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Haloperidol ameliorates androgen-induced behavioral deficits in developing male rats

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Abstract

The purpose of present study was to infer the potential effects of testosterone increase in some male-based childhood-onset neuropsychiatric disorders, such as Tourette syndrome. Thus, the influence of early postnatal androgen exposure upon the neurobehaviors and its possible neural basis were investigated in the study. Male pup rats received consecutive 14-day testosterone propionate (TP) subcutaneous injection from postnatal day (PND) 7. The TP treatment produced the hyperactive motor behavior and grooming behavior as well as the increased levels of dopamine, tyrosine hydroxylase and dopamine transporter in the mesodopaminergic system and the elevated levels of serotonin in the nucleus accumbens, without affecting the levels of glutamate, γ -aminobutyric acid, norepinephrine and histamine in the caudate putamen and nucleus accumbens of PND21 and PND49 rats. Dopamine D2 receptor antagonist haloperidol was administered to the early postnatal TP-exposed PND21 and PND49 male rats 30 min prior to open field test. Haloperidol significantly ameliorated the motor behavioral and grooming behavioral defects induced by early postnatal TP exposure. The results demonstrated that early postnatal androgen exposure significantly disturbed the brain activity of developing male rats via enhancing the mesodopaminergic activity. It was suggested that abnormal increments of testosterone levels during the early postnatal development might be a potential risk factor for the incidence of some male-based childhood-onset neuropsychiatric disorders by affecting the mesodopaminergic system.

Key Words

- ▶ haloperidol
- ▶ testosterone propionate
- ▶ neurobehavior
- ▶ dopaminergic system
- ▶ male rats

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Introduction

Sex differences in the incidence, onset or severity have been documented in the studies on childhood-onset neuropsychiatric disorders, such as Tourette syndrome (TS) (Sandyk *et al.* 1988). Male TS patients have a higher predominance, with the sex ratio of males to females reported ranging from around 3:1 to 10:1 (Freeman *et al.*

2000, Khalifa & von Knorring 2003, Jin *et al.* 2005). Tourette syndrome has a distinctive natural history with onset of tics in childhood, common exacerbation of tics near the onset of puberty and frequent moderation or resolution of tics as TS patients enter adulthood (Bloch & Leckman 2009). The increased testosterone is detected in

an extremely severe TS boy (Zou *et al.* 2011). Anti-androgen treatment alleviates the symptoms of TS patients (Peterson *et al.* 1998, Bortolato *et al.* 2007, Muroi *et al.* 2011) and administration of exogenous androgens exacerbates tic symptom (Leckman & Scahill 1990). The natural history, marked male predominance and symptom relief via anti-androgen treatment of TS suggest that the hyperandrogen environment during the early development might be involved in the regulation of the brain activity of male TS patients.

Brain activity depends on the balanced neurotransmissions. An imbalance in dopamine (DA) neurotransmissions leads to either hyperactive or hypoactive neuropsychiatric disorders (Mehler-Wex *et al.* 2006, Howes & Kapur 2009, Leckman *et al.* 2010, Chen *et al.* 2013). Elevation of DA, as a driver of psychosis, induces schizophrenia (Howes & Kapur 2009, Kegeles *et al.* 2010) and reduction of DA within the nigrostriatal pathway results in Parkinson disease (Mehler-Wex *et al.* 2006). The studies on TS patients show that dopaminergic (DAergic) dysfunction is implicated in the pathophysiology of TS (Steeves & Fox 2008, Wong *et al.* 2008, Leckman *et al.* 2010). Increased dopamine transporter-binding sites and amphetamine-induced increased release of DA are detected in TS patients or postmortem (Singer *et al.* 1991, 2002, Albin *et al.* 2003, Wong *et al.* 2008). Anti-DAergic agents reduce effectively tics (Scahill *et al.* 2006). The studies above demonstrate that the central DAergic activity is enhanced in TS patients.

Abnormal elevation in testosterone levels during the early postnatal development might account for a higher predominance and severity in some male-based childhood-onset neuropsychiatric disorder patients. Hyperandrogen environment in developing males might induce neurobehavioral deficits by altering the activity of central DAergic system, which might be retrieved by haloperidol (Hal) treatment. Haloperidol, a D2 receptor antagonist, is widely used for the treatment of schizophrenia, affective disorders and TS (Silay & Jankovic 2005, Termine *et al.* 2013, Egolf & Coffey 2014). It can control the tics and vocal utterances associated with TS (Tyler *et al.* 2017). Therefore, to infer the potential effects of testosterone increase in some male-based childhood-onset neuropsychiatric disorders, such as TS, the present study analyzed the alterations of neurobehaviors and neurotransmitters as well as the influence of Hal upon the neurobehavioral deficits in male rats affected with early postnatal androgen exposure.

