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Original Article

Modifications of anxiety-like behavior in prenatally stressed male offspring with imbalance of androgens

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Abstract: Gonadal hormones have been well-known to affect brain regions known to be involved in the modulation of mood and affective-related behavior. Prenatal stress might alter hypothalamic-pituitary-gonadal axis, it could be a target for development of affective-related disorders in male offspring. The present study was designed to examine an anxiety-like behavior in the adult male offspring with low levels of endogenous androgens delivered from pregnant dams exposed to prenatal stress from gestation day 15 to gestation day 19. The non-stressed and prenatally stressed intact, gonadectomized (GDX) and GDX male offspring treated with oil solvent or testosterone propionate (TP, 0.5 mg/kg, s.c., 14 days, once daily) were used in all experiments. Anxiety-like behavior was assessed in the elevated plus maze (EPM) and the open field test (OFT), respectively. Also, testosterone levels in the blood serum were measured in all experimental groups of offspring. Prenatally stressed GDX offspring demonstrated a significant decrease for time spent into the open arms and increase for time spent into the closed arms as compared to the non-stressed offspring. Administration of TP to the prenatally stressed GDX offspring resulted in a more markedly decrease of the time spent into the open arms and significantly raised the time spent into the closed arms as compared to the non-stressed GDX offspring treated with TP, non-stressed/prenatally stressed GDX offspring. Prenatally stressed GDX offspring showed a significant increase of crossing, rearing, grooming and defecation as compared to the prenatally stressed control offspring. On the contrary, administration of TP to the prenatally stressed GDX offspring significantly decreased crossing behavior, frequency of rearing and grooming behavior as compared to the non-stressed GDX offspring treated with TP, non-stressed/prenatally stressed GDX offspring. Prenatally stressed GDX offspring demonstrated a significant decrease of testosterone levels as compared to the non-stressed/prenatally stressed intact offspring, as well as non-stressed GDX offspring. Administration of TP significantly increased testosterone levels when prenatally stressed GDX offspring were compared with the prenatally stressed intact offspring, non-stressed/prenatally stressed GDX offspring. Thus, the results of the study clearly suggest that gonadectomy and TP supplementation profoundly changed an anxiety-related behavior in prenatally stressed male offspring in the EPM. Our current findings suggest that androgen deficiency in the prenatally stressed male offspring produces the high anxiety level and induces a marked anxious-like state. TP supplementation provokes development of profoundly anxious-like state in the prenatally stressed male offspring, Furthermore, this is the first study to show anxiogenic-like effect of TP administration on anxiety-related states in prenatally stressed male offspring with androgen deficiency.

Keywords: Prenatal stress, anxiety, behavior, gonadectomy, testosterone, male offspring

Introduction

Numerous clinical and preclinical studies clearly demonstrate that exposure to maternal stress during early life programmes the brain and subsequent behavior [1-5]. The offspring exposed to prenatal stress show the abnormal psychiatric behaviors such as increased fear

and anxiety, persistent paradoxical sleep alterations, deficits of learning and memory, depressive-like behavior and schizophrenia-like behavior [1, 2, 6-9]. Evidence suggests that altered neurobiology of prenatally stressed rats develops from exposure of the fetus to high plasma levels of endogenous glucocorticoids released by the mother under chronic stress (Brunton, Russell, 2010) [10]. Nonetheless, the precise mechanisms accounting for impact of prenatal stress on the life of adulthood have not fully understood.

The hypothalamo-pituitary-adrenal (HPA) axis is particularly sensitive to programming [11, 12], which may link fetal programming and increased susceptibility of offspring to adulthood cardiometabolic and neuropsychiatric pathologies [11-15]. It is generally accepted that a dysregulation of the HPA axis is one of the most commonly described alterations that correlate with symptoms of mood disorders and other neuropsychiatric and neurodegenerative diseases [16]. On the other hand, gonadal steroids can modulate HPA axis activity [17, 18]. Effects of the gonadal hormones on HPA function have been demonstrated at different levels of the axis [19]. In general, androgens inhibit HPA activity. Androgens inhibit corticotrophin releasing hormone (CRH) expression, and gonadectomy of adult male rats increases both adrenocorticotropin (ACTH) and CORT responses to physical and psychological stressors [20], an effect reversed by replacement with testosterone or the non-aromatizable androgen, 5β-dihydrotestosteone (5β-DHT). GDX rats also show greater stress-induced c-Fos expression and higher arginine vasopressin hnRNA levels than intact males, both of which are negatively correlated with plasma testosterone levels [21-23]. The gonadal and stress hormone systems have been shown to interact with each other, resulting in reciprocal modulation of each other's expression and function. These interactions are likely to be particularly important for development of numerous neuropsychiatric disorders.

