



Gene expression of synaptosomal-associated protein 25 (SNAP-25) in the prefrontal cortex of the spontaneously hypertensive rat (SHR)

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ARTICLE INFO

Article history:

Received 8 August 2008

Received in revised form 20 May 2009

Accepted 21 May 2009

Available online 28 May 2009

Keywords:

Attention-deficit/hyperactivity disorder (ADHD)

Spontaneously hypertensive rat (SHR)

Prefrontal cortex (PFC)

Functional MRI (fMRI)

D-amphetamine

Synaptosomal-associated protein 25 (SNAP-25)

ABSTRACT

Dopamine is believed to play an important role in the etiology of attention-deficit/hyperactivity disorder (ADHD). In our previous study, we showed that gene expression of dopamine D4 receptor decreased in the spontaneously hypertensive rat (SHR) in the prefrontal cortex (PFC). In the present study, we explored the potential causes of dysfunction in the dopamine system in ADHD. It is the first time that neuronal activities in both juvenile SHR and WKY rats have been measured by functional MRI (fMRI). Our results showed that in PFC the Blood Oxygenation Level Dependent (BOLD) signal response in SHR was much higher than WKY under stressful situations. We tested the effects of acute and repeated administration of amphetamine on behavioral changes in SHR combined with the expression of the neuronal activity marker, *c-fos*, in the PFC. Meanwhile dopamine-related gene expression was measured in the PFC after repeated administration of amphetamine. We found that potential neuronal damage occurred through deficit of D2-like receptor protective functions in the PFC of the SHR. We also measured the expression of synaptosomal-associated protein 25 (SNAP-25) in SHR in PFC. The results showed decreased expression of SNAP-25 mRNA in the PFC of SHR; this defect disappeared after repeated injection of D-AMP.

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1. Introduction

Attention-deficit hyperactivity disorder (ADHD) in humans is characterized by age-inappropriate inattention, impulsiveness, and hyperactivity [1]. Dopamine is believed to play an important role in the pathophysiology of ADHD. Genes involved in regulating the dopamine system play a key role in the etiology of ADHD. In our previous study, we showed that expression of dopamine D4 receptor decreased in the spontaneously hypertensive rat (SHR) in the prefrontal cortex (PFC) [2]. Several current studies have demonstrated synaptosomal-associated protein 25 (SNAP-25) polymorphisms that have been associated with ADHD [3,4]. SNAP-25 is involved in the exocytosis of neurotransmitters from storage vesicles into synaptic spaces [5]. Given these findings, it is possible that SNAP-25 might have a role in dopamine hypofunction in the prefrontal cortex in SHR, but to date, this idea has not been assessed. Also, the relationship between SNAP-25 and stimulant responses in ADHD is unclear. Therefore, it is important to clarify the role of SNAP-25 in the regulation of the dopamine system in ADHD and its relationship between stimulants

frequently used as treatments for ADHD, and to explore the causes of dysfunction of the dopamine system in ADHD. Specifically, is the dysfunction of the dopamine system a *cause* or *result* of the etiology of ADHD?

With the improvements in imaging technology, more detailed structural, functional and neurochemical profiles can be visualized across the entire brain. Magnetic resonance imaging (MRI) morphometric studies of ADHD have identified reductions in total brain volume and in various regions of the brain (such as the prefrontal lobe, caudate nucleus, corpus callosum and cerebellum) that are known to be involved in the regulation of attention and impulsivity [6]. Also, during functional magnetic resonance imaging (fMRI), ADHD children have shown differences in frontostriatal activity, particularly in the right prefrontal cortex and anterior cingulate [7–9]. Other relevant neuroimaging studies have shown dysfunction of the dopaminergic system in ADHD children [10]. Animal models can help to elucidate and further the understanding of ADHD. Juvenile spontaneously hypertensive rats (SHR) (before hypertension develops) show many behavioral characteristics similar to ADHD [11,12]. SHR, therefore, are the most frequently used model of ADHD [12], however there is no evidence as yet of neuron activities in the PFC in juvenile SHR using fMRI.

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In clinical trials, psychostimulants (e.g., methylphenidate, *D*-amphetamine, and pemoline) are the most common treatments for ADHD [13], even though the precise etiology and pathological mechanisms underlying ADHD are poorly understood. The mechanism of action of *D*-amphetamine (*D*-AMP or AMPH) is to release dopamine from presynaptic nerve terminals in addition to inhibiting reuptake of dopamine [14]. However, there has been no evidence to show the effects of repeated administration of amphetamine or the effects of systemic administration of amphetamine on expression of dopamine-related genes. In the present study, we measured these effects in the juvenile spontaneously hypertensive rat, the most frequently used model of ADHD [12], to examine any neurobiological alterations.

As the prefrontal cortex is the major affected brain area in ADHD [15], we focused our study on the biochemical alterations of the prefrontal cortex in SHR after administration of psychostimulant drugs. We tested the effects of acute and repeated administration of amphetamine on behavioral changes in SHR combined with the expression of the neuronal activity marker, *c-fos*, in the PFC after an acute administration of amphetamine. This is the first time the neuronal activities in the PFC of SHR have been demonstrated under fMRI. Also, the expression of dopamine-related genes was measured in the PFC after repeated administration of amphetamine. Furthermore, we measured the expression of SNAP-25 in SHR in PFC and expression of SNAP-25 with repeated amphetamine treatment.

