zones (30 × 20 cm) and the test lasted 20 min under red-light illumination during the first half of the dark phase of their cycle. The number of visits to each of the stimulus animal zones and the duration of each visit to each stimulus animal’s zone were measured (adapted from Ref. [32]). After each test, a partner preference score was calculated by subtracting the time spent in the zone containing the sexually active male from the time spent in the zone containing the estrus female. Thus, a positive score indicates a preference for the estrous female, a negative score a preference for the sexually active male [25].

Plasma testosterone quantification

The plasma T quantification was designed to evaluate whether alteration of the hypothalamic axis could have occurred.

The adult male rats were anesthetized with sodium pentobarbital (40 mg/kg, ip). Blood from the abdominal aorta was collected (between 8:00 and 10:00 h), centrifuged (2500 rpm for 20 min at 2°C), and the plasma stored at −20°C until assayed. The level of the hormone was measured by radioimmunoassay, by use of the Coat-A-Count Total Testosterone Kit (Diagnostic Products, Los Angeles, CA, USA) in accordance with the manufacturer’s instructions. The assay detection limit was 4.0 ng/dl, the intra-assay coefficient of variation was 4.8%, and all samples were analyzed in a single assay.

Statistical analysis

The results were analyzed by descriptive statistics for determination of normal data distributions. The median (IQ25–IQ75%) values were compared by use of the two-tailed Mann–Whitney nonparametric test (male sexual behavior data, fertility assessment data). Mean ± SD values were compared by use of Student’s t test (body weight, AGD, puberty onset, sexual partner preference data, and testosterone level data). Data expressed as pure proportions were compared by use of Fisher’s exact test (AGDI, fertility and gestational index). In all experiments, sample sizes were calculated to ensure statistical power of at least 80% and a significance level of 95%. Differences between groups were considered significant if \( p < 0.05 \).

Results

Body weight, AGD, AGDI and puberty onset of male pups

Prenatal treatment with T did not alter body weight (BW) and AGD at birth or at weaning (post-natal day, PND, 22), \( p > 0.05 \), or AGDI at weaning \( p > 0.05 \) (Table 1). The mean time from birth to puberty onset was 41.55 ± 0.32 for the control group versus 45.83 ± 0.22 for the testosterone group (\( p < 0.0001 \)) (Fig. 1).

Aggressive behavior

Results from measurement of aggressive behavior variables (attack latency, attack total, and attack duration) are listed in Table 2. Values were higher for animals exposed to T prenatally (\( p < 0.05 \)). CAS data are shown in Fig. 2. As a

Table 1 Body weight and anogenital distance, immediately after birth and at 22 days old, and anogenital index at PND 22, for control and T-treated male pups

| Variable                | Control          | Testosterone     |
|-------------------------|------------------|------------------|
| Body weight at birth (g)| 6.88 ± 0.23      | 6.96 ± 0.15      |
| Body weight at PND 22 (g)| 51.90 ± 1.86     | 53.07 ± 1.03     |
| AGD at birth (mm)       | 4.56 ± 0.14      | 4.42 ± 0.27      |
| AGD at PND 22 (mm)      | 18.51 ± 0.30     | 18.26 ± 0.30     |
| AGDI                    | 36.34            | 34.63            |

Values are expressed as mean ± SEM. Student’s t test and Fisher’s exact test were used for comparison of proportions. There was no significant difference between the groups, \( p > 0.05 \)

Fig. 1 Puberty onset of male pups exposed prenatally to testosterone. Values are expressed as mean ± SEM for 8 animals/group. \(* p < 0.0001\), Student’s t test
consequence of the increase in all behavior variables, CAS was increased for animal exposed to T \((p < 0.05)\).

Fertility assessment

As shown in Table 3 and Fig. 3, the results obtained from assessment of fertility were not statistically different for male rats from the T group \((p > 0.05)\).

Male sexual behavior

Results from evaluation of male sexual behavior are shown in Table 4. Prenatal exposure to T did not induce any alterations in sexual behavior \((p > 0.05)\).

Evaluation of sexual partner preference in adulthood

Results from evaluation of sexual partner preference are shown in Table 5. Sexual partner preference of male rats from the T group, in terms of the total number of visits to the female or male zone, was not statistically different. However, these males spent significantly less time \((228.28 \pm 93.47, p < 0.05)\) in the female zone than their controls \((693.14 \pm 114.19, p > 0.05)\) and more total time in the male zone \((352.75 \pm 68.49, p < 0.05)\) than their controls \((234.62 \pm 68.49)\). Thus, prenatal exposure to T led to a negative score \((-124.5 \pm 173.23, p < 0.05)\) relative to the control group \((458.5 \pm 143.12)\).

