

Testosterone enhancement during pregnancy influences social coping and gene expression of oxytocin and vasopressin in the brain of adult rats

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Steroid hormones are important mediators of prenatal maternal effects and play an important role in fetal programming. The aim of our study was to investigate how testosterone enhancement during pregnancy influences neurobehavioral aspects of social coping of rat offspring in adulthood. Pregnant rat dams were exposed to depot form of testosterone during the last third of pregnancy (i.e., beginning on the 14th day of pregnancy). Their adult offspring were later tested in a social interaction test and expression of oxytocin and arginine-vasopressin mRNA in the hypothalamic nuclei was evaluated. Our research showed that prenatal exposure to higher levels of testosterone activated socio-cohesive and socio-aversive interactions, but only in males. The testosterone-exposed group also showed decreased oxytocin mRNA expression in the supraoptic and paraventricular nuclei of the hypothalamus, and increased arginine-vasopressin mRNA expression in the supraoptic and suprachiasmatic nuclei as compared to controls. However, we did not observe any sex differences in the expression of oxytocin and arginine-vasopressin mRNA in these regions. Our findings show that testosterone enhancement in pregnancy could have long-lasting effects on oxytocin and arginine-vasopressin levels in the brain of adult animals, but lead to changes in behavioral aspects of coping strategies only in males.

Key words: testosterone, social coping strategy, oxytocin, arginine-vasopressin

INTRODUCTION

Adaptation through maternal effects has become a key concept in modern evolutionary biology (Groothuis et al. 2005, Mousseau and Fox 1998) and numerous studies have focused on better understanding the mechanism underlying this phenomenon (Dloniak et al. 2006, Groothuis et al. 2005, Kaiser and Sachser 2009, Kemme et al. 2007).

Steroid hormones, such as gonadal hormones, play an important role in fetal programming (Lombardo et al. 2012, O'Connor and Barrett 2014) and are considered to be important mediators of prenatal maternal effects (Groothuis et al. 2005). Steroids have a substantial influence on prenatal and early postnatal brain develop-

ment (Auyeung et al. 2013, Bao and Swaab 2010, Filova et al. 2013, Geschwind and Galaburda 1985) and contribute to variation in neural and behavioral phenotypes (Baron-Cohen et al. 2015, Celec et al. 2015, Knickmeyer and Baron-Cohen 2006, Steinman and Trainor 2017). Thus, understanding the influence of key hormones such as testosterone on brain development is important for understanding how it might program neural circuitry for diverse responsiveness later in life, and potentially lead individuals down multiple developmental paths (Bao and Swaab 2010, Baron-Cohen et al. 2005, Lombardo et al. 2012, Martel 2013, Martel and Roberts 2014).

Prenatal testosterone plays a key role in the development of many aspects of mammal behavior including social behavior, as well as aggressive and stress reactions

(Dela Cruz and Pereira 2012, Pivina et al. 2007, Xu et al. 2015). An imbalance in testosterone during prenatal development may lead to changes in the hypothalamic-pituitary-adrenal (HPA) axis (Kapoor and Matthews 2011, Weinberg et al. 2008), that can, in turn, result in changes in coping strategies (Koolhaas et al. 1999). Specific coping styles are not only characterized by differences in behavior but also by differences in physiological coping responses and neurobiological characteristics (Koolhaas et al. 2010).

The neuropeptides arginine-vasopressin (AVP) and oxytocin (OT) are among the major neurotransmitters involved in social behavioral network signalling, contributing to modulation of emotionality and social behavioral and physiological responses that are reflected in a coping style of an individual (Coppens et al. 2010, Heinrichs et al. 2009, Koolhaas et al. 2010). While AVP is generally known to increase anxiety-like behavior, stress, and aggressiveness, OT on the other hand, is thought to have the opposite effect, facilitating social attachment, care, and affiliation (Heinrichs et al. 2009, Insel et al. 1998, Neumann and Slattery 2016). Both neuropeptides are produced by magnocellular and parvocellular neurosecretory neurons in the paraventricular (PVN), supraoptic (SON), and other nuclei of hypothalamus. AVP is also produced by parvocellular cells in the suprachiasmatic nucleus (SCN) (Dhakar et al. 2013).

