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Testosterone enhancement during pregnancy influences the 2D:4D ratio and open field motor activity of rat siblings in adulthood

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ABSTRACT

In humans, the relationship between the prenatal testosterone exposure and the ratio of the second and the fourth digits (2D:4D) has been extensively studied. Surprisingly, data on this relationship have thus far been lacking in experimental animals such as rats. We studied the effect of maternal testosterone enhancement during pregnancy on the digit ratio and open field activity of adult progeny in Wistar rats. Elevated levels of maternal testosterone resulted in lower 2D:4D ratios and an elongated 4D on the left and right forepaws in both males and females. We found no sex difference in 2D:4D in control animals. In the open field test, control females were more active than control males and testosterone females, while the activity of testosterone females did not differ from that of control males. We found a positive correlation between motor activity and the right forepaw 2D:4D ratio of control males and females. Prenatal exposure to testosterone resulted in the disappearance of this correlation in both males and females. Our results show that elevated levels of testosterone during the prenatal period can influence forepaw 4D length, 2D:4D ratio, and open field motor activity of rats, and that these variables are positively correlated. Thus, this approach represents a noninvasive and robust method for evaluating the effects of prenatal testosterone enhancement on anatomical and physiological parameters.

Introduction

Testosterone is a dominant androgen controlling not only genital masculinization (Baron-Cohen et al., 2004), but also the masculinization of the central nervous system (CNS) (Morris et al., 2004). Elevated levels of the plasma testosterone during sensitive periods of development result in masculinizing effects (Garcia-Falgueras et al., 2005), while testosterone deficiency feminizes the CNS (Hotchkiss et al., 2002; Casto et al., 2003). Testosterone concentrations in developing male rats are not constant over the whole intrauterine period. The peak in testosterone concentration during days 18 and 19 of intrauterine development has been proposed to be a critical period for the sexual differentiation of the brain (Weisz and Ward, 1980; Rhees et al., 1997). When pregnant female rats are exposed to stress, testosterone levels reaches its peak before the critical period for male offspring, and this situation results in desynchronization between hormonal levels and the development of the CNS (Ward and Weisz, 1980). The absence of testosterone during critical developmental periods may result in behavioral changes after birth.

In addition to sexual differences and CNS differentiation, testosterone can affect other morphological characteristics like the ratio between the length of the second and fourth digit (2D:4D). In humans, both digit length and digit ratio are sexually dimorphic. Males have a longer 4D than females (Peters et al., 2002), and this difference is especially apparent on the right hand (Manning et al., 1998). Males have also a significantly lower 2D:4D ratio on the right hand than females, and this difference persists from age two until adulthood (Manning et al., 1998). There has been some suggestion of a relationship between plasma testosterone levels and the 2D:4D ratio in adult males, with testosterone concentrations negatively related to right hand 2D:4D ($P = 0.03$). However, this relationship lost its significance when controlling for weight and age ($P = 0.07$; Manning et al., 1998). The 2D:4D ratio is also lower in male than female baboons (right hand; Roney et al., 2004) and mice (right hindpaw; Brown et al., 2002a).

Lower 2D:4D ratios have also been found in studies on autistic patients (Manning et al., 2001) and patients with attention-deficit/hyperactivity disorder (ADHD; de Bruin et al., 2006). Autism and ADHD share some common patterns of behavior, such as stereotypies, inattention and hyperactivity (American Psychiatric Association, 1994; World Health Organization, 1992). Our previous results have suggested that elevated levels of maternal testosterone during pregnancy influence behavior of rat offspring in the open field test after weaning, and male rats in particular show behavioral similarities to autistic children (Kršková and Talarovičová, 2005). Some authors have also suggested that 2D:4D ratio can serve as a predictor of future tendency toward some personality trait behaviors (Hampson et al., 2008). Normally, in the open field test female rats are more active than males (Fitch and Denenberg, 1998). Perinatal testosterone

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administration leads to masculinization of this behavior (Gray et al., 1969; Stewart et al., 1975). In the present study we were interested in the relationship between open field behavior and 2D:4D digit ratio in rats after prenatal administration of testosterone.

The 2D:4D digit ratio probably depends on the effects of androgens during development (Romano et al., 2005). Clinical studies in humans have also raised the possibility of prenatal influences of androgens on the digit ratio. Male and female patients with congenital adrenal hyperplasia have 2D:4D ratios significantly lower than the population average, (i.e., males on both hands, females on the right hand; Brown et al., 2002b). There are limited data on sex differences in forepaw 2D and 4D length in rats. McMechan et al. (2004) found that 30 day old males had longer left 2D and 4D lengths, and right 4D lengths, than females.

However, possible prenatal influences of androgens on digit ratio have never been tested experimentally in laboratory rats. Since there are limited data about 2D:4D ratios in rats, in the context to their individual personal histories and subsequent behavior in adulthood, the present study investigated the effect of experimentally elevated testosterone concentration in female rats during pregnancy on the digit ratios and behavior of progeny in adulthood.

We tested two specific hypotheses: 1) that prenatal exposure to testosterone influences the length of digits and the 2D:4D ratio in rats; and 2) that prenatal exposure to testosterone influences locomotor activity in the open field test.