On the other hand, gonadal hormones have been shown to affect brain regions known to be involved in the modulation of mood and affective-related behavior [24, 25]. Since, prenatal stress might alter hypothalamic-pituitary-gonadal (HPG) axis, it could be a target for development of affective-related disorders in male offspring [1, 2, 15, 26]. There is growing evidence that exposure to prenatal stress reduces the capacity of the brain for neurosteroidogenesis and may also alter the ability of gonadal steroids to exert their actions [27, 28].

Data from animal studies show that prenatally stressed male offspring with normal androgen levels have demonstrated anxiety-like behavior alterations in the different models of anxiety [1-3, 29-32]. However, little is known about anxiety-like behavior in the adult prenatally stressed male offspring with androgens imbalance. Based on the interaction between HPA and HPG systems, we hypothesized that castration or testosterone supplementation would change anxiety-like behavior in prenatally stressed male offspring. The role of androgens in anxiety-related disturbances for prenatally stressed male offspring during postnatal development warrant further study.

The present study was conducted to investigate an anxiety-like behavior in the adult male offspring with low levels of androgens delivered from pregnant females exposed to prenatal stress.

Materials and methods

Animals

Twelve adult female Wistar rats (180-200 g; Koltuschi, St. Petersburg, Russia) were used in the present study. Rats were kept under standard laboratory conditions in a 12 h light/dark schedule (lights on 8:00 a.m.) with *ad libitum* access to rat chow and tap water, constant temperature of 23 ± 1°C and 60% humidity. All experiments were carried out in accordance with the guide for care and use of Laboratory Animals published by the National Institute of Health (National Research council, publication No 85-23, revised in 1996). The rationale, design and methods of this study have been approved by the Ethical Committee for Animal Research, I.P. Pavlov Institute of Physiology. All efforts were performed to minimize the pain and stress levels experienced by the animals.

All animals were gently handled by experienced keepers from the facility each day for a week prior to experimental procedures. Any environmental or physical stress was avoided in order

to habituate the rats to manipulation. Animals were randomly assigned to experimental groups and were used only once in the behavioral experiments. The behavioral tests were conducted between 09:00 AM and 13:00 PM. Experiments were carried out in a soundproof and air-regulated experimental room, to which animals were habituated at least 30 min before each test.

Pregnancy

Females were group-housed (six per cage for one week) to co-ordinate their estrous cycle. They were then placed with a sexually experienced male for an estrous cycle (5 days). In the sixth day, (considered gestational day one), the male was removed and the females were transferred to individual cages until delivery. Pregnant dams were then randomly assigned to either the control or the prenatal stressed group and were individually housed with *ad libitum* access to rat chow and tap water, constant temperature of 23 ± 1°C and 60% humidity. Control pregnant dams were left undisturbed in the home cage, while other pregnant dams were subjected to a prenatal stress procedure. During pregnancy, cage cleaning procedures were performed three times a week.

Prenatal stress procedure

Prenatal stress was conducted as previously described [29-32] from gestation day 15 to 19, pregnant female rats were randomly subjected to stress session starting between 09 am and 13 pm daily, during which they were placed in plastic transparent cylinders (diameter 7 cm; length 20 cm) and exposed to bright light for 60 min. This time period during pregnancy is when stress can affect offspring outcomes resulting in anxiety-like behavior [29-32]. No other subjects were present in the experimental room during stress exposure. At the end of the stress session, the rats were returned to the animal housing room and were then individually housed with free access to food and water.

The day after delivery, all the offspring were sexed. Only male offspring together with prenatal stressed dams were weaned at postnatal 30 days, the remaining males being disposed. Then, 30 days old male offspring were removed from their mother and grouped for the behavioral experiments. Male offspring from each experimental group were housed in groups of 5 and maintained under the same laboratory conditions throughout the study. At 3 months of age, female offspring from prenatal stressed groups were subjected to the castration.