Increased AVP activity in discrete brain regions is strongly linked to stress-coping regulation that is associated with increased levels of aggressiveness and, perhaps in general, proactive/active coping styles. On the other hand, increased OT activity in the same neural structures is implicated in more reactive/passive coping (Koolhaas et al. 2010).

The aim of the present study was to investigate how testosterone enhancement during pregnancy influences neurobehavioral aspects of social coping of rat offspring in adulthood. We hypothesized that testosterone enhancement during pregnancy could have long-lasting effects on OT and AVP levels in the brain, resulting in proactive social coping strategies in adulthood.

METHODS

Animals

In this study, eight nulliparous female and three virgin male Wistar rats (obtained from the Institute of Experimental Pharmacology, Slovak Republic) were housed in a room with light:dark cycle 12:12 (lights on at 6 a.m.), with food (Dos — 2b OVO, Dobrá Voda, SR) and water *ad libitum*. After a one-week acclimatization

period, rats were mated overnight and the presence of spermatozoa in vaginal smear was considered as a day zero of gestation.

On gestational day (GD) 14, pregnant females were randomly assigned to one of two groups. One group was assigned to a control (Ctrl, $n=4$) and received a single intramuscular injection of vehicle (sesame oil, 0.1 ml). The second group was assigned to a treatment (T, $n=4$) and received a single intramuscular injection of 2.5 mg testosterone (testosteroni isobutyras) in 0.1 ml of microcrystalline aqua suspension on day 14 (Agovirin Depot, Biotika, Slovenská Ľupča, Slovak Republic). GD14 was selected to cover the last third of pregnancy, as this interval represents a critical period for differentiation of male and female phenotypes (Baum et al. 1991, Bayer and Altman 2004, Weisz and Ward 1980). By using a depot form of testosterone, we exposed females and their developing offspring to a slowly metabolizing form of testosterone that acts over a period of approximately 14 days, while minimizing the stress of daily injection for pregnant females. The dose of a 2.5 mg of testosterone was chosen to reflect possible natural variation in increase of testosterone that does not induce adverse effects on delivery, number of pups, or morphological changes in progeny (Wolf et al. 2002).

Pregnant females were housed in groups until GD20. One day before delivery at GD21, testosterone and control females were housed individually and allowed to raise their offspring until weaning at postnatal day (PND) 23. On PND1, the litters were culled to 8 animals per litter (4 males, 4 females). The culled pups were used to determine testosterone levels after birth that did not differ between the control and testosterone group in either males or females (Talarovičová et al. 2009). After weaning, rats of either sex were housed separately in groups of 4 animals per cage.

At PND80, 64 animals (Ctrl: $n=32$, males $n=16$, females $n=16$; T: $n=32$, males $n=16$, females $n=16$) were tested in a social interaction test. At PND85, animals were euthanized with CO₂ and sacrificed by decapitation. Brains were quickly removed, placed into frozen medium (Cryomount, Histolab AB, Sweden) frozen on dry ice and stored until sectioning at -80°C . The brains of all animals tested in social interaction test were evaluated by *in situ* hybridization.

Social Interaction Test

The social interaction test was adapted from File (1980). Each rat was tested for social interaction with an unknown test partner that did not differ by more than 10 g in weight. Both members of a pair were of the same sex and same treatment group. Pairs were tested

in a random order between 14:00 and 15:00 p.m. (light phase of light:dark cycle). The test chamber was 72 cm long, 34 cm wide and 39 cm high.

One day before the social interaction test, animals were habituated to the test chamber by placing them individually into the test chamber for 20 min. During the testing period the next day, each of the tested animals was placed in the opposite corners of the test chamber and their behavior was recorded for 5 min using a digital camera SONY DCR-DVD 92 E.