Materials and methods

Animals

Pregnant Wistar rats were obtained from the Experimental Animal Center of Hebei Medical University during their first week of gestation and housed singly in an air-conditioned room ($22\pm 2^{\circ}\text{C}$) on a 12-h light-darkness cycle (lights on 06:00h). Food and water were available *ad libitum*. Pregnant females were monitored and the day of birth for pups was defined as postnatal day (PND) 0. In order to avoid any cohort effects, male pup rats of the same litter were randomly assigned to different treatment groups. Six male and 3 female pups were kept in the same cage with a dam and weaned on PND21. After weaning, the male rats were housed with four rats per cage and the female rats were not used in the study. All of the experimental procedures followed the rules in the 'Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research' and approved by the Committee of Ethics on Animal Experiments at Hebei Medical University.

Experiment 1

The purpose of Experiment 1 was to investigate the effects of early postnatal TP exposure on the neurobehaviors and neurochemicals of male rats. Twelve pregnant Wistar rats were used to collect 72 male pup rats. The male offspring were treated by the subcutaneous injection of TP (0.5 mg, 20 μL) once daily or sesame oil from PND7 to PND20 and were designated as TP group or control (C) group. Twelve rats in each group were killed at PND21, PND49 or PND168, which corresponded to 3-week (3 w) old, 7-week (7 w) old and 24-week (24 w) old, respectively. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used to measure the levels of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), glutamate (Glu), γ -aminobutyric acid (GABA), norepinephrine (NE) and histamine (His) in the caudate putamen (CPu) and nucleus accumbens (Acb). Based on the neurochemical results, real-time quantitative polymerase chain reaction (qPCR) and Western blot were used to detect the alterations of mRNA and protein levels of related markers in corresponding neural system. Furthermore, body weight, seminal vesicle and testis weights were documented when the rats were killed.

Experiment 2

The aim of Experiment 2 was to analyze if haloperidol might ameliorate the neurobehavioral defects in early postnatal TP-treated male rats based on the results of Experiment 1. Haloperidol was dissolved in physiological saline (Sal) and given to experimental animals according to the following experimental design. Eighty-four male pup rats from 14 mother rats were used. As described in Experiment 1, they received the subcutaneous injection of TP or sesame oil and fell into TP group or C group. At PND21 or PND49, prior to the open field test, they were further divided into following groups: C-Sal, TP-Sal, C-0.02Hal, C-0.15Hal, TP-0.02Hal and TP-0.15Hal. In each group, 7 rats were used. They received Sal treatment or Hal injection (0.02 or 0.15 mg/kg, i.p.) 30 min before the open field test at 1 mL/kg body weight (Hidaka *et al.* 2010).

Experiment 3

The aim of Experiment 3 was to analyze the effects of Hal treatment concurrent with TP treatment on the neurobehaviors of developing male rats. PND21 and PND49 male rats were used. They were randomly divided into C, C-TP, C-Hal and Hal-TP groups. There were 7 rats in each group. Thirty minutes before the open field test, the rats in C-TP, C-Hal and Hal-TP groups received one injection of TP (0.5 mg and 20 μ L), Hal (0.15 mg/kg) and Hal concurrent with TP treatment respectively. The rats in C group received corresponding vehicle treatment.

Open field test

Open field test was performed according to the procedures used in our previous study (Zhang *et al.* 2011). Prior to the behavioral test, all the rats for open field test were tail-marked and handled for 5 days and received 2-day accustomization to the open field apparatus for 5 min and 15 min respectively. The test was performed between 08:00 h and 11:00 h. Each rat was placed in the center of an open field chamber (100 $\times$ 100 $\times$ 40 cm) under a luminance of 20 lux. Fifteen-minute open field behavior of each rat was recorded for later analysis. Two types of behavioral patterns, motor behavior and grooming behavior, were analyzed in the study.

Sample preparation

Following open field test, 12 rats in each group of Experiment 1 were killed by decapitation. The brains

were removed quickly. The tissue block containing the substantia nigra (SN), ventral tegmental area (VTA), CPu or Acb was dissected according to The Rat Brain in Stereotaxic Coordinates (Paxinos & Watson 1997) on an ice cold plate using a scalpel for ophthalmic surgery and stereomicroscope. The tissue blocks were processed for LC-MS/MS, qPCR or Western blot analysis based on the study purposes.