Plasma testosterone quantification

Prenatal exposure to T led to a significant reduction in plasma testosterone level in adulthood \((\text{mean} \pm \text{SEM} \text{ for } 8 \text{ males/group}, *p < 0.05)\) (Fig. 4).

Discussion

The main objective of this study was to evaluate whether administration of T at the time of the first peak of sexual differentiation of the hypothalamus [18] would modify the social and sexual behavior of male offspring in adulthood. The main findings of this study were that onset of puberty was delayed, aggressive behavior was increased, pattern of sexual preference was altered, and T plasma levels were reduced in adulthood for male rats exposed prenatally to T.

The developing “sex brain” is, similar to the reproductive system, feminine by default unless specific stimuli drive it to a male phenotype. Therefore, to achieve a male-specific brain, it is necessary to activate two independent processes—development of neural circuits enabling expression of male-specific behavior, when activated by testicular hormones (masculinization), and the loss of those able to respond to ovarian hormones (defeminization) during adulthood [33]. The AGD is defined as the distance between the genital papilla and the anus; male rodents have AGDs
approximately twice those of females [34] and AGD correlates closely with prenatal exposure to androgens [22]. Puberty is known to be a sensitive period during which exposure to gonadal hormones can have permanent effects on brain structure and function [35]. In our study alteration in body weight, AGD, and AGDI were not observed for T-treated animals. In contrast, however, onset of puberty was delayed compared with control animals. Despite this, animals treated prenatally with T were efficient in carrying out typical male sexual behavior and were efficient at producing descendants; females rats mated with males of the testosterone group had normal pregnancies and, as consequence, fertility was normal. These results indicate that supra-physiological levels of testosterone administered prenatally do not significantly alter male behavior [36], in contrast with results obtained by others when the hormonal manipulations occurred during the postnatal period [5, 36]; thus prenatal exposure to T did not reduce motivation to approach a female [36]. Additionally, the unaltered gestational index suggests that testosterone supplementation was not toxic to the fetuses; for all pregnant females, termination revealed all fetuses were alive [25].

After T links to its receptor it is aromatized into estrogens within the neurons [18]. In addition, estrogen or aromatizable androgens (that are converted to estrogen by the enzyme aromatase in cells) are very important in regulating neuronal development and neural circuit formation during the perinatal period, and this organizational activity of the

Table 4 Effects of prenatal treatment with testosterone on the sexual behavior of adult male rats

| Variable                        | Experimental groups |
|---------------------------------|---------------------|
|                                 | Control (n = 8)     | Testosterone (n = 8) |
| Latency to first intromission (s) | 235.00 (214.15–236) | 229.5 (165–328) |
| No. of intromissions until ejaculation | 14.50 (12.5–15.75) | 16.5 (15.25–17.75) |
| Latency to first ejaculation (s) | 556 (450.5–612.75) | 662.5 (604.75–712.75) |
| Postejaculatory intromission latency (s) | 997 (774.25–1285.75) | 927 (798–930) |
| No. of postejaculatory intromissions | 11.5 (10–13.75) | 6.5 (4.75–8.5) |
| Total number of intromissions | 26.5 (23.75–24) | 21 (18–24) |
| Total number of ejaculations | 2.5 (2–3) | 2 (2–3) |

Values are expressed as median (IQ25–IQ75%). There was no significant difference between the groups, p > 0.05, Mann–Whitney U test

Table 5 Results from evaluation of sexual partner preference of adult male rats supplemented prenatally with testosterone

| Variable                        | Experimental groups |
|---------------------------------|---------------------|
|                                 | Control (n = 8)     | Testosterone (n = 8) |
| Number of visits to the female zone | 9.12 ± 1.82        | 8.5 ± 1.97 |
| Number of visits to the male zone | 6.62 ± 1.68        | 9.0 ± 2.12 |
| Total time spent within female zone (s) | 693.14 ± 114.19 | 228.28 ± 93.47* |
| Total time spent within male zone (s) | 234.62 ± 68.49 | 352.75 ± 127.21* |
| Preference score | +458.5 ± 143.12 | −124.5 ± 173.23* |

Values are expressed as mean ± SEM for 8 animals/group, *p < 0.05, Student’s t test