2. Materials and methods

2.1. Animals

Juvenile male spontaneously hypertensive rats (SHR) aged 4–6 weeks old and age- and gender-matched genetic control Wistar-Kyoto rats (WKY) were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong (CUHK). The rats had free access to standard laboratory rodent chow and water, and were housed in a room with 12 h light–dark cycle. Temperature and humidity were maintained at 22 ± 1 °C and 45–55%, respectively. The experiments were approved by the Animal Experimentation Ethics Committee, CUHK.

2.2. Behavioral testing

2.2.1. Experiment 1: effects of an acute systemic administration of *D*-AMP on locomotor activity

Four-week-old SHR and WKY ($n=5$ /group/strain) were maintained in the Laboratory Animal Services Centre, the Chinese University of Hong Kong (CUHK). Rats of the same strain were housed five per cage and after at least 3 days of acclimation, were divided into the following five treatment groups for: (1) *D*-AMP (gift from the Department of Pharmacology, CUHK) diluted with 0.9% saline to 0.1 mg/kg intraperitoneal (IP) injection; (2) *D*-AMP 0.3 mg/kg IP; (3) *D*-AMP 1 mg/kg IP; (4) *D*-AMP 3 mg/kg IP; and (5) *D*-AMP 10 mg/kg IP injection. The saline administration rats were the control group. The drugs and saline were administered in the home cages once between 4 pm and 6 pm, and all the rats were pretreated with saline by intraperitoneal injection for 2 days before the drug and saline administration in order to adapt them to the stress of injection.

The apparatus and methods were the same as mentioned in our previous study [2]. The open-field (OF) box was a 50-cm³ cube constructed from black Plexiglas. Animals were placed into the box and were videotaped for 5 min using an Animal Behavior Recognition System (Institute of Psychology, Chinese Academy of Sciences, Beijing, China). We recorded locomotion activities for 5 min in SHR and WKY after 15-min injection of *D*-AMP. We measured locomotor activities on rats after 0 min, 15 min and 30 min injection of *D*-AMP (data not shown).

2.2.2. Experiment 2: effects of the repeated administration of *D*-AMP on locomotor activity

The strains of rats, 4-week-old SHR and WKY ($n=5$ /group/strain), were each divided into two groups respectively: 1) *D*-AMP 3 mg/kg IP for 7 days; 2) 0.9% saline 1 ml/kg IP for 7 days. All rats were pretreated with saline by intraperitoneal injection for 2 days before drug administration to reduce the stress from injection. The drugs were administered in the home cages each day between 4 pm and 6 pm to coincide with the recording of locomotion activities.

2.2.3. Experiment 3: effects of an acute systemic administration of *D*-AMP on prepulse inhibition (PPI)

Four-week-old SHR and WKY ($n=5$ /group/strain) were housed five per cage. After at least 3 days of acclimation, each strain was divided into four groups: (1) 0.9% saline 1 ml/kg IP; (2) *D*-AMP 1 mg/kg IP; (3) *D*-AMP 3 mg/kg IP; and (4) *D*-AMP 5 mg/kg IP injection.

The drugs were administered in the home cages once between 4 pm and 6 pm, and the rats were pretreated with saline by intraperitoneal injection for 2 days before the drug administration. The apparatus and methods were the same as described in our previous studies [2]. Prepulse inhibition was measured in one startle chamber (San Diego Instruments, San Diego, CA). Startle responses were measured by an accelerometer in response to acoustic stimuli delivered by a white noise generator (4–19 kHz; SR-Lab, San Diego, CA). A test session consisted of placing the animals in the startle chamber under 70 dB background noise for a 5 min acclimatization period after which they were exposed to a total of 37 trials separated by variable inter-stimulus intervals that averaged 15 s [16,17]. The intensity of the prepulse stimulus varied from 3 to 15 dB above the background noise level of 3 dB increments. These trials were presented randomly, with the restriction that no more than two trials of the same type could be run in succession. For data analysis, the average of the last 10 startle trials was taken as the measure of startle reactivity for each animal. We also averaged the 5 trials taken at each of the 5 prepulse intensities, and then expressed these values as a percentage of the average reactivity for the 10 startle trials, using the formula: $[(\text{startle} - \text{prepulse}) / \text{startle}] \times 100$. We started prepulse inhibition tasks 15 min after they were treated with *D*-AMP.

2.3. Functional magnetic resonance imaging (fMRI)

There were 5 rats in each of the SHR and WKY groups. They were anesthetized with a mixture of ketamine (100 mg/kg, IP) and xylazine (10 mg/kg, IP) and placed into a head and body restrainer. Experiments were performed on a 1.5 T clinical MR scanner (Intera NT, Philips Medical Systems, Best, the Netherlands) a microscopy radiofrequency coil (M-23, Philips) of 2.3 cm diameter was used for signal reception. The coil was carefully placed over the head of each animal and a standardized imaging protocol was applied. For structural imaging, a turbo spin echo T2-weighted sequence [repetition time (TR), 446 ms; echo time (TE) 100 ms; imaging matrix, 256 × 256; field of view (FOV), 60 mm] was employed and this scan was performed for each animal before functional scanning. Functional imaging of the animal brains was performed using a single shot, gradient-echo, echo planar imaging (EPI) sequence with the following parameters: FOV, 60 mm; slice thickness, 0.8 mm; TR, 2000 ms; TE, 33 ms; flip angle 90°; matrix, 64 × 64. Sixty-four dynamic scans were acquired using the latter imaging sequence in the transverse plane with a scanning time of 2 min and 16 s. Each run consisted of dummy scan (dynamic 1–4), “off” scan (dynamic 5–19), “on” scan (dynamic 20–34), “off” scan (dynamic 35–49) and lastly “on” scan (dynamic 50–64). First, we put the rat's tail between two plates, then put a 500 g weight onto the plate (the area of the plate is about 14 × 12 cm). The area of a rat's tail is approximately 5 cm² and the gravity of a 500 g mass is 4.9 N. Therefore, 100 g/cm² weight was given during the “on” scan interval and the weight was taken off during the “off” scan interval.