Gonadectomy

The non-stressed and prenatally stressed male offspring were anesthetized with a mixture of ketamine/xylazine (70 mg/kg of ketamine and 10 mg/kg xylazine; i.p.) and bupivicaine (0.25% solution; 0.4 mL/kg) was applied topically as analgesic. The non-steroidal anti-inflammatory drug meloxicam (1.0 mg/mL) was injected subcutaneously. Following disinfection of the skin (with alcohol and betadine), a 1-2 cm ventral midline incision was made in the scrotum of adult male rats to expose the tunica. The tunica was pierced and both testes were extracted to expose the underlying blood vessels, which were ligated with silk suture. The testes were excised and all vessels and ducts were placed back into the tunica prior to suturing. Sham-operated animals received incisions with no testicle removal. Rats were allowed to recover for 2 weeks before any behavioral testing was performed. The effectiveness of castration or exogeneous administration of TP were determined after behavior upon sacrifice, and bulbospongiosus muscles were dissected from the penile bulb and immediately weighed.

Experimental groups

After gonadectomy, non-stressed and prenatally stressed GDX male offspring were retrieved in a community cage with other rats with free access to food and water. A non-stressed (NS/GDX) and prenatally stressed (PS/GDX) male offspring were allowed to have 14 days for post-operative recovery before administration of oil solvent or TP. After two weeks, all NS/GDX and PS/GDX male offspring were randomly assigned to each of the experimental group and subjected to behavioral testing. All male offspring were divided into 6 groups (n = 10 per group). The first group consisted of NS/sham-operated intact males (control). The two other groups were of NS/GDX male offspring treated with oil solvent daily and NS/GDX male offspring injected with TP in a dose of 0.5 mg/kg, daily. The next group was consisted of PS/sham-operated intact males (control). The two other groups were of PS/GDX male offspring treated with oil

solvent daily and PS/GDX male offspring injected with the same dose of TP, daily.

Testosterone propionate supplementation

Testosterone propionate (TP) (T-1875, Sigma Chemical Co) was dissolved in sterile sesame oil. TP was injected subcutaneously (s.c.) at a dose of 0.5 mg/kg body weight, in a volume of 0.1 mL. After the 14 days of postoperative recovery after gonadectomy, TP and oil solvent were injected once daily for 28 days.

Behavioral tests

Animals were handled daily for 1 week before testing to adapt them to handling and introduce them to experimental room. Behavioral experiments were carried out in a soundproof and air-regulated experimental room, to which animals were habituated, at least 30 min before each test. Any environmental or physical stress was avoided in order to habituate the rats to manipulation for behavioral tests. One hour after the last injection, testing in the elevated plus maze (EPM) and the open field test (OFT) was carried out as described below. During all behavioral tests the control and experimental groups of rats were also treated with TP or oil solvent.

Elevated plus maze test

To investigate the changes in anxiety-like behavior, all experimental groups of non-stressed and prenatally stressed offspring were subjected to the elevated plus maze (EPM) [33]. EPM is a widely used test of anxiety-like behavior and was used to assess an anxiety-like behavioral response [34]. This test is sensitive to putative anxiogenic-like and anxiolytic-like drugs [35]. It is designed to present the animal with a conflict between its natural tendency to explore a novel environment and its reluctance to move away from the sheltering walls and into the open environment in which the risk of falling or exposure to predators is much higher. The maze was made of grey Plexiglas and consisted of four arms (50 cm long and 10 cm wide); two arms had 40-cm-high dark walls (closed arms), and two arms had 0.5-cm-high ledges (open arms). In the center of the arms of EPM located crosswise there was an open area in the size of 10 × 10 cm. The floor of the apparatus was 50 cm high. The experimental room was lit by a 60-W bulb placed 1.75 m above the central square of the maze (22 lx in the maze central square). For testing, rats were placed individually into the center of the maze facing a closed arm and removed after a 5-min period. The number of entrances and the time spent into the open or closed arms were registered during time of testing. A video camera was installed above the cage to record the activity of the rats. Two independent observers measured the behavioral variables. After each test session, the EPM was carefully cleaned and deodorized with a cleaning solution (Vekton, Russia, composition is ammonia 0.5%, ethanol 15%, extran 10%, isopropyl alcohol 5%, citrus aromatizing 19%, and distilled water 50.5% as v.v%).

Open field test

To investigate the changes in spontaneous locomotor activity, grooming, and rearing, all experimental groups of offspring were submitted to a 5-min period to the open field test (OFT). The OFT consisted of a square platform (80.0 cm × 80.0 cm; wall height 36.0 cm) [36]. The floor of the platform was divided into 16 equal squares of 19.5 cm × 19.5 cm. The platform was illuminated by a light source (lamp 60 W). Two independent observers measured the behavioral variables. A video camera was installed above the cage to record the activity of the rats. After each test session, the OFT was carefully cleaned and deodorized with a cleaning solution (Vekton, Russia, composition is ammonia 0.5%, ethanol 15%, extran 10%, isopropyl alcohol 5%, citrus aromatizing 19%, and distilled water 50.5% as v.v%).