A total duration of socio-cohesive (play – play wrestling with partner, following – follows the partner while the other is walking away, chasing – chasing the partner, mutual sniffing – sniffing the partner's body, genital investigation – sniffs the partner's anogenital area, climbing over – walks over the partner, crawling under – walks under the partner, allogrooming contact – grooming the partner's body, neck or face) and socio-aversive (escape – initiation of partner fleeing, mounting – hold the other rat's trunk with the forelimbs, defence of the rump, tail biting – biting of partner's tail) interactions during the 5 min interval was manually scored from the video recording. Observed behavior forms were analyzed individually for each animal.

In Situ Hybridization

In situ hybridization was performed as described previously (Dzirbikova et al. 2011). Briefly, serial 14 μ m thick coronal sections of the hypothalamus were cut on a cryostat at -24°C , transferred onto slides (SuperFrost[®] Plus, MenzelGläser, Thermo Fisher Scientific Inc), and stored at -80°C until further processing. Before the start of the hybridization process the sections were warmed up at room temperature, fixed with 4% formaldehyde in phosphate-buffered saline (PBS), pH 7.4, washed twice in PBS, acetylated, dehydrated in increased ethanol concentration and delipidated in chloroform. The oligodeoxyribonucleotide probes used for *in situ* hybridization were 3'-end labelled with [³⁵S]dATP using terminal deoxynucleotidyltransferase (TdT). The following probes were used: OT 41-mer (Patisaul et al. 2003) and AVP 48-mer (Jac et al. 2000). The hybridization was performed at 41°C for 16–20 hours with purified labelled oligoprobe (172,000 cpm in 70 μ l of hybridization buffer per one slide). The sections were then washed twice in 1x saline-sodium citrate (SSC) at room temperature (for 5 min), three times in 1xSSC at 55°C (for 15, 15 and 30 min), twice again in 1xSSC at room temperature (30 and 30 min), and dehydrated and air dried. Sections were then exposed to autoradiography film (BioMax MR, Kodak) for 24 hours. Follow-

ing the exposure, the slides were counterstained with cresyl violet. The hybridization signal was assessed using Image J 1.50i software (<http://rsbweb.nih.gov/ij/index.html>) as a difference between the optical density of the hypothalamic nuclei (SCN, PVN, SON) and adjacent brain regions. Results were expressed as relative optical density. The mean of the left- and right-side values from at least four slices per animal was taken as final results.

Statistical Analysis

Statistical analysis was performed using STATISTICA 7.0 (StatSoft, Inc., Tulsa, USA). A Kolmogorov-Smirnov test was used to check for normality of the data and a logarithmic transformation was applied where necessary. A linear model analysis of variance (ANOVA) was fitted to test for effects of group, sex, and their interactions. Additionally, to control for variation between siblings, the effect of litter nested within group was included in the model. Differences between individual groups were evaluated using the Fisher LSD *post hoc* test (behavioral data) and the Tukey's *post hoc* test (*in situ* hybridization data).

Ethics Statement

Methods and procedures of the present study were approved by State Veterinary and Food Administration of the Slovak Republic and local Ethics Committee of the Comenius University in Bratislava and were in the accordance with Directive of the European Parliament and of the Council on the protection of animals used for scientific purposes.

RESULTS

Social Behavior

During the social interaction test, we found a significant interaction of sex and group ($F_{1,54}=4.14$, $P<0.05$) in time spent in socio-cohesive interaction. Males prenatally exposed to testosterone spent more time in socio-cohesive interaction than control males ($P<0.05$), while testosterone-exposed females did not differ from control females (Fig. 1A). Similarly, we found a significant interaction of sex and group ($F_{1,54}=8.71$, $P<0.01$) in time spent in socio-aversive interaction. Again, testosterone-exposed males spent more time in socio-aversive interaction than control males ($P<0.05$), while no difference was observed among females (Fig. 1B).