Statistical parametric maps were generated with the scanner's activation processing tool (View Forum release 4.2, Phillips Medical Systems, Best, The Netherlands). For each voxel in a given slice, *t*-tests between the "on" and "off" periods, including a Bonferroni correction for the total number of voxels in a slice, were performed and *Z*-scores were computed. Clusters of activated voxels were determined using a *Z*-score threshold of 1.6 and a minimum cluster size of 10. Activated regions of interest were matched with the anatomical T2-weighted images of identical slice thickness and location. The *Z*-scores of clusters contained in the transverse T2-weighted slices showing the prefrontal cortex brain zone were calculated.

2.4. Determination of dopamine concentration by using an enzyme-linked immunosorbent assay (ELISA) in mesocortical and mesolimbic dopamine system between SHR and WKY

Rats ($n=6$ per group) were killed by decapitation and the brains were removed and dissected. Total protein samples were prepared from the midbrain, prefrontal cortex, nucleus accumbens and amygdala, and were immediately immersed in liquid nitrogen. Afterwards, the specimens were stored at -80°C until future use.

Brain tissue samples (50 mg wet weight) were homogenized in 0.1 N HCl and 1 mM EDTA. The homogenate was centrifuged at 15,000 *g* for 15 min at 4°C and the supernatant obtained was used freshly or stored at -80°C . Dopamine concentration was evaluated by using dopamine ELISA kit (IBL, RE59 161, Hamburg, Germany). The optimal standard curve with dopamine at known concentrations (0, 12, 35, 115, 450, 2250 ng/ml) was obtained by using a cubic spline curve-filling technique [18]. Optical density was recorded for the standard samples, controls, and experimental samples. The concentration was calculated according to the cubic formulae.

2.5. *c-fos* protein expression in the prefrontal cortex after an acute systemic administration of D-AMP

The method and dosage of D-AMP usage were the same as mentioned above in 2.2.1. **Experiment 1.**

SHR and WKY rats ($n=5$ /group/strain) were killed 24 h after the last drug injection. In order to analyze the protein expression of *c-fos*, Western blot was used with enhanced chemical luminescence (ECL). 100 mg brain tissue samples was homogenized in 0.5 ml of 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 0.1 mg/ml PMSF, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstain A, and 5 $\mu\text{g}/\text{ml}$ aprotinin. The homogenate was centrifuged at 14,000 rpm for 3 min at 4°C and the supernatant obtained was either used immediately or stored at -80°C until use. The protein concentration of the extract was determined by DC protein assay (DC protein assay kit, Bio-Rad, CA). All the samples were equalized to 70 μg from the prefrontal cortex of SHR and WKY. Tissue homogenates were mixed with $2\times$ loading buffer (100 mM Tris-HCl of pH 6.8, 4% sodium dodecyl sulphate, 200 mM dithiothreitol, 0.2% bromophenol blue, and 20% glycerol). The mixture was then boiled for 6 min before being loaded onto a 12% SDS-polyacrylamide gel. Electrophoresis was performed at 60 V for 120 min followed by semi-dry transfer onto nitrocellulose membrane at 10 V for 1 h. The membrane was then blocked for 1 h in blocking solution containing 5% non-fat dry milk, 0.05% Tween-20, and phosphate buffered saline. The blocked membrane was incubated overnight with rabbit polyclonal *c-fos* (1:1000) (Santa Cruz Biotechnology, INC, SC-253). After 30 min washing with 0.05% Tween-20 and phosphate buffered saline, the membrane was incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody for 1 h (1:3000 for anti-rabbit IgG-HRP, sc-2077). Bound antibody was detected by the ECL-plus kit from Amersham. The optical density of the immunoreactive bands was detected with a Multimage™ II light cabinet (DE-500) that was equipped with

Fluorochem Beta 1.1 software (Alpha Innotech Corp., CA, USA). Band density values were normalized to β -Actin. Equal loading samples were normalized to β -Actin. Mean band densities were normalized to the corresponding values for the control samples of WKY.

2.6. Expression of *c-fos* and dopamine-related genes in the prefrontal cortex after repeated use of D-AMP

SHR and WKY ($n=5$ /group/strain) were divided into two groups for each strain: 1) D-AMP 3 mg/kg i.p. for 7 days; and 2) 0.9% saline 1 ml/kg i.p. for 7 days. The drugs and saline were administered in the home cages each day between 4 pm and 6 pm, and all the rats were pretreated with saline by intraperitoneal injection for 2 days before the drug and saline administrations.