Testosterone ELISA

Approximately 5 ml of blood samples were drawn from animals anesthetized with ketamine (5.0-10 mg/kg, i.m.). After centrifugation, plasma samples were used for the measurement of T levels using a commercially available ELISA kit (DRG Diagnostics, Marburg, Germany). Technical variability was low with coefficients of variation of 3% intra-assay and 5% inter-assay. The sensitivity of the ELISA was 0.083 ng/ml.

Statistical analysis

All values were expressed as mean ± S.E.M. Comparisons between values were performed using two-way ANOVA test with between sub-

Table 1. Effects of prenatal stress procedure on behavior of GDX male offspring and GDX male offspring treated with testosterone propionate in the elevated «plus» maze

Groups	Time spent into the open arms	Time spent into the closed arms	The number of entries into the open arms	The number of entries into the closed arms
NS/intact offspring	86.4 ± 6.2	213.6 ± 2.4	1.8 ± 0.2	2.1 ± 0.4
NS/GDX offspring (NS/GDX/oil solvent)	44.0 ± 3.2*	256.0 ± 5.6*	0.5 ± 0.2*	4.0 ± 0.2*
NS/GDX offspring + TP (NS/GDX/TP)	78.0 ± 4.4**	222.0 ± 4.6**	1.4 ± 0.2**	2.2 ± 0.2**
PS/intact offspring	108.6 ± 3.4*	191.4 ± 4.6*	1.5 ± 0.2	2.2 ± 0.2
PS/GDX offspring (PS/GDX/oil solvent)	27.5 ± 3.2$^{*,**,\#}$	272.5 ± 18.8$^{*,**,\#}$	0.7 ± 0.2$^{*,\#}$	5.7 ± 0.6$^{*,**,\#}$
PS/GDX offspring + TP (PS/GDX/TP)	12.8 ± 2.2$^{*,**,\#,\#\#}$	287.2 ± 22.6$^{*,**,\#,\#\#,\#\#\#}$	0.4 ± 0.2$^{*,\#}$	6.3 ± 0.6$^{*,**,\#,\#\#,\#\#\#}$

*P<0.05 as compared to the control group of NS/intact offspring, **P<0.05 as compared to the NS/GDX/oil solvent offspring, $^{\#}$P<0.05 as compared to the control group of PS/intact offspring, $^{\#\#}$P<0.05 as compared to the PS/GDX/oil solvent offspring, $^{\#\#\#}$P<0.05 as compared to the NS/GDX/TP offspring. Each group comprised a minimum of 10 rats. TP was given at 0.5 mg/kg, s.c., once daily during 14 days. The obtained results show the mean ± S.E.M.

ject factors for hormone treatment (oil or TP) and stress procedure (non-stressed or prenatally stressed) followed by Dunnett's test for multiple comparisons post-hoc test. Statistical analysis was performed using SPSS version 11.5.

Results

We investigated the effects of prenatal stress on anxiety-like behavior in GDX male offspring with oil or TP supplementation. In order to perform this, the source of endogenous androgens was removed by gonadectomy in male offspring and the behavioral results from NS and PS GDX male offspring were compared. Anxiety-like behavior was assessed using the EPM and the behavioral reactivity was tested in the OFT. Our previous studies have found that PS/intact males demonstrated a low levels of anxiety-like behavior in the EPM and the OFT as compared to the NS/intact offspring [29-32]. Moreover, our studies have demonstrated that administration of sesame oil alone failed to cause any effect on behavioral values of NS/GDX rats (data are not shown).