Oxytocin and Arginine-Vasopressin mRNA Expression in the Hypothalamus

We observed a significant decrease in levels of OT mRNA expression in the SON and PVN in rats prenatally exposed to high maternal testosterone as compared to controls (autoradiographs, Fig. 2A, B). A more than 30% decrease in mRNA expression was observed in the PVN ($F_{1,27}=34.246$, $P<0.001$, Fig. 3A), and an almost 10% decrease was observed in the SON ($F_{1,27}=5.702$, $P<0.05$, Fig. 3A). There was no sex difference or interaction between sex and treatment in the levels of OT mRNA expression. AVP mRNA expression levels were elevated in the brain of testosterone-exposed rats as compared to the control group (autoradiographs, Fig. 2C, D). An almost 12% stronger signal was observed in the SON ($F_{1,28}=7.04$, $P<0.05$, Fig. 3B), and almost 30% stronger signal in the SCN ($F_{1,24}=14.455$, $P<0.001$, Fig. 3B). No difference in expression was found in the PVN. Expression of AVP mRNA in these hypothalamic nuclei was not influenced by sex or the interaction between sex and treatment.

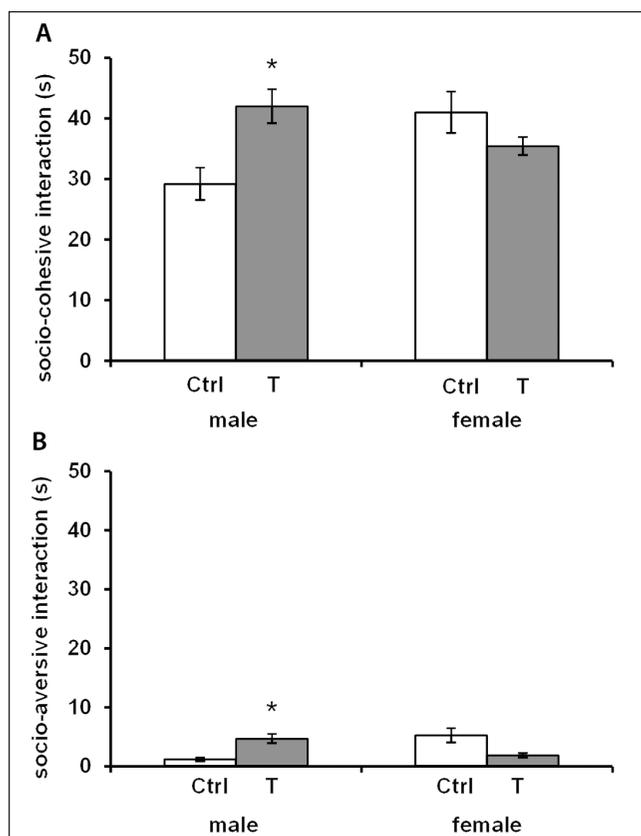


Fig. 1. Socio-cohesive (A) and socio-aversive (B) interaction of control (Ctrl, male: $n=16$, female: $n=16$) and testosterone (T, male: $n=16$, female: $n=16$) rats during the 5 min long social interaction test. Data are expressed as mean \pm SEM. An asterisk indicates a significant difference between Ctrl and T group ($*p<0.05$).

DISCUSSION

Maternal hormonal responses to environmental stimuli represent a potential tool by which the development of offspring can be influenced (Kaiser and Sachsler 2009). Steroid hormones, including testosterone, are important mediators of prenatal maternal effects and play an important role in fetal programming (Groothuis et al. 2005, Lombardo et al. 2012, O'Connor and Barrett 2014). In our study, we investigated whether testosterone enhancement during pregnancy influences neuro-behavioral aspects of social coping of rat offspring in adulthood. Social behavioral strategy of experimental animals was tested in a social interaction test. Our results showed that T males spent more time in socio-cohesive and socio-aversive interaction than Ctrl males, while T females did not differ from Ctrl females. These results support the idea that prenatal exposure to elevated hormones during a critical period of development permanently alters the structure of the developing brain, which in turn influences behavioral outcomes during the lifespan. High levels of testosterone leads to neural lateralization and slower overall development of the brain. Slower development of the brain in boys/males has traditionally been viewed as making them more vulnerable to environmental insults (Baron-Cohen et al. 2015, Geschwind and Galaburda 1985, Martel 2013, Martel and Roberts 2014). Higher levels of testosterone may form a more systemizing brain – with a higher ability to understand systems including structured social systems (e.g., dominance hierarchies). The increased social interactions observed in our prenatally testosterone-exposed males may support this idea (Knickmeyer and Baron-Cohen 2006). Male behavioral phenotypes are generally more consistent with proactive coping strategies in which stressors are more directly confronted in comparison with females (Steinman and Trainor 2017). If we apply Koolhaas's two-tier model (Koolhaas et al. 2010) of coping style and stress reactivity as two independent dimensions of stable trait characteristics to our results, we see that T males appeared to be more proactive and bold in social interaction with an unknown rat. The proactive coping is characterized as high levels of aggression (Koolhaas et al. 1999), which corresponds with our finding of increased time spent in socio-aversive interaction by T males and increased in the number of animals exhibiting this form of behavior in the T group as compared to Ctrl group of animals (81% vs. 50%). Socio-positive interaction was observed in all males of both groups (T: 100%, Ctrl: 100%). However, aggression is only one of a larger set of behavioral characteristics that make up the proactive coping style (Koolhaas et al. 1999). More time spent with socio-cohesive interactions also indicates a more active strategy