Rats were killed by decapitation 24 h after the last drug injection and the brains were removed immediately. Total RNA samples were prepared from the prefrontal cortex. It was important that the total RNA was free from genomic DNA contamination. The A260/A280 ratio was at least 1.8 and the agarose gel showed that 28S band was more intense than 18S ribosomal RNA. TRIZOL reagent (Invitrogen™, USA) was used for RNA extraction. cDNA was synthesized from 5 μg of total RNA in a 40 μl reaction containing Oligo(dT) 15 and Moloney murine leukaemia virus (MMLV) reverse transcriptase (Invitrogen™, USA). Real-time quantitative PCR was performed using a 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). SYBR Green chemistry was used to perform real-time PCR analysis [19–21], which provided the simplest and most economical format for detecting and quantifying PCR products in real-time reactions. Each 25 μl reaction mixture contained SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), the primers (300 mmol/l for β -Actin and 900 mmol/l for *c-fos*, dopamine D1-5 receptors, DAT and TH), and 0.5 μl cDNA sample. Amplification was performed in 96-well plates and the PCR cycle consisted of 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. All primers were designed and checked with the Blat (<http://genome.ucsc.edu/index.html?org=Human&db=hg18&hgsid=80649300>) so that no non-specific product would be amplified. Specificity was also verified by electrophoresis of the PCR products that showed only one band for each PCR on the gel, and by one single peak in the melting curve analysis in the real-time PCR reaction (data not shown). Each sample was analyzed in triplicate. β -Actin was used to normalize results of the targeted genes [22]. Changes in gene expression between SHR and WKY brain samples were calculated from the differences in threshold cycles (CT values). The $\Delta\Delta\text{CT}$ method was used to determine the relative gene quantity (Fold induction = $2^{-\Delta\Delta\text{CT}}$). However, before using this method, the efficiencies of target and reference genes were demonstrated to be approximately equal [23,24].

2.7. SNAP-25 gene expression in the PFC

2.7.1. Basal SNAP-25 gene expression in the PFC between SHR and WKY

SHR and WKY ($n=5$ /group/strain) were killed and processed using real-time PCR as described above (see 2.5). SYBR Green chemistry was used to perform real-time PCR analysis [18–21], which provided the simplest and most economical format for detecting and quantifying PCR products in real-time reactions. Each 25 μl reaction mixture contained SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), the primers (300 mmol/l for β -Actin and 900 mmol/l for SNAP-25), and 0.5 μl cDNA sample. Amplification was performed in 96-well plates and the PCR cycle consisted of 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min.

2.7.2. SNAP-25 gene expression in the PFC after the repeated use of D-AMP

SHR and WKY ($n=5$ /group/strain) were divided into two groups for each strain: 1) D-AMP 3 mg/kg i.p. for 7 days; and 2) 0.9% saline 1 ml/kg i.p. for 7 days. The drugs were administered in the home

cages each day between 4 pm and 6 pm, and all rats were pretreated with saline by intraperitoneal injection for 2 days before the drug administration.

Animals were killed 24 h after the last drug injection and real-time PCR was used to measure the expression of dopamine-related genes in the PFC. RT-PCR method was the same as described above in 2.7.1.

2.8. Statistical analysis

Where appropriate, data analysis was followed by the independent sample *t*-test, one-way ANOVA, and general linear model ANOVA (SPSS version 13.0 and Sigmaplot version 9.0). When significant difference ($p < 0.05$) was found, a multiple comparison Post Hoc Test (Bonferroni) was applied to identify groups differing significantly from each other. Data were reported as mean \pm SEM unless otherwise stated.

3. Results

3.1. Behavioral testing

3.1.1. Experiment 1: effects of an acute systemic administration of D-AMPH on locomotor activity

The 2 \times 6 (groups \times different treatments with saline and 5 different dosages of D-AMPH) ANOVA univariate analysis showed a significant difference in the different treatments ($F = 24.043$, $p < 0.0001$). Also the interaction between groups and different treatments showed significant difference ($F = 4.06$, $p < 0.005$). Post-hoc Bonferroni tests revealed that these differences mainly came from the saline compared to all the different dosages of D-AMPH. Both of SHR and WKY showed significant decreased locomotor activities after an acute systemic amphetamine treatment (see Fig. 1).

3.1.2. Experiment 2: effects of the repeated administration of D-AMPH on locomotor activity

Increased locomotor activity was due to administration of amphetamine which is independent of rat strain but dependent on the time of treatment. 2 \times 4 \times 7 (strains \times treatment \times days) ANOVA analysis showed the differences between strains only in saline treatment group ($F = 66.52$, $p < 0.0001$). Post Hoc Tests (Bonferroni) showed

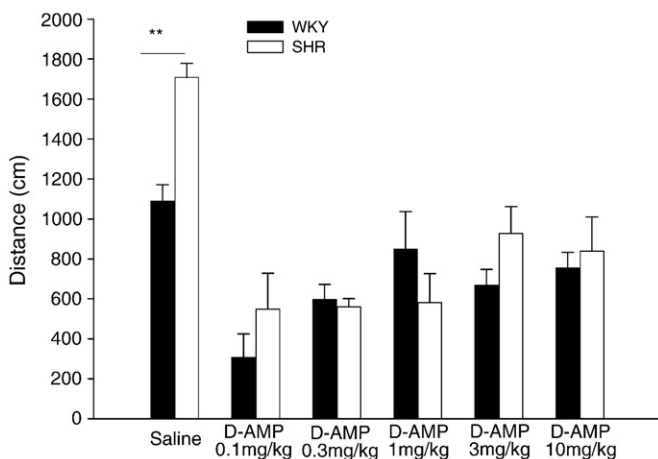


Fig. 1. Locomotor activity was measured after acute injection 15 min of different dosages of D-AMPH. Rats were treated with saline or different dosages of amphetamine. The total distance for a 5 min period in an open-field box was measured after drug injection. 2 \times 6 (groups \times different treatment) ANOVA univariate analysis showed the significant difference in different treatments ($F = 24.043$, $p < 0.0001$). Post hoc comparisons by Bonferroni, the differences in locomotion under the saline and systematic amphetamine administration mainly came from that of saline compared to all the different dosages of D-AMPH. ** $p < 0.0001$ represented the significant differences in saline when compared with different dosages of D-AMPH treatment both in SHR and WKY.