Anxiety-like behavior of non-stressed and prenatally stressed GDX offspring in the elevated plus maze

A two-way ANOVA revealed the significant differences in the time spent into the open arms and closed arms between stress procedure ([F(3,24) = 4.9, P<0.05 and [F(3,24) = 7.24, P<0.01], respectively), between hormone treatment [F(3,24) = 24.44, P<0.0003 and F(3,24) = 5.26, P<0.05], respectively), and an interaction between stress procedure and hormone treatment ([F(3,24) = 1.84, P<0.001] and [F(3,24) = 11.18, P<0.05], respectively) in the GDX offspring. The post-hoc test revealed differences among the groups for the time spent into the open arms and closed arms in the EPM (P< 0.05). The NS/GDX male offspring demonstrated a significant decrease of the time spent into the open arms and increase the time spent into the closed arms as compared to the NS/control rats (**Table 1**, P<0.05). TP (0.5 mg/kg, s.c.) supplementation in NS/GDX offspring increased the time spent into the open arms and decreased the time spent into the closed arms as compared to the NS/GDX/oil solvent offspring. Prenatal stress procedure in sham-operated rats induced a significant increase of the time spent into the open arms and decrease the time spent into the closed arms as compared to the NS/intact offspring (**Table 1**, P<0.05). PS/GDX/oil solvent offspring demonstrated a significant decrease of the time spent into the open arms and increase of the time spent into the closed arms as compared to the NS/GDX/oil solvent offspring (**Table 1**, P<0.05). Administration of TP in the PS/GDX offspring resulted in a more markedly decrease of the time spent into the open arms and significantly raised time spent into the closed arms as compared to the NS/GDX/oil solvent offspring, NS/GDX/TP offspring and PS/GDX/oil solvent offspring (**Table 1**, P<0.05).

The significant differences in the number of entrances into the open and closed arms between hormone conditions between stress procedure ([F(3,24) = 7.40, P<0.05 and [F(3,24) = 11.04, P<0.05], respectively), between hormone treatment [F(3,24) = 7.44, P<0.05 and F(3,24) = 5.22, P<0.001], respectively), and an interaction between stress procedure and hor-

Table 2. Effects of prenatal stress procedure on behavior of GDX male offspring and GDX male offspring treated with testosterone propionate in the «open field» test

Groups	Crossing	Rearing	Grooming	Defecation
NS/intact offspring	57.6 ± 2.8	10.1 ± 1.2	1.8 ± 0.2	2.1 ± 0.4
NS/GDX offspring (NS/GDX/oil solvent)	62.4 ± 2.2	12.6 ± 2.0	0.5 ± 0.2*	4.0 ± 0.2*
NS/GDX offspring + TP (NS/GDX/TP)	60.2 ± 2.2	11.9 ± 2.2	1.4 ± 0.2**	2.2 ± 0.2**
PS/intact offspring	63.7 ± 3.4	4.4 ± 0.4*	1.5 ± 0.2	1.0 ± 0.2*
PS/GDX offspring (PS/GDX/oil solvent)	73.6 ± 2.4$^{*,**,\#}$	8.3 ± 0.8$^{*,**,\#}$	3.6 ± 0.2$^{*,**,\#}$	1.1 ± 0.2*,**
PS/GDX offspring + TP (PS/GDX/TP)	47.3 ± 2.2$^{*,**,\#,\#\#}$	2.8 ± 0.6$^{*,**,\#,\#\#}$	1.6 ± 0.2$^{**,\#\#}$	0.5 ± 0.2$^{*,**,\#,\#\#}$

*P<0.05 as compared to the control group of NS/intact offspring, **P<0.05 as compared to the NS/GDX/oil solvent offspring, $^{\#}$P<0.05 as compared to the control group of PS/intact offspring, $^{\#\#}$P<0.05 as compared to the PS/GDX/oil solvent offspring. Each group comprised a minimum of 10 rats. TP was given at 0.5 mg/kg, s.c., once daily during 14 days. The obtained results show the mean ± S.E.M.

mone treatment ([F(3,24) = 5.84, P<0.001] and [F(3,24) = 9.40, P<0.05], respectively) in the GDX offspring. The post-hoc test revealed differences among the groups for the number of entrances into the open and closed arms in the EPM (P<0.05). A significant decrease of the number of entrances into the open arms and enhanced number of entrances into the closed arms were registered in the PS/GDX/oil solvent offspring as compared to NS/GDX offspring (**Table 1**, P<0.05). Animals in the NS/GDX/TP group demonstrated the increased number of entrances into the open arms and the decreased number of entrances into the closed arms as compared to the NS/GDX/oil solvent offspring (**Table 1**, P<0.05). However, PS/GDX/TP offspring demonstrated profoundly reduction in the number of entrances into the open arms and increase for the number of entrances into the closed arms as compared to the NS/GDX/oil solvent offspring, NS/GDX/TP offspring and PS/GDX/oil solvent offspring (**Table 1**, P<0.05).