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**Fig. 3** Frequency of pre and post-implantation losses observed for untreated female rats when mated with male rats from the control or testosterone groups. Values are expressed as median (IQ25%–IQ75%). There was no significant difference between the groups, p > 0.05, Mann–Whitney U test
sex steroids can induce permanent sexual dimorphisms in specific brain regions, in synapse formation, in dendritic length, in the distribution patterns of serotonergic fibers, and in neuronal connectivity [37]. Although the animals prenatally supplemented with T had reduced plasma T levels, our results showed that the behavior of these animals was more aggressive than that of control animals. Some authors have suggested that estrogens are more effective than testosterone in inducing aggression [38–40]. Researchers who used male aromatase knockout mice, which lacked a functional aromatase enzyme, observed a marked reduction in aggression [41, 42], and when these male aromatase knockout mice were treated with estradiol their aggressive behavior was partially restored [41]. In another experiment, castrated mice received doses of estrogen, T, or dihydrotestosterone (androgen, not aromatized), and the results showed an increase in aggressive behavior in all experimental animals [38]. By use of a similar procedure others have compared the aggressive behavior of adult male rats that received anabolic steroid or T, and concluded that both were able to increase the aggressive behavior of these animals [43]. In the same sense, researchers evaluated the effects of nandrolone decanoate on rats in a dose–response study and observed that the aggressive behavior of these animals was dose-dependent [23]. Given the data presented and the controversies found in the literature, factor such as the source of androgen, expression of different estrogen receptor types, and social experience can have important effects on how aromatization affects aggressive behavior [12]. In this sense, more studies are needed to evaluate how T, estradiol and its receptors, and the aromatase enzyme act on the brain and become responsible for aggressive behavior.

In our study the pattern of sexual preference of animals exposed prenatally to T was altered, reflecting the decrease in T levels. In a study performed by Henly et al. [36], who used similar procedures to evaluate sexual behavior and sexual preference, no differences were found. An important difference between these experiments was the dose of TP and the period of treatment; in our study we used 1 mg/animal/day on GD 17, 18, and 19 whereas Henly et al. [36] used 2 mg/0.1 ml/day on GD 16, 17, 18, 19, and 20. Also in this study, males treated with TP from the day of birth through PND 21 and tested as intact adults spent more time with a sexually active male than with control males. Our study revealed that prenatally androgenized animals were completely defeminized, because they did not show typical female sexual behavior, for example lordosis; although male-specific behavior was observed for the T-treated animals, and these animals were able to produce descendants, our results suggest that their masculinization was not complete, because the pattern of sexual preference was shifted. Thus, it could be argued that, in contrast with estrogenic stimulation during development, exposure to elevated androgenic stimulation selectively prevents the defeminization of partner preferences without affecting its masculinization [44]. In addition, an explanation of this behavior is related to the low plasma T level of these animals compared with the control group.

This study examined the effect of T supplementation on behavior (social and sexual) and our results showed that prenatal androgen supplementation caused alteration of production and release of T, and consequently changed the pattern of social behavior and sexual preference in testosterone-supplemented animals. Finally, these results suggest that further studies could investigate the molecular mechanisms of androgen hormones and the involvement of aromatase enzyme and estrogen in sexual differentiation of the hypothalamus and evaluate its effect on behavior in adulthood. It is interesting to note that girls with congenital adrenal hyperplasia, who were exposed to high testosterone levels in the womb, tend to choose boys as playmates, prefer boys’ toys, and exhibit some male-typical personality features [45, 46]. This is a strong indication of the crucial importance of T levels during pregnancy in the development of such sexual differences in behavior [46]. In addition, the doses of androgens given in animal models result in androgen levels that far exceed those seen in pregnant women with hyperandrogenemia. It is, therefore, unclear whether the more subtle elevations in androgens typical of PCOS and congenital adrenal hyperplasia reach the fetus or have any effect on its development [6].

**Conclusions**

In conclusion, our results showed that prenatal exposure to testosterone could alter important sexual and social
behavior, although these animals were able to produce descendants. In this sense, more studies should be conducted to evaluate the effect, on humans, of such hormonal alteration in the critical period of sexual differentiation, because exposure of pregnant women to hyperandrogenemia, and thus potential exposure of their unborn children to elevated androgen levels in the uterus, could lead to alteration of normal levels of testosterone during the period of sexual differentiation of the children, and, as a consequence, affect their reproductive and behavior patterns in adulthood.

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