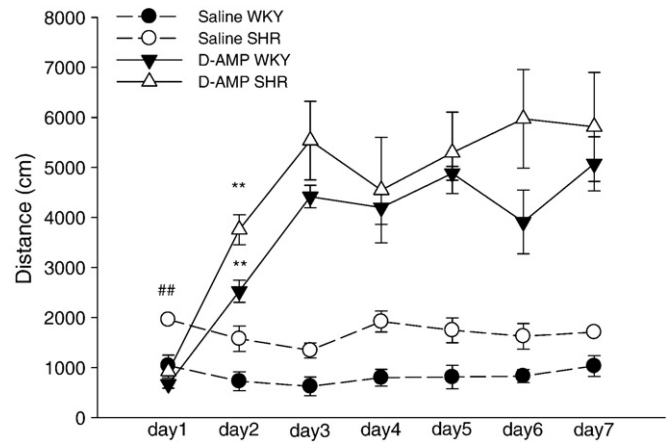


Fig. 2. Locomotor activity was measured after repeated injection 15 min of D-AMPH and saline. Four groups of rats were treated with saline and 3 mg/kg D-AMPH for 7 days. The total distance during a 5 min period in an open-field box was measured after drug injection. During the 7 days of injection of amphetamine, both SHR and WKY showed significantly greater increase in locomotor activity (ANOVA multi-variate, $F = 8.716$, $p < 0.0001$), but there was no difference after saline treatment in 7 days in SHR and WKY. SHR showed much more locomotor activity than WKY after saline administration (ANOVA multi-variate, $F = 66.52$, $p < 0.0001$). Post Hoc Tests (Dunnett, 2-sided) showed significant differences between day 1, day 2 and day 7 after administration of AMPH. ** $p < 0.0001$ represented the significant differences in SHR or WKY after amphetamine injection, when compared to saline injection in SHR or WKY. ## $p < 0.0001$ represented the significant differences between SHR and WKY after saline injection.

significant differences in day 1, day 2 and day 7 after administration of D-AMPH ($p < 0.05$). Our data showed that amphetamine can induce hyper-locomotor activity in both SHR and WKY ($F = 8.716$, $p < 0.0001$) and eliminate the differences of locomotor activities before amphetamine administration between SHR and WKY (see Fig. 2).

3.1.3. Experiment 3: effects of an acute systemic administration of D-AMPH on prepulse inhibition (PPI)

It was obvious that with the increase of pre-pulse intensities, the inhibition ability increased. But after AMPH administration, this trend became variable except for the behavioral changes after 3 mg/kg AMPH administration in WKY and 3 mg/kg, 5 mg/kg in SHR (see Fig. 3). The 2 \times 4 \times 5 (strains \times treatments \times prepulse intensities) ANOVA multi-variate analysis showed a main effect on interaction between different pre-pulse intensities and different treatments ($F = 2.477$, $p < 0.01$). Also we found the main effects came from interaction between different pre-pulse intensities and different rat strains ($F = 4.41$, $p < 0.01$). There was a significant difference of inhibition ability between SHR and WKY ($F = 7.31$, $p < 0.05$). After saline administration, SHR showed inhibition deficit in 79, 82, and 85 dB pre-pulse intensities when compared with WKY, but on the other hand better inhibition ability in lower pre-pulse intensities – 73 and 76 dB. There was a significant difference between the saline and different AMPH dosages for inhibition ability ($F = 2.89$, $p \leq 0.05$). After AMPH administration, inhibition ability showed a deficit in both WKY and SHR in the different pre-pulse intensities except for 73 dB in WKY which had better PPI ability. We also found that SHR had a similar inhibitory ability to WKY after administration of 3 mg/kg amphetamine (see Fig. 3).

3.2. Functional magnetic resonance imaging (fMRI)

The functional MRI imaging showed positive BOLD signals (red) in the cortex, striatum, cerebellum, hippocampus, caudate putamen (CPU), thalamus, hypothalamus, midbrain, and frontal cortex of SHR when a 500-g weight was put onto a rat's tail (see Fig. 4B). In WKY, the fMRI showed high activity of positive BOLD signals (red) in the cortex, cerebellum, CPU, thalamus and the frontal cortex during pain