Behavior of non-stressed and prenatally stressed GDX offspring in the open field test

In the experimental groups of GDX offspring, a significant difference in the crossing, rearing and grooming was observed between hormone conditions ([F(3,24) = 4.17, P<0.008], [F(3,24) = 7.40, P<0.01], [F(3,24) = 11.04, P<0.01], respectively), between drug treatments [F(3,24) = 15.4, P<0.001], [F(3,24) = 11.56, P<0.05], [F(3,24) = 8.01, P<0.002], respectively), and an interaction between hormone condition and treatments ([F(3,24) = 3.8, P<0.001], [F(3,24) = 5.46, P<0.0003], [F(3,24) = 4.02, P<0.008], respectively). The post-hoc test revealed differences among the groups for all behavioral parameters in the OFT (P<0.05). Treatment with TP in NS/GDX male offspring led to increase of grooming behavior and decrease of defecation as compared to the control NS/GDX male offspring. PS/intact rats showed a significant decrease of the frequency of rearing and defecation as compared to the NS/control offspring (**Table 2**, P<0.05). The post-hoc test revealed that PS/GDX/oil solvent offspring demonstrated a significant increase of crossing, rearing, grooming and defecation as compared to the PS/intact offspring (**Table 2**, P<0.05). Also, the crossing and the grooming in PS/GDX rats were higher while the rearing and defecation were lower as compared to the NS/intact and NS/GDX offspring. On the contrary, administration of TP to the PS/GDX offspring significantly decreased crossing behavior, frequency of rearing and grooming behavior as compared to the NS/intact offspring, NS/GDX/oil solvent offspring, NS/GDX/TP offspring or PS/GDX/oil solvent offspring (**Table 2**, P<0.05).

Testosterone levels of non-stressed and prenatally stressed GDX offspring

A two-way ANOVA revealed significant differences in T levels between stress procedure ([F(3,24) = 7.9, P<0.05 and [F(3,24) = 17.4, P<0.05], respectively), between hormone treatment [F(3,24) = 28.12, P<0.05 and F(3,24) = 5.84, P<0.001], respectively), and an interaction between stress procedure and hormone treatment ([F(3,24) = 5.84, P<0.001] and [F(3,24) = 9.40, P<0.05], respectively) in the GDX offspring. The post-hoc test revealed differences among the groups for T levels in the ELISA test (P<0.01). This statistical test found that NS/GDX/oil solvent rats showed a pro-

Table 3. Effects of prenatal stress procedure on testosterone levels of GDX male offspring and GDX male offspring treated with testosterone propionate in ELISA test

Groups	T, nmol/l
NS/intact offspring	5.9 ± 0.6
NS/GDX offspring (NS/GDX/oil solvent)	2.5 ± 0.2*
NS/GDX offspring + TP (NS/GDX/TP)	5.3 ± 0.6**
PS/intact offspring	3.1 ± 0.4*
PS/GDX offspring (PS/GDX/oil solvent)	1.4 ± 0.2*,**,#
PS/GDX offspring + TP (PS/GDX/TP)	5.2 ± 0.6**,#,##

*P<0.05 as compared to the control group of NS/intact offspring, **P<0.05 as compared to the NS/GDX/oil solvent offspring, #P<0.05 as compared to the control group of PS/intact offspring, ##P<0.05 as compared to the PS/GDX/oil solvent offspring. Each group comprised a minimum of 10 rats. TP was given at 0.5 mg/kg, s.c., once daily during 14 days. The obtained results show the mean ± S.E.M.

found decrease in the T levels as compared to the NS/intact control offspring (P<0.05, **Table 3**). TP supplementation caused a significant increase of T levels when NS/GDX/TP offspring were compared with the NS/GDX/oil solvent rats (P<0.05, **Table 3**). T levels was significantly lower in PS/intact rats than that of the NS/intact control offspring (P<0.05, **Table 3**). PS/GDX/oil solvent offspring demonstrated a significant decrease of T levels as compared to the NS/intact or PS/intact offspring, as well as NS/GDX/oil solvent offspring (**Table 3**, P<0.05). Administration of TP significantly increased T levels when PS/GDX offspring were compared with the PS/intact offspring, PS/GDX/oil solvent or NS GDX/oil solvent offspring (**Table 3**, P<0.05).

Discussion

The major findings of this study are following: PS/GDX offspring showed higher levels of anxiety-related behavior than NS/GDX, TP supplementation does not have similar effects on anxiety-related behavior in NS/GDX and PS/GDX offspring, as well as TP administration produces anxiogenic-like effect in PS/GDX offspring in the EPM.