LC-MS/MS assay

The tissue block of the CPu or Acb was weighed and homogenized in 80% acetonitrile containing 0.1% formic acid (5 μ L/mg). The homogenates were centrifuged at 21,100 *g* and 4°C for 10 min. The supernatants were collected and stored at -80°C. Dopamine, DOPAC, HVA, 5-HT, Glu, GABA, NE and His were determined by LC-MS/MS. The LC separation was performed on an Agilent 1200 LC system (Agilent) using a Synergi Fusion-RP C18 column (50 $\times$ 3.0 mm, 4 μ m) provided by Phenomenex. MS/MS detection was carried out using a 3200 QTRAP LC-MS/MS system (Applied Biosystems). The multiple-reaction monitoring mode was used for the quantification.

qPCR analysis

Total RNA (2 μ g) from the SN or VTA tissue block was subjected to reverse transcription using random primer to obtain the first-strand cDNA template. qPCR was performed with 0.8- μ L cDNA (diluted 1:10), 2- μ L specific primers and 2X GoTaq Green Master Mix (Promega) with a final volume of 20 μ L. PCR was performed as follows: an initial cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 58°C for 20 s and 72°C for 27 s. The products were then analyzed by melting curve to confirm the specificity of amplification. The expression of tyrosine hydroxylase (TH) and dopamine transporter (DAT) genes was analyzed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. For all samples, qPCR was performed in triplicate and the data were averaged. The relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method. The sets of primers were as follows: *Th* (5'-GCTTCTCTGACCAGGTGTATCG-3' and 5'-GCAATCTCTTCCGCTGTGTAT-3'), *Dat* (5'-ACTCTGTGAGGCATCTGTGTG-3' and 5'-TGTAAGTGGAGAAGGCAATCAG-3'), *Gapdh* (5'-TGAACGGGAAGCTCACTG-3' and 5'-GCTTCAACACCTTCTTGATG-3').

Western blot analysis

The CPU or Acb tissue blocks for detection of TH and DAT protein levels were homogenized in radioimmunoprecipitation assay (RIPA) buffer containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate and protease inhibitors (100- μ g/mL phenylmethanesulfonyl fluoride, 30- μ g/mL aprotinin and 1-mM sodium orthovanadate), and then sonicated for 4 $\times$ 10s. After centrifugation at 12,000g for 20 min at 4°C, the supernatant was collected and centrifuged again as described earlier. The resulting final supernatant was stored at -80°C until use. Samples from the CPU (50 μ g) or Acb (50 μ g) were diluted with 2 $\times$ sample buffer (50-mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β -mercaptoethanol) and heated for 5 min at 95°C before sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel, and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane was incubated for 2 h with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) (20-mM Tris-Cl, 137-mM NaCl, 0.1% Tween 20, pH 7.6) at room temperature. The membrane was rinsed three times with TBST and then incubated overnight with mouse anti-TH monoclonal antibody (Sigma, T2928, 1:10,000) or rabbit anti-DAT polyclonal antibody (Millipore; AB2231, 1:4000) at 4°C based on the experimental aims. After washing thrice, the membrane was incubated for 1 h in IRDye 800-conjugated goat anti-mouse second antibody (1:3000, Rockland, Gilbertsville, PA, USA) or goat anti-rabbit second antibody (1:3000, Rockland) at room temperature. The relative density of bands was analyzed on an Odyssey infrared scanner (LI-COR Biosciences, Lincoln, NE, USA). Following stripping, each PVDF membrane was subsequently immunoblotted with mouse anti- β -actin monoclonal antibody (Santa Cruz Biotechnology; sc-47778). The densitometry values of TH, or DAT were normalized with respect to those of β -actin, which were the endogenous control. For all samples, Western blots were performed in triplicate and the data were averaged.

Hormone assays

The trunk blood was collected when the rats were killed by decapitation in Experiment 1. The levels of serum testosterone were determined by radioimmunoassay using radioimmunoassay kit (Cat. PID RG4, Tianjin Nine Tripods Medical and Bioengineering Co. Ltd, China).

Statistical analysis

All data were presented as mean $\pm$ s.d. Between-group differences in Experiment 1 were evaluated using independent sample *t*-tests. The data in Experiment 2 and Experiment 3 were analyzed by the tests of normality (Kolmogorov-Smirnov test) and homogeneity variance (Levene's test). Then, parametric test was performed by one-way ANOVA followed by a Student-Newman-Keuls (SNK) *post hoc* test for multiple comparisons or nonparametric statistics were done by Kruskal-Wallis test followed by Mann-Whitney *U* test for *post hoc* analysis between groups. A criterion for statistical confidence of $P < 0.05$ was used.

Results

Body weight as well as seminal vesicle and testis weights

There was no significant difference in the body weight of rats between C group and TP group at 3-, 7- or 24-week old. In addition to the body weight, the seminal vesicle and testis weights were also analyzed between C group and TP group at the end of Experiment 1 to confirm peripheral effects of androgen during early postnatal TP treatment at the dose of 0.5 mg/day. The TP exposure increased the seminal vesicle weight of rats at 3 weeks old ($P < 0.01$) and reduced the testis weight of rats at 3 weeks and 7 weeks old ($P < 0.01$). There was no significant difference in the testis weight and seminal vesicle weight of rats between C group and TP group at 24 week old (Table 1).