Materials and methods

In our experiment, we used six nulliparous female Wistar rats, obtained from the Institute of Experimental Pharmacology, Slovak Republic. One group of pregnant females was used as a control (C; $n=3$) and received a single intramuscular application of sesame oil on gestation day 14. A second group of pregnant females ($n=3$) received a single intramuscular injection of 2.5 mg testosterone (T; *testosteroni isobutyras*) in 0.1 ml of microcrystalline aqua suspension on day 14 (Agovirin Depot, Biotika, Slovenská Ľupča, Slovak Republic). We selected gestation day 14 to cover the last trimester of pregnancy, because this interval is a critical period for differentiation of male and female phenotypes (Weisz and Ward, 1980; Baum et al., 1991; Bayer and Altman, 2004). We used a depot form of testosterone to minimize the stress to pregnant female rats from daily administration. We chose the dose so as not to induce adverse effects on delivery, number of pups or morphological defects of progeny (Wolf et al., 2002). To ensure that elevated levels of maternal testosterone had potential organizational effects without elevating plasma levels of testosterone in offspring, we also measured hormone levels of the offspring within 1 day after birth.

During the first day after birth, litters were randomly adjusted to seven pups per nest without any similarity of sex ratio in litters. The culled pups were used to determine testosterone levels after birth. Testosterone concentrations in plasma were measured by direct radioimmunoassay using [1,2,6,7-^{3}H]-testosterone (Amersham Biosciences, UK, specific activity 3.25 TBq/mmol) and antiserum raised against testosterone linked at position 3 by carboxymethyloxine to bovine serum albumin (Zeman et al., 1986). All samples were run in a single assay. The intra-assay coefficient of variation was 9.6%, assay sensitivity was 0.9 pg/tube.

Plasma testosterone levels of newborn rats did not differ between the control and testosterone group in either males (mean±SEM; control 1241.8±205; testosterone 1032.8±203; $P=0.479$) or females (mean±SEM; control 165.8±35; testosterone 135.8±7; $P=0.543$). Pups (total $n=21$ from three litters per treatment group) were housed with their mothers in plastic cages (57×37×19 cm) with wood shavings, 12 h:12 h light/dark cycle and free access to water and commercial food pellets. Young rats were weaned on postnatal day 23 and divided into cages according to sex and birth nest. Only animals of the same sex and from one nest were caged together. At postnatal day 56 animals were randomly (but equally from every nest) selected from each cage, and 4 groups were formed ($n=8$ per sex/group combination) to maintain the equal number of animals for every group.

Methods and procedures of the present study were approved by the local Ethics Committee of the Comenius University in Bratislava, Slovak Republic.

Digit measurement

In adulthood (postnatal day 80) we measured the 2nd (2D) and 4th (4D) digits on both left and right forelimbs of randomly selected control (male: $n=8$, female: $n=8$) and testosterone (male: $n=8$, female: $n=8$) treated male and female rats. The digits were measured directly on restrained animals with calipers (0.1 mm precision). Each animal's forelimb was positioned on a level surface. Measurements were done from the upper side of the forepaws by placing the zero point of the caliper on the basal crease proximal to the palm and then measuring to the tip of the digit, excluding toenails. The person performing the measuring was blind to the sex and treatment condition of the animals. Measurements were made three times for each digit, and mean values were used in evaluation. The length of all digits was highly repeatable between three independent measurements (left 2D: $r=0.98$, $F_{1,31}=164.3$; $P<0.001$; left 4D: $r=0.99$, $F_{1,31}=533.3$; $P<0.001$; right 2D: $r=0.98$, $F_{1,31}=170.5$; $P<0.001$; right 4D: $r=0.99$, $F_{1,31}=695.5$; $P<0.001$).

Open field test

Between postnatal days 76–84 animals underwent an open field test. Each rat was individually tested for 20 min in a testing chamber (72×34×39 cm) according to the procedure used in our previous study (Kršková and Talarovičová, 2005). The bottom of the testing chamber was divided into 32 equal squares (8×4 configuration) by painted dark lines. The rats were tested during the light phase (14.00 h–17.00 h) and frequency of locomotor activity (ambulation + rearing) was registered in protocols by shorthand. Ambulation was scored when animals were moving actively through the squares of the observation area and rearing when animals reared up on their hind limbs. The observer was blind to the sex and treatment of the animals.

Statistics

All data were square root transformed to fit the normal distribution. Outliers were estimated as variables two standard deviations above or below the mean. Differences between groups in plasma levels of testosterone in offspring were determined by *t*-test for independent variables. The repeatability of digit measurements was performed by a one-way ANOVA with individual as a factor (Lessells and Boag, 1987). The effect sizes for sex and treatment differences were calculated as the difference between the mean values of the two groups, divided by the standard deviation (Cohen, 1977). Statistical analysis was performed by a two-way ANOVA with fixed factors treatment (control and testosterone) and sex. Differences between groups were determined by LSD post-hoc test. The correlation between the 2D:4D ratio and total locomotor activity was determined by a Pearson's correlation. A comparison of the correlation coefficients for right 2D:4D and motor activity was evaluated by *p*-value based on two sided difference test for correlation coefficients.

Results

Left and right 2D and 4D

We found significant effects of treatment (for 2D $F_{1,28}=5.241$, $P<0.05$; for 4D $F_{1,28}=49.865$, $P<0.001$) and sex (for 2D $F_{1,28}=11.383$,