for obtaining information about the social environment (i.e., a strong tendency to act), which often occurs in the individuals with proactive strategy. On the other hand, this pattern may indicate weak cognitive flexibility, which is also a characteristic trait in active coping strategy (Koolhaas et al. 2010, Steinman and Trainor 2017). It is commonly accepted that behavioral, neuroendocrine, and autonomic physiological reactivity is the ultimate output of a complex organization of the brain at the level of structural and functional activity in various neuronal networks, neurotransmitter systems, and the molecular processes of signal transduction in neurons. Animals with different coping styles can also be expected to show consistent differences in the putative neurobiological characteristics that underlie the respective behavioral and physiological coping responses (Koolhaas et al. 2010). The neuropeptides AVP and OT modulate emotionality, social behavior, and physiological responses that are reflective of coping styles (Heinrichs et al. 2009, Koolhaas et al. 2010, Meyer-Lindenberg et al. 2011). Our results showed that the T group exhibited higher AVP mRNA expression levels in the SCN and SON (but not in the PVN) as compared with the Ctrl group. On the other hand, expression of OT mRNA was lower in T rats in PVN and SON hypothalamic nuclei in comparison with Ctrl group. These neurophysiological data correspond with our behavioral data and support the hypothesis that the prenatal T enhancement induces a proactive social behavior strategy and alters levels of social neurotransmitters in the brain, at least in males. The question arising from our study is that, despite changes in expression of AVP and OT mRNA observed in both males and females, changes in social strategies were observed only in males. However, no sex differences

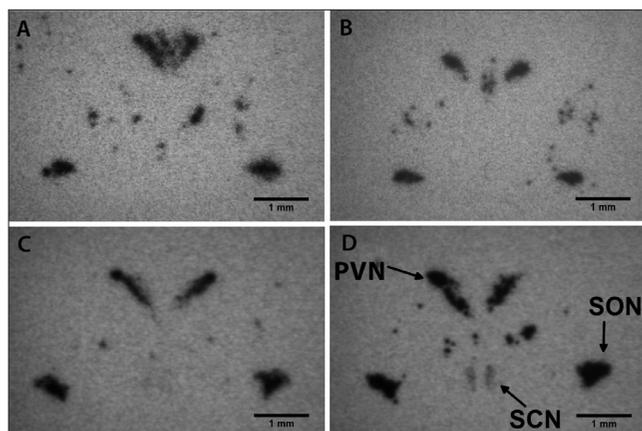


Fig. 2. Representative autoradiographs from *in situ* hybridization. Oxytocin mRNA expression in control rats (A) and in prenatally testosterone-exposed rats (B), and arginine-vasopressin mRNA expression in control rats (C) and in prenatally testosterone-exposed rats (D) in the hypothalamic nuclei. Structures: (SON) supraoptic nucleus, (SCN) supra-chiasmatic nucleus, (PVN) paraventricular nucleus.