Serum testosterone levels

Consecutive 14-day TP exposure to PND7 male rats resulted in the high levels of serum testosterone in the rats at 3 weeks old ($P < 0.01$) and the low levels of serum testosterone in the rats at 7 weeks old ($P < 0.01$). There was no significant difference in the serum testosterone levels of rats between C group and TP group at 24 weeks old (Table 1).

Motor behavior and grooming behavior

The effects of early postnatal TP exposure on brain activity were investigated by analyzing the altered parameters of motor behavior and grooming behavior, which included vertical activity, horizontal activity and total path length

Table 1 Effects of early postnatal TP exposure on body weight as well as seminal vesicle and testis weights and serum testosterone of male rats ($n = 12$).

	C	TP
3 week old		
Body weight (g)	47.4 ± 7.0	49.0 ± 4.0
Seminal vesicle weight (g)	0.009 ± 0.001	0.097 ± 0.011*
Testis weight (g)	0.11 ± 0.02	0.08 ± 0.01*
Testosterone (ng/mL)	1.26 ± 0.22	55.87 ± 10.26*
7 week old		
Body weight (g)	193.3 ± 22.3	184.6 ± 29.6
Seminal vesicle weight (g)	0.14 ± 0.06	0.13 ± 0.03
Testis weight (g)	1.01 ± 0.08	0.76 ± 0.09*
Testosterone (ng/mL)	3.16 ± 0.58	1.47 ± 0.34*
24 week old		
Body weight (g)	439.7 ± 27.0	421.9 ± 33.6
Seminal vesicle weight (g)	1.06 ± 0.15	0.97 ± 0.14
Testis weight (g)	1.40 ± 0.11	1.37 ± 0.12
Testosterone (ng/mL)	4.26 ± 0.65	4.12 ± 0.77

* $P < 0.01$ vs C.

for motor behavior, as well as the latency, number and duration of grooming for grooming behavior (Zhang *et al.* 2011). Except for the latency of grooming, all other behavioral parameters were elevated in the rats of TP group compared with the rats of C group at 3 weeks old (Figs 1A, B and C and 2B and C, $P < 0.01$) or at 7 weeks old (Figs 1D, E and F and 2E and F, $P < 0.01$). There was no significant difference in behavioral parameters of the rats between TP group and C group at 24 weeks old (Figs 1G, H and I and 2G, H and I).

Alterations of neurochemical levels

The changes of neurochemical levels were analyzed in the CPu and Acb of rats affected with early postnatal TP exposure. Both brain regions are related to the regulation of motor behavior and grooming behavior. There was no significant difference in the levels of Glu, GABA, NE and His in the CPu and Acb of rats between TP group and C group at 3 weeks, 7 weeks and 24 weeks old (Table 2). The significant increments of DA levels in the CPu and Acb (3 weeks old, CPu: $P < 0.05$; Acb: $P < 0.01$; 7 weeks old, CPu: $P < 0.05$; Acb: $P < 0.01$), HVA in the CPu (7 weeks old: $P < 0.01$) and 5-HT in the Acb (3 weeks old: $P < 0.05$; 7 weeks old: $P < 0.01$), as well as the reduction of the DOPAC+HVA/DA ratio in the Acb (3 weeks old: $P < 0.01$) were found in the rats of TP group, as compared to C group (Table 2). There was no significant difference in the levels of DA, DOPAC, HVA and 5-HT, or in the DOPAC+HVA/DA ratio in the CPu and Acb of rats between TP group and C group at 24 weeks old (Table 2).

TH and DAT expression

Based on the revealed alterations of neurochemical levels in the CPu and Acb of the rats between TP group and C group, the mesoDAergic system became our main concern and their markers, TH and DAT, were further studied. Elevated TH and DAT at mRNA levels in the SN and VTA (Fig. 3A, B, D and E, $P < 0.01$) as well as at protein levels in the CPu and Acb (Fig. 3G, H, I, J, K and L, $P < 0.01$) were

Figure 1

Effects of early postnatal androgen exposure on motor behavior. Early postnatal TP exposure increased the vertical activity and horizontal activity as well as total path length of PND21 (A, B and C) and PND49 (D, E and F) male rats. (G, H and I) = PND168. The results were expressed as mean ± s.d. ($n = 12$). * $P < 0.01$.

Figure 2

Effects of early postnatal androgen exposure on grooming behavior. Early postnatal TP exposure increased the number of grooming and duration of grooming of PND21 (B and C) and PND49 (E and F) male rats. There was no significant difference of early postnatal androgen exposure on the latency of grooming of PND21 (A) and PND49 (D) male rats. (G, H and I)=PND168. The results were expressed as mean $\pm$ s.d. ($n=12$). * $P<0.01$.

observed in the rats of TP group at 3 weeks and 7 weeks old. There was no significant difference in TH and DAT expression of rats between TP group and C group at 24 weeks old.

Effects of Hal on motor behavior and grooming behavior

In Experiment 2, group differences among C-Sal, TP-Sal, C-0.02Hal, C-0.15Hal, TP-0.02Hal and TP-0.15Hal were observed in the behavioral parameters of motor behavior (Figs 4A, B and C and 5A, B and C, $P<0.01$) and grooming behavior (Figs 4E and F and 5E and F, $P<0.01$) at 3-week and 7-week old. For motor behavior, the *post hoc* test showed the significant increments of behavioral parameters in TP-Sal (3-week old, vertical activity: $P<0.05$, horizontal activity and total path length: $P<0.01$; 7-week old, $P<0.01$) and the marked decrements of them in C-0.15Hal (3-week and 7-week old, $P<0.01$) compared with C-Sal. The rats in TP-0.15Hal demonstrated the reduced levels of behavioral parameters compared with TP-Sal (3-week old, vertical activity: $P<0.01$, horizontal activity: $P<0.05$, total path length: $P<0.01$; 7-week old, $P<0.01$) (Figs 4A, B and C and 5A, B and C). For the grooming behavior, *post hoc* test revealed the more number and longer duration of grooming in TP-Sal (3-week old, number of grooming: $P<0.01$, duration of grooming: $P<0.05$; 7-week old, $P<0.01$), as well as the less number and shorter duration in C-0.15Hal (3-week and 7-week old, number of grooming: $P<0.05$; duration of grooming: $P<0.01$) than that in

C-Sal. Compared with TP-Sal, the number and duration of grooming significantly reduced in TP-0.15Hal (3-week old, number of grooming: $P<0.01$, duration of grooming: $P<0.05$; 7-week old, $P<0.01$) (Figs 4E and F and 5E and F). There was no significant difference in motor behavior and grooming behavior between C-Sal and TP-0.15Hal at 3-week and 7-week old (Figs 4 and 5).

In Experiment 3, significant reduction of motor behavior and grooming behavior was found in the rats of C-Hal and Hal-TP compared with the rats of C and C-TP at PND21 (Fig. 4, $P<0.01$) and PND49 (Fig. 5, $P<0.01$). There was no significant difference in motor behavior and grooming behavior between C and C-TP, as well as C-Hal and Hal-TP (Figs 4 and 5).

Discussion

The present study showed that consecutive 14-day TP exposure to PND7 male rats induced the hyperactive motor behavior and grooming behavior, the increments of DA levels as well as the upregulation of TH and DAT expression at PND21 and PND49. Administration of Hal markedly ameliorated the early postnatal TP exposure-induced behavioral defects in PND21 and PND49 male rats. The results demonstrated that early postnatal androgen exposure disturbed the brain activity of developing male rats via enhancing the mesoDAergic activity. It was suggested that the abnormal increments of testosterone levels during the early postnatal development might be a

Table 2 Neurochemical alterations in the CPu and Acb of male rats induced by early postnatal TP exposure ($n=12$, ng/g).

	CPu		Acb	
	C	TP	C	TP
3 week old				
DA	714.1±154.4	864.4±111.7*	149.2±63.0	418.5±54.1**
DOPAC	1889.0±581.9	2255.3±634.8	1348.1±294.5	1543.6±303.8
HVA	905.8±225.0	1093.4±299.6	909.9±186.2	1013.9±191.7
DOPAC+HVA/DA	4.0±0.9	3.9±1.2	17.7±7.5	6.2±1.3**
5-HT	90.8±22.8	95.0±16.3	80.2±16.2	99.7±19.1*
5-HIAA	686.2±160.6	689.0±94.2	618.4±148.4	690.7±209.2
NE	3155.5±553.8	3413.8±361.2	2700.2±531.5	2798.8±347.6
GABA	44768.4±11647.5	50976.4±6041.7	81944.2±17609.9	82341.5±15701.8
His	633.1±124.5	715.6±99.1	687.0±117.1	730.8±103.9
Glu	34410.4±6813.6	38226.6±4955.5	28252.4±9664.4	26323.9±5796.2
7 week old				
DA	1417.3±196.6	1686.5±326.3*	761.7±164.8	942.7±99.9**
DOPAC	2059.3±722.7	2434.8±752.0	1309.0±256.8	1599.3±434.6
HVA	539.5±153.9	719.1±135.3**	372.5±74.3	442.8±126.0
DOPAC+HVA/DA	1.9±0.5	1.9±0.3	2.3±0.6	2.2±0.6
5-HT	61.0±11.4	70.0±13.2	111.2±18.3	146.7±19.9**
5-HIAA	373.7±110.5	415.2±80.7	446.8±57.1	484.1±97.1
NE	4646.0±601.4	5099.3±744.7	4042.2±567.4	4137.1±642.7
GABA	33390.2±9343.5	39635.6±9181.9	78998.8±17192.7	81361.0±13677.0
His	444.7±83.1	511.4±100.4	541.1±85.4	581.1±121.7
Glu	40184.3±4326.0	43601.8±5825.3	32287.1±8031.8	30383.3±6718.2
24 week old				
DA	901.7±68.8	825.9±165.7	85.0±19.2	79.6±12.3
DOPAC	6673.0±1437.8	6877.7±1689.8	3195.5±524.4	3013.9±902.1
HVA	1626.0±490.2	1740.8±323.6	1368.1±203.8	1261.0±242.3
DOPAC+HVA/DA	9.2±2.0	10.7±2.8	56.5±14.7	56.0±20.9
5-HT	834.7±56.4	751.5±166.7	397.2±68.1	384.8±71.5
5-HIAA	2848.7±493.9	2904.0±443.8	2492.5±583.8	2382.2±562.1
NE	4480.5±431.4	4767.0±534.1	4210.0±543.0	4221.5±574.1
GABA	38660.1±7119.6	38858.8±6023.8	94048.5±16105.7	96960.4±15728.6
His	562.7±83.6	600.7±98.0	698.4±92.3	692.9±133.2
Glu	44241.3±4550.6	47241.4±5379.0	35383.2±8482.2	34914.3±7088.2

* $P<0.05$; ** $P<0.01$ vs C.

potential risk factor for the incidence of some male-based childhood-onset neuropsychiatric disorders by affecting the mesoDAergic system.

Testosterone produced by the testes regulates brain activity including cognitive, motor and motivational behaviors (Adler *et al.* 1999, Frye *et al.* 2008, Zhang *et al.* 2011, Celec *et al.* 2015). Gonadectomy decreased the ambulations of adult male rats in the open field (Adler *et al.* 1999, Zhang *et al.* 2011). Supplementation of TP to castrated rats recovered the reduced ambulation activity (Adler *et al.* 1999, Zhang *et al.* 2011) in the open field. Administration of testosterone to aged intact male animals elevates motor behavior (Frye *et al.* 2008, Cui *et al.* 2012). In the present study, we found that the motor behavior and grooming behavior of developing male rats were able to be manipulated via early postnatal testosterone exposure. The exposure of excessive testosterone during the early postnatal development produced the motor

behavioral and grooming behavioral defects of PND21 and PND49 male rats. The increments of motor behavioral and grooming behavioral parameters were detected among them, which indicated the alteration of the brain function of PND21 and PND49 male rats to some extent.

Brain function is based on a balance of neurotransmissions (Steeves & Fox 2008, Wong *et al.* 2008, Hidaka *et al.* 2010, Leckman *et al.* 2010). Abnormality in the neurotransmitter levels of the brain induces neurological or psychotic disorders, such as schizophrenia, Huntington's disease, Parkinson disease and TS (Mehler-Wex *et al.* 2006, Howes & Kapur 2009, Kegeles *et al.* 2010, Leckman *et al.* 2010, Chen *et al.* 2013). Changes in brain DA content contribute to abnormal movements and cognitive deficits in Huntington's disease (Chen *et al.* 2013) and in Parkinson disease (Mehler-Wex *et al.* 2006). In the present study, among the neurochemicals detected by LC-MS/MS assay, the main findings were the increments of DA

Figure 3

Effects of early postnatal androgen exposure on the mesoDAergic system. Early postnatal TP exposure increased TH mRNA (A and B) and DAT mRNA (D and E) levels in the SN and VTA, as well as the expression of TH and DAT protein levels in the CPu and Acb of PND21 (G, H and I) and PND49 (J, K and L) male rats. (M, N and O)=PND168. The results were expressed as mean $\pm$ s.d. ($n=12$). $*P < 0.01$.

levels in the CPu and Acb of TP-treated rats at PND21 and PND49, as well as the reduction in the DOPAC+HVA/DA ratio in the Acb of TP-treated rats at PND21. It was suggested that early postnatal androgen exposure induced the alteration of the mesoDAergic system in developing male rats. Androgen has been shown to influence DAergic system (Thiblin *et al.* 1999, Kindlundh *et al.* 2002, de Souza Silva *et al.* 2009). Anabolic androgenic steroids increase DAT density (Kindlundh *et al.* 2002) as well as DAergic metabolism (Thiblin *et al.* 1999) in the striatum of male rats. The administration of testosterone to castrated male rats prevents the castration-induced reduction of striatal TH activity (Abreu *et al.* 1988) and enhances DAergic activity in the neostriatum and Acb of male adult rats (de Souza Silva *et al.* 2009). In the present study, we found that early postnatal exposure to high levels of TP facilitated the activity of the mesoDAergic system and upregulated the expression of TH and DAT at mRNA and protein

levels in PND21 and PND49 male rats. Furthermore, early postnatal TP exposure might increase the expression of vesicular monoamine transporters 2 (VMAT2). It was found in the previous study that TP supplements elevated VMAT2 levels in aged male rats (Wang *et al.* 2016). VMAT2 transports DA from the cytoplasm into synaptic storage vesicles to prevent DA from being metabolized. Thus, the increased DA content with reduction or without change in the DOPAC+HVA/DA ratio indicated there was more DA present in synaptic vesicles of DAergic neurons in TP-treated PND21 and PND49 rats. Enhanced mesoDAergic activity by early postnatal exposure to excessive testosterone might underlie the PND21 and PND49 behavioral deficits of early postnatal TP-exposed male rats.

The DAergic neurons in the SN and VTA are the sources of the nigrostriatal and mesocorticolimbic DAergic projections, respectively. They form the nigrostriatal and mesocorticolimbic circuits and regulate the function of

Figure 4

Effects of Hal on early postnatal TP exposure-induced behavioral defects in experiment (Exp) 2 and effects of Hal treatment concurrent with TP treatment on the neurobehaviors of developing male rats in Exp 3. Administration of Hal significantly ameliorated the early postnatal TP exposure-induced defects in motor (A, B and C) and grooming (D, E and F) behaviors of PND21 male rats (Exp 2). There was no significant difference in motor behavior and grooming behavior of PND21 male rats between C and C-TP, as well as C-Hal and Hal-TP (Exp 3). The results were expressed as mean $\pm$ s.d. ($n=7$). * $P<0.05$, ** $P<0.01$.

the basal ganglia (Blandini *et al.* 2000, Mehler-Wex *et al.* 2006), the subcortical structures that are considered to be of central relevance in the pathophysiology of TS (Peterson *et al.* 1993, Singer *et al.* 1993, Bronfeld & Bar-Gad 2013). It is known that the basal ganglia play an important role in motor control including simple and complex motor functions, such as grooming behavior in rodent. Grooming behavior is natural and ubiquitous. It consists of complex strings of movements. Due to its complex repetitive nature, rodent grooming behavior is a logical phenotype to be observed in putative models of TS (Berridge *et al.* 2005). Dopamine, a major modulator in the mesoDAergic systems, is involved in the regulation of motor and grooming behaviors in rodents (Hidaka *et al.* 2010, Taylor *et al.* 2010, Kalueff *et al.* 2016). The activation of central DAergic system markedly increases locomotor activity (Hidaka *et al.* 2010) as well as grooming behavior (Taylor *et al.* 2010) of rats. The increased locomotor activity and grooming behavior can be suppressed by Hal treatments (Hidaka *et al.* 2010, Taylor *et al.* 2010). Haloperidol, a formally approved medication in the United States (Egolf & Coffey 2014), is used to treat behavioral disorders and hyperactivity (Tyler *et al.* 2017) and to control the tics and vocal utterances associated with TS (Termine *et al.* 2013, Tyler *et al.* 2017). It is a

high-affinity antagonist of dopamine D2 receptors (Kapur & Mamo 2003) and one of the most effective anti-DAergic agents for the reduction of tics (Silay & Jankovic 2005, Scahill *et al.* 2006). In the present study, we found that Hal treatment to PND21 and PND49 male rats ameliorated the motor behavioral and grooming behavioral deficits induced by early postnatal TP exposure. Previous study revealed that Hal treatment prevented male hamsters from displaying conditioned place preference to vaginal secretions, which is dependent on testosterone levels (Bell & Sisk 2013). It has been reported that conditioned place preference induced by testosterone can be blocked by dopamine receptor antagonist (Schroeder & Packard 2000). Thus, the amelioratory effects of Hal on the defects of motor behavior and grooming behavior in our study suggested that the mesoDAergic system might be the targets through which early postnatal TP exposure induced the behavioral impairments of PND21 and PND49 male rats.