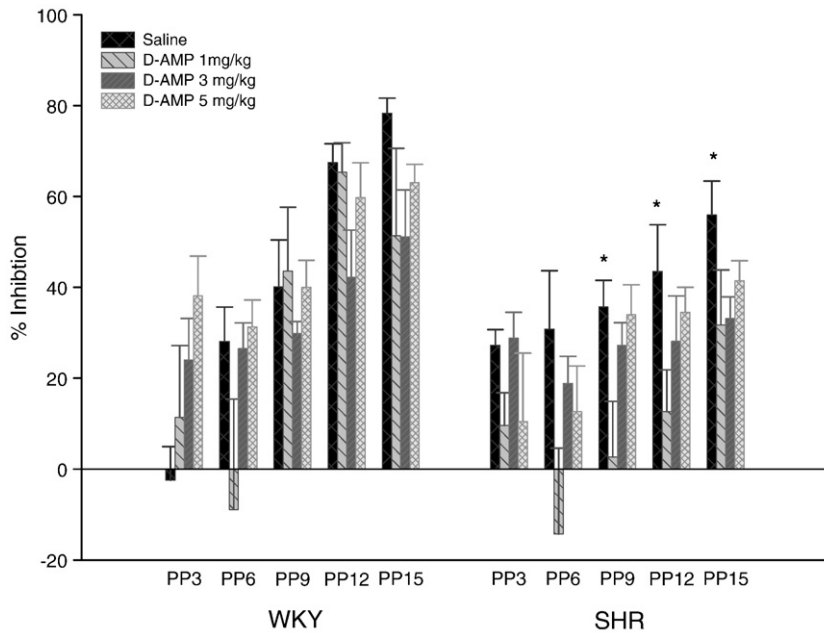


Fig. 3. Prepulse inhibition (PPI) after an acute systemic amphetamine (D-AMP) treatment. There were four types of treatment: saline, 1 mg/kg D-AMP, 3 mg/kg D-AMP and 5 mg/kg D-AMP. The intensity of the prepulse stimulus varied from 3–15 dB above the background noise level (70 dB) of 3 dB increments. % inhibition was measured under the 73 dB (PP3), 76 dB (PP6), 79 dB (PP9), 82 dB (PP12) and 85 dB (PP15) prepulse stimulants. There was a significant difference of inhibition ability between SHR and WKY (ANOVA, $F=7.31$, $p<0.05$). Meanwhile, there was a significant difference between the saline and different AMPH dosages in inhibition ability ($F=2.89$, $p\leq 0.05$). The interaction between different pre-pulse intensities and different treatments had a statistic significance within-subjects ($F=2.477$, $p<0.01$). Also the interaction between different pre-pulse intensities and different rats' strains showed a statistic significance ($F=4.41$, $p<0.01$). * $p<0.05$ represented the significant differences between SHR and WKY after saline treatment.

stimulation on the tail (see Fig. 4C). Identification of the specific brain areas was according to Pellegrino's 'A stereotaxic atlas of the rat brain' and DiFranze's work [25] (see Fig. 4A).

Compared to WKY, SHR showed multiple-system activation under this stressful stimulus, not only in the sensory-discriminative brain areas but in the limbic system as well. The number of positive BOLD voxels measured in PFC in SHR was 23.60 ± 5.22 and in WKY was 8.66 ± 2.85 ($p<0.05$). In addition, there was a significant difference between SHR and WKY after normalization of data (SHR 0.00097 ± 0.00022 , WKY 0.00032 ± 0.000095 , $p<0.05$) (see Fig. 5).

3.3. Determination of dopamine concentration by using an enzyme-linked immunosorbent assay (ELISA) in mesocortical and mesolimbic dopamine system between SHR and WKY

The cubic formulae was $y = -0.729 + 10.817x - 13.688x^2 + 6.435x^3$. This cubic curve provided a good fit of the data obtained from the positive control and standard.

There were significantly lower dopamine concentrations (ng/ml) in prefrontal cortex, midbrain and amygdala in SHR compared to WKY (dopamine concentration in the prefrontal cortex: WKY: 146.40 ± 13.71 , SHR: 104.33 ± 4.81 , $p<0.05$; in the midbrain: WKY: 138.20 ± 18.27 , SHR: 88.00 ± 9.98 , $p<0.05$; in the amygdala: WKY: 25.00 ± 2.04 , SHR: 13.25 ± 1.65 , $p<0.01$) (see Fig. 6). There was no significant difference in nucleus accumbens (NAc) between SHR and WKY using an independent sample *t*-test.

3.4. c-fos protein expression in the prefrontal cortex after the systemic D-AMP administration

In prefrontal cortex (PFC), there was a significant difference in c-fos protein synthesis between SHR and WKY after injection of 3 mg/kg amphetamine. SHR showed a significantly higher level of expression of c-fos in PFC than WKY (SHR 3.27 ± 0.28 , WKY 2.25 ± 0.15 , $p<0.05$) (see Fig. 7). There were no differences in c-fos synthesis with other amphetamine dosages using independent sample *t*-test.