The EPM is recognized as a valuable model able to predict anxiolytic- or anxiogenic-like effects of drugs in rodents [37, 38]. The EPM demonstrated that gonadectomy induces an anxiety-like profile in the EPM in our study. Two weeks of TP supplementation had an anxiolytic-like effect in the GDX rats, but it was not able to diminish anxiety-like state level up to the level of control sham-operated animals. Our findings confirm previous numerous studies indicating that androgens have anti-anxiety effects [39-42]. Testosterone and its metabolites have been shown to possess anxiolytic-like properties in several animal models of anxiety, reducing anxiety-like behavior in male rodents [41, 43]. Moreover, testosterone replacement in castrated males ameliorates anxiety-like behavior in the EPM) and OFT [43, 44]. Testosterone may either be converted to dihydrotestosterone by 5α-reductase or aromatized to estradiol by the enzyme aromatase [41]. Estradiol stimulates estradiol receptor α and β and produces anxiolytic-like behavior in rats [45, 46]. The non-aromatizable androgen receptor agonist dihydrotestosterone also exerts anxiolytic-like properties [43, 47]. Moreover, dihydrotestosterone can be metabolized by the enzyme 3α-hydroxysteroid dehydrogenase to 3a-diol (5α-androstane-3α, 17β-diol), which induces anxiolytic-like behavior [43]. The non-steroidal androgen receptor antagonist flutamide, antagonizes the anxiolytic-like effect of dihydrotestosterone and testosterone in the GDX rats, indicating that androgen receptors may be involved in the underlying mechanisms [47-49]. However, the precise mechanisms by which testosterone produces anxiolytic-like behavior are not well known.

The PS/GDX male offspring demonstrated a marked decrease of the time spent on and the number of entrances into the open arms of the EPM, indicating in a more significant anxiogenic-like effect of androgens deficiency in PS/GDX offspring than that in NS/GDX male rats. Moreover, it should be emphasized that TP supplementation failed to produce anxiolytic-like effect in PS/GDX offspring, like it was noted in NS/GDX male offspring. Importantly, that TP supplementation in the PS/GDX offspring resulted in highest level of anxiety-related behavior than that in NS/GDX offspring. This fact suggests that TP in PS/GDX offspring behaves itself as anxiogenic-like treatment, rather than as anxiolytic-like treatment in NS/GDX offspring.

When the results from the NS and PS/GDX offspring treated with oil or TP from the EPM and the OFT are considered together, they indicate

that prenatal stress influences anxiety-related profile rather than motor, grooming or emotional behaviors. There is evidence that grooming abnormally increases in rats with low levels of stress, while in animals submitted to high stress (such as the electric footshock or exposure to predator odors) grooming is significantly diminished [50, 51]. This reduction of grooming is prevented by anxiolytic or antidepressant drugs, which return grooming to control values [50]. In addition, increased rearing is interpreted as an indicator of exploration; an effect enhanced by anxiolytic-like substances in the OFT [50]. The results of the OFT in our study indicated that PS/GDX offspring showed the increasing of general locomotor activity (crossing and rearing), as well as grooming behavior. Nevertheless, the PS/GDX male offspring did not demonstrate a low anxiety level in the EPM. Furthermore, this fact additionally proves that anxiogenic-like state of PS/GDX offspring is associated with direct or indirect actions of prenatal stress on the pathophysiological mechanisms of anxiety-related behavior in the brain of the GDX male offspring. However, we may suppose that decreasing total behavioral reactivity of PS/GDX offspring treated with TP in the OFT could promote the anxious-like to some extent, however, further experimental studies should be carried out to further evaluate the interaction between anxiety-like state and modifications of behavioral reactivity in the PS/GDX offspring given with TP. Different dosages and duration of TP treatment in the PS/GDX offspring should be tested in the future study.

Hormonal data showed that PS/intact males demonstrated a low T levels in the blood as compared to the NS/intact offspring, as we have found previously [31, 32]. Gonadectomy per se more significantly decreased T levels in the PS/GDX male offspring than that in the NS/GDX offspring. Interestingly, administration of TP in the PS/GDX male offspring completely restored the T levels in the blood up to value in the NS/GDX rats. Although, T levels in NS and PS/GDX offspring treated with TP supplementation were practically similar, we observed different anxiety-like state in NS and PS/GDX offspring given with TP in the EPM (low or high levels of anxiety, respectively). Thus, we can conclude that modifications of anxiety-related behavior after gonadectomy or TP supplementation of the PS male offspring are not determined the hormonal status of these rats. Our future investigations will aim to clarify how prenatal stress alters LH and corticosterone levels in the blood and the brain in male offspring with an imbalance of androgens.

Affective disorders are commonly associated with a dysregulation of the HPA axis [16-18]. Further, prenatal stress and prenatal overexposure to glucocorticoids can impact adult behaviors and neuroendocrine responses to stress [1-4, 52]. An anxiety-like phenotype is frequently observed in animals exposed to early life stress. This has been demonstrated by increased ultrasonic vocalisations in neonates, reduced social play during adolescence, reduced open arm entries on the elevated-plus maze test and decreased exploration in an open field [4, 5, 14, 53-55]. Organizational and activating effects of gonadal steroid hormones have been shown for the regulation of HPA axis function and may underlie increased risk of affective disorders [20]. Testosterone suppresses HPA responses to acute stress [20, 22]. In male rats, the suppressive actions of testosterone on stress-induced HPA axis activity are mediated by the 5α-reduced metabolite of testosterone, DHT and its metabolite, 3β-diol [56, 57]. Whether this is the result of a direct action of testosterone or an indirect action via one of its metabolites, e.g. DHT, 3α-diol or 3β-diol, is not definitely known. Moreover, treatment with the 5α-reductase inhibitor, finasteride results in enhanced/prolonged ACTH and corticosterone responses to acute stress [58]. Further, gonadectomy of male rats increases neuroendocrine responses to stress and correspondingly, c-fos mRNA expression, an indicator of neuronal activity, is elevated in PVN neurons indicating an involvement of testosterone [16, 22, 23, 59]. These effects of androgens in reducing the reactivity of the HPA axis are not through the aromatization to estrogens since the nonaromatizable androgen, dihydrotestosterone (DHT) also reduces ACTH and corticosterone responses to stress [22, 23]. Androgens also affect CRH-ir and vasopressin mRNA expression within the PVN. Androgens might regulate PVN neuropeptide expression and secretion trans-synaptically, through projections from the preoptic area and bed nucleus of the stria terminalis, with the caveat that the direction of this regulation is conflicted by studies showing both enhancement and inhibition of the expression

of neuropeptides that control the drive of PVN neuroendocrine neurons [22, 23, 60-62].

Thus, animal studies clearly demonstrate that exposure to stress during early life programs the brain and subsequent behaviour. HPA axis dysregulation is a common feature of the 'programmed' phenotype and may contribute to heightened anxiety behaviour and cognitive deficits. Similarly, stress exposure during development in humans seemingly increases the propensity for psychiatric disorders and cognitive impairments. Neuroactive steroids, including testosterone, play a critical role in brain development and can modulate HPA axis activity and influence anxiety behaviour and cognitive performance. There is growing evidence that exposure to stress in early life reduces the capacity of the brain for neurosteroidogenesis and may also alter the ability of neuroactive steroids to exert their actions. Given the role of testosterone in modulating neuroendocrine stress responses, anxiety behaviour as well as the findings that stress during early pre- or post-natal life alters neurosteroidogenesis, it can be hypothesised that altered neuroactive steroid production and/or action may modulate and alleviated some of the adverse effects of early life stress.

Conclusion

Our current findings suggest that androgen deficiency in the prenatally stressed male offspring produces the high anxiety level and induces a marked anxious-like state. TP supplementation provokes development of profoundly anxious-like state in the prenatally stressed male offspring, even than androgen deficiency per se. The anxiety-related state of prenatally stressed male offspring with androgen deficiency may not be explained only by its direct and/or indirect different actions on emotional functions of the brain and hormonal status. Taken together, it can be proposed that behavioral effects of TP in prenatally stressed male offspring with androgen deficiency on mood-related brain function after are connected with its modulating and complex action on the HPG and/or HPA systems. Furthermore, this is the first study to show anxiogenic-like effect of TP administration on anxiety-related states in prenatally stressed male offspring with androgen deficiency. The role of androgens imbalance in the development of anxiety-related disturbances of prenatally stressed male offspring during postnatal period warrant further study.

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Disclosure of conflict of interest

None.

Abbreviations

GDX, gonadectomized; EPM, elevated plus maze; HPA system, hypothalamo-pituitary-adrenal system; HPG system, hypothalamic-pituitary-gonadal system; OFT, open field test; TP, testosterone propionate.

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