During the early postnatal development, high levels of testosterone exist in males compared with females (Slob *et al.* 1980, Baum *et al.* 1998). These high testosterone levels contribute to the organizational effects of androgen on the sexual dimorphic brain regions (Arnold & Breedlove 1985) as well as the reproduction and energy homeostasis (Nohara

Figure 5

Effects of Hal on early postnatal TP exposure-induced behavioral defects in Exp 2 and effects of Hal treatment concurrent with TP treatment on the neurobehaviors of developing male rats in Exp 3. Administration of Hal significantly ameliorated the early postnatal TP exposure-induced defects in motor (A, B and C) and grooming (D, E and F) behaviors of PND49 male rats (Exp 2). There was no significant difference in motor behavior and grooming behavior of PND49 male rats between C and C-TP, as well as C-Hal and Hal-TP (Exp 3). The results were expressed as mean $\pm$ s.d. ($n=7$). * $P<0.05$, ** $P<0.01$.

et al. 2013). Administration of exogenous androgen after the critical periods of developing nervous system does not produces the enduring changes in behaviors (Diaz *et al.* 1995), which shows the activational effects of androgen on developing nervous system. In the present study, we found that early postnatal TP exposure enhanced the activity of the mesoDAergic system in PND21 male rats, and moreover, the enhanced mesoDAergic activity was still maintained in PND49 male rats although at this time PND49 male rats had much low serum testosterone levels due to the feedback inhibition of early postnatal TP exposure on testis development (low serum testosterone levels and light testis weight). When they were adults, the elevated mesoDAergic activity in the rats of the same treatment group was restored to the control level. The activational effects of androgen might explain the restoration of the mesoDAergic activity as well as behavioral parameters in the early postnatal TP-treated adult male rats. Testosterone propionate exposure to male pup rats started at PND7 in the present study. It has been found that injection of TP or androgen receptor antagonist (flutamide) to pup rats at this time does not induce the enduring changes of behavior (Diaz *et al.* 1995, Yamada *et al.* 2015).

Testosterone is known to mediate its effects by two different pathways of action, classical pathway of

cytosolic/nuclear androgen receptors and non-classical pathway of membrane-associated androgen receptors (Deng *et al.* 2017). The classical effects of testosterone occur slowly because of the translation of newly synthesized proteins and the non-classical effects of testosterone occur fast due to the rapid phosphorylation of signal molecules (Deng *et al.* 2017). In Experiment 3 of the present study, we did not find the differences of motor behavior and grooming behavior between the rats receiving Hal treatment and the rats receiving Hal concurrent with TP treatment. Combining the results of no behavioral difference between C rats and C-TP rats, it seemed that TP treatment 30 min prior to open field test did not work via non-classical pathway of androgen receptors, which is worthwhile being investigated in the future.

The subcutaneous injection of TP at the dose of 0.5 mg was adopted based on the previous study (Rhees *et al.* 1990). In the present study, 2-week TP treatment to PND7 male pup rats at this dose did not produce the alterations of body weight from 3-week old to adult. However, it created hyperandrogen milieu to early postnatal male pup rats, which resulted in the increased seminal vesicle weight and extremely high serum testosterone concentrations at 3-week old, low serum testosterone concentrations at 7-week old and lighter testes at both ages. The behavioral

deficits and the enhanced mesoDAergic activity in early TP-treated rats were induced under the early postnatal hyperandrogen milieu, which presented the concerns of the undetermined physiological relevance of this testosterone effects. Therefore, to evaluate the physiological relevance of this testosterone effect, it is necessary in the future study to determine whether injection of TP at low dose or implantation of testosterone capsules could produce the similar effects on early postnatal male pup rats.

In addition to DA, early postnatal TP exposure induced the alteration of 5-HT levels in the Acb of PND 21 and PND49 male rats. The central serotonergic system innervates most of the brain regions and controls affection, cognition and movement along with DAergic system (Steeves & Fox 2008). Dysfunction of serotonergic system is found in TS patients (Heinz *et al.* 1998, Müller-Vahl *et al.* 2005, Behen *et al.* 2007), such as the abnormality of tryptophan metabolism (Behen *et al.* 2007) and the reduced availability of serotonin transporter (Heinz *et al.* 1998, Müller-Vahl *et al.* 2005). Therefore, how much extent the central serotonergic system was influenced by early postnatal androgen exposure deserves further investigation in the future.

In conclusion, hyperandrogen environment during the early postnatal development induced the hyperactive motor as well as grooming behaviors and enhanced the activity of the mesoDAergic system of developing male rats. Dopamine D2 antagonist, haloperidol, could ameliorate the motor behavioral and grooming behavioral deficits induced by hyperandrogen environment. It is suggested that abnormal increments of testosterone levels during the early postnatal development might be a potential risk factor for the incidence of some male-based childhood-onset neuropsychiatric disorders through modulation of the mesoDAergic system.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Q C, J X and Z G performed PCR, Western blot experiments, analyzed the data and wrote the manuscript. K Y performed LC–MS/MS assay and contributed to drafting. H Y and C R carried out behavioral experiments and the measurement of serum testosterone levels. L S and C H interpreted

data and revised the manuscript. S G designed the study, analyzed the data and revised the manuscript. All authors have read and approved the final version of the manuscript.

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