3.5. Expression of c-fos and dopamine-related genes in the prefrontal cortex after repeated use of D-AMP

SHR showed significant upregulation of c-fos by a factor of 9.264 compared to WKY after amphetamine injection ($p<0.05$). Measurements of dopa decarboxylase (DDC) and dopamine beta-hydroxylase (DBH), enzymes involved in dopamine synthesis, did not show any differences. Also there were no significant differences in dopamine-related gene expression (including dopamine D1–5 receptors and the two key enzymes for dopamine synthesis and transportation, respectively TH and DAT) between SHR and WKY in the PFC after repeated use of amphetamine (see Fig. 8A). However, there were significant increases in dopamine 1, 2 and 3 receptors in WKY treated with amphetamine compared to treatment with saline. The relative gene expression (saline compared to AMPH in WKY) of dopamine 1 receptor (D1R) was 3.151, while β -Actin was 0.706. D1R was upregulated 4.47 times in WKY treated with amphetamine ($p<0.05$). The relative expression of dopamine 2 receptor (D2R) was 3.492, β -Actin was 0.706. Therefore, the D2R was upregulated by a factor of 4.95 in WKY treated with amphetamine ($p<0.01$). The relative expression of dopamine 3 receptor (D3R) was 4.093, β -Actin was 0.706. Therefore, the dopamine 3 receptor was upregulated by a factor of 5.8 in WKY treated with amphetamine ($p<0.05$) (see Fig. 8B). In SHR, there was a significant increase in dopamine 1 receptor after treatment with amphetamine compared to treatment with saline. The relative expression of dopamine 1 receptor (D1R) was 1.763, β -Actin was 0.869. The dopamine 1 receptor was upregulated by a factor of 2.03 ($p<0.05$) (see Fig. 8B).

3.6. SNAP-25 gene expression in the PFC between SHR and WKY

3.6.1. Basal SNAP-25 gene expression in the PFC between SHR and WKY
There was a significant difference in SNAP-25 gene expression in PFC between SHR and WKY. In the PFC, the normalized relative expression of SNAP-25 was 0.683, β -Actin was 0.745 (SHR compared to WKY). Therefore, SNAP-25 gene expression was downregulated in SHR compared to WKY by a factor of 1.414 ($p<0.05$) (see Fig. 9).

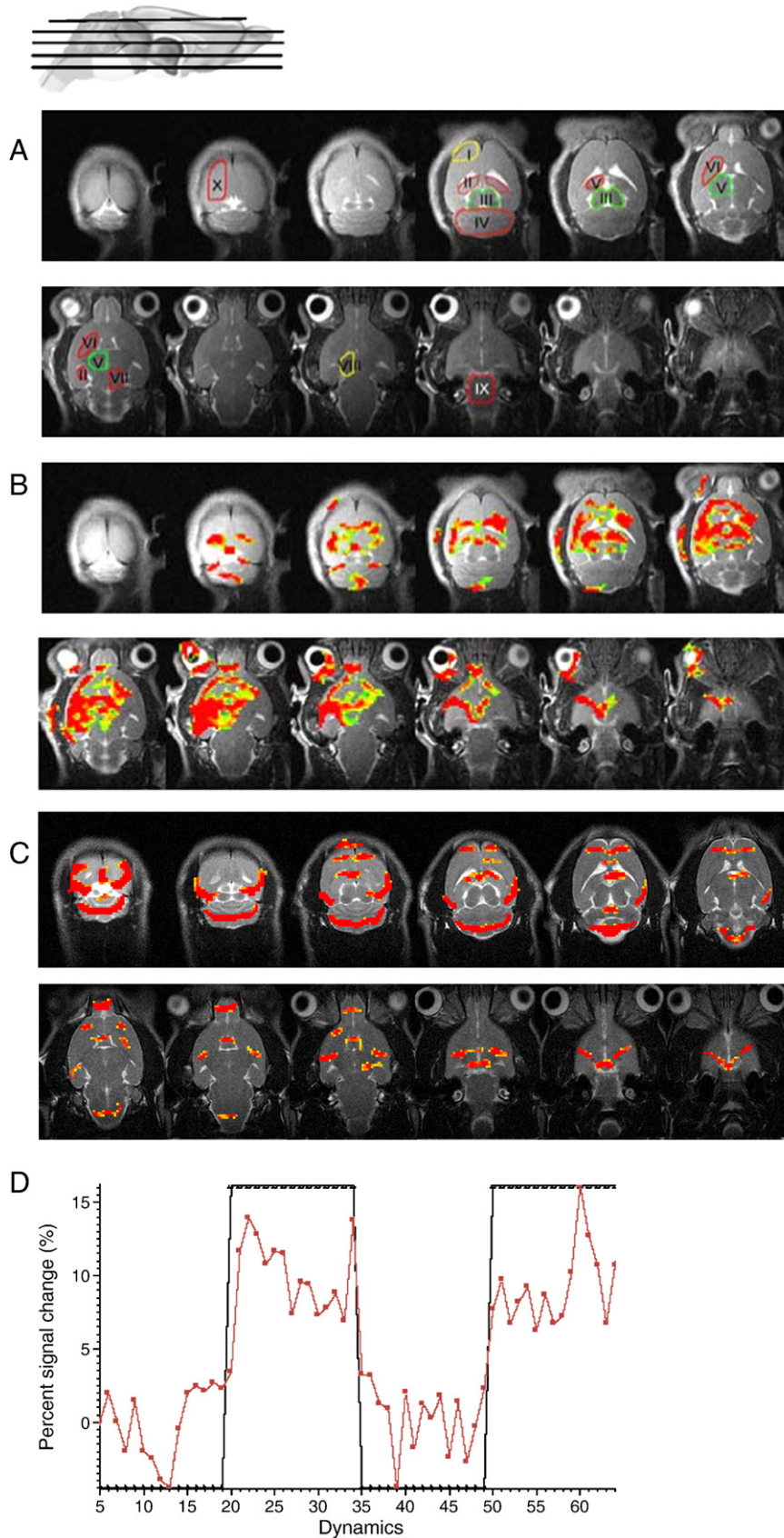


Fig. 4. (A) Anatomical images (MRI) showing 10 regions of interest. These brain areas were targeted according to Pellegrino's 'A stereotaxic atlas of the rat brain' [55]. **I** PFC (prefrontal cortex), **II** Hippocampus, **III** SC (superior colliculus) and **IC** (inferior colliculus), **IV** Cerebellum, **V** Thalamus, **VI** CPu (caudate putamen), **VII** Midbrain, **VIII** Hypothalamus, **IX** Pons, **X** Cortex. (B) SHR showing Blood Oxygenation Level Dependent (BOLD) activation in many brain structures in response to a 500-g weight onto a rat's tail. The positive BOLD signals were present in the cortex, cerebellum, hippocampus, CPu, thalamus, hypothalamus, midbrain, and prefrontal cortex (PFC). (C) Region-specific activation maps of WKY induced by a 500-g weight onto a rat's tail. Positive BOLD signals are shown in the cortex, cerebellum, CPu, thalamus, hypothalamus, and prefrontal cortex. (D) Signal intensity changes from the prefrontal cortex during stimulation period.

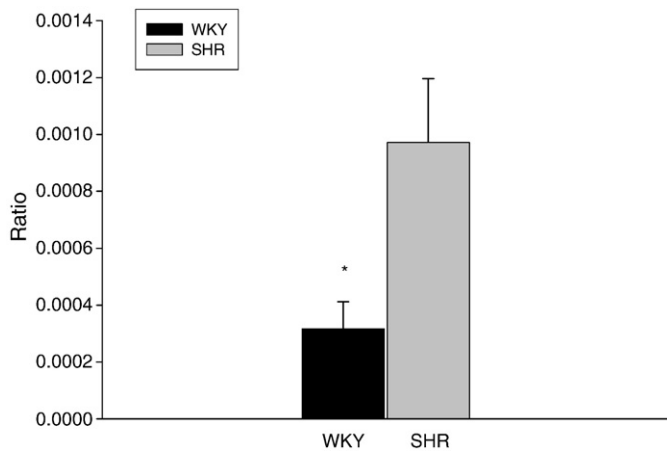


Fig. 5. The volume of positive Blood Oxygenation Level Dependent (BOLD) signal in the prefrontal cortex was normalized by the whole brain volume. Significant differences are indicated by * $p < 0.05$ between SHR and WKY.

3.6.2. SNAP-25 gene expression in the PFC after the repeated administration of D-AMP

Although SHR showed lower SNAP-25 expression in PFC when compared to WKY, there were no significant differences in SNAP-25 gene expression in PFC between SHR and WKY after D-AMP administration for 7 days (SNAP-25 gene expression was down-regulated in SHR compared to WKY by a factor of 1.309, $p = 0.65$). Further, there were no significant differences in SNAP-25 expression in the PFC in SHR with amphetamine administration when compared to saline-treated SHR. The normalized relative expression of SNAP-25 was 1.560, β -Actin was 1.11 (SHR saline treatment compared to AMPH treatment) (SNAP-25 upregulated by factor 1.405, $p = 0.189$). Similar results were found in WKY. The normalized relative expression of SNAP-25 was 1.105, while β -Actin was 0.922. Therefore, SNAP-25 was upregulated by a factor of 1.2, $p = 0.053$.

4. Discussion

In this study, dopamine concentration was found to be significantly lower in the prefrontal cortex, midbrain, and amygdala in the SHR. This would suggest that dopamine storage or release is hypofunctional in mesocortical and mesolimbic dopamine pathways in the SHR. Attention deficit and poor behavioral planning may result from decreased dopaminergic activity in the mesocortical dopamine pathway [26]. Also less dopamine concentration was found in the amygdala of SHR. A recent fMRI study found that amygdala showed slower response latencies when approaching negative emotional content [27]. This represents dysfunction in the amygdala, which might be correlated with less dopamine release or storage in amygdala. However, the cause-effect relationship should be demonstrated through further studies. We did not find any differences in dopamine concentration in nucleus accumbens (NAc) between SHR and WKY in this experiment. Carboni et al. [28] reported that the extracellular dopamine concentration in the NAc shell was higher in SHR. A possible reason is that it was difficult to precisely dissect NAc into shell and core *in vitro*, so it was difficult to determine the differences in the whole NAc.

One competing hypothesis about the etiology of ADHD is the "frontal lobe hypothesis", which suggests that ADHD is caused by problems of inhibition mediated by genetically-based abnormalities in those frontal structures of the brain responsible for executive functions [29,30]. Our studies suggest that lower levels of dopamine concentration and lower dopamine D4 receptor gene expression in the frontal cortex may translate into a relatively lower inhibitory dopaminergic influence, leading to hyperactivity in SHR. The

prefrontal cortex was the major affected brain area in SHR, hence, in our studies we focused on the prefrontal cortex alterations in SHR.

When neural activity increases in a region of the brain, the local MR signal produced in that part of the brain increases by a small amount due to changes in blood oxygenation. Functional MRI based on the BOLD effect is now a widely used tool for probing the working brain. The goal of fMRI studies is to map patterns of local changes in the MR signal in the brain as an indicator of neural activity associated with particular stimuli. Using fMRI, we tried to discover whether there were any differences in neuronal activity under stressful stimulation. A 500-g weight was put onto the rat's tail as an acute stressful stimulus, which might include pain and pressure stimuli. We found that both rat strains had similar bilateral activated brain areas, including striatum, caudate, putamen, thalamus, cerebellum, and frontal cortex. These cortical and subcortical areas were consistent with those activated by other acute and chronic pain stimuli [31,32]. As we compared the