were observed in AVP and OT expression in hypothalamic nuclei in the Ctrl group, and these results are in accordance with previously published studies (Dumais et al. 2013, Dumais and Veenema 2016, Paul et al. 2014, Taylor et al. 2012). The testosterone treatment also did not influence AVP and OT expression in hypothalamic nuclei in a sex-specific way in our study. Changes in neuropeptide expression resulting from developmental exposure to testosterone were observed in a similar fashion in both sexes. OT and AVP are able to act via both oxytocin as well as vasopressin receptors (Kimura et al. 1994, Koehbach et al. 2013, Song et al. 2014). We observed a decrease in OT mRNA expression in the PVN and SON and an increase in AVP mRNA expression in SCN and SON in the T group of rats of both sexes. It is therefore possible that a sex difference observed in behavior is a result of an interaction between sex-specific receptor density in the brain (Dumais and Veenema 2016) and a possible compensatory mechanism of elevated AVP expression acting via OT receptors. However, further research is necessary to examine this idea.

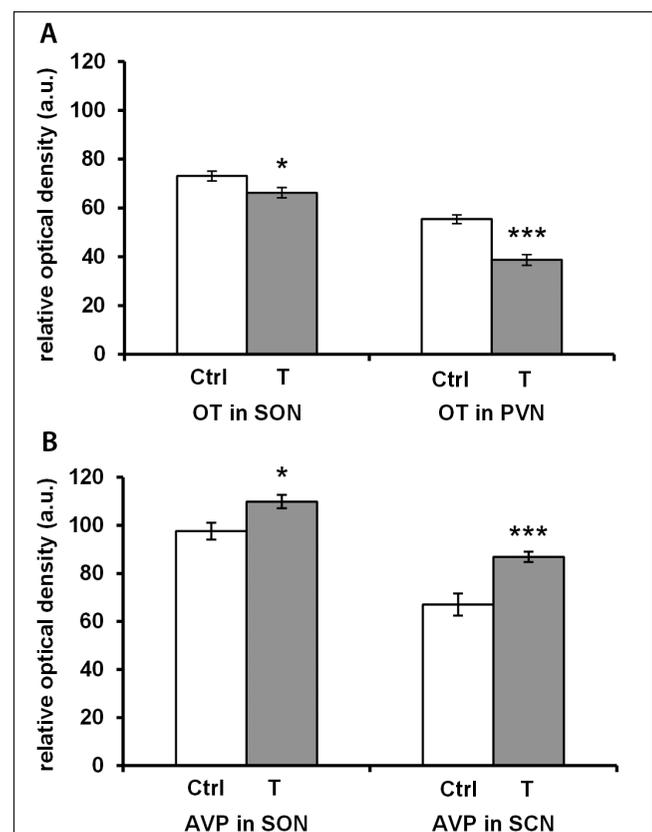


Fig. 3. Oxytocin (OT, A) and arginine-vasopressin (AVP, B) mRNA expression in selected brain regions (SON – supraoptic nucleus, PVN – paraventricular nucleus, SCN – supra-chiasmatic nucleus) of control (Ctrl, $n=32$) and prenatally testosterone-exposed (T, $n=32$) rats. Data represent mean \pm SEM. Asterisks indicate significant differences between Ctrl and T group ($*p<0.05$, $***p<0.001$).

CONCLUSIONS

Application of testosterone during the third part of rat gravidity had an impact on social coping strategies in adult males but not females, as measured during a social interaction test. Changes in AVP and OT expression, however, were found in the hypothalamic nuclei in both sexes during adulthood. Data obtained in this study support the commonly-accepted theory that testosterone is an important mediator of maternal effects in mammals, and has long-lasting effects on the development of rat offspring.

ACKNOWLEDGMENTS

This study was supported by the VEGA 2/0166/16 and VEGA 1/0557/15 grants from Scientific Grant Agency of Ministry of Education, Science, Research and Sport of the Slovak Republic and APVV-0291-12 from Slovak Research and Development Agency. We thank to M.Sc. Petra Mikušová and M.Sc. Katarína Bučková for their help with behavioral analysis and professor D. Ježová (Institute of Experimental Endocrinology, Biomedical Research Centrum, Slovak Academy of Sciences, Bratislava, Slovakia) for providing the AVP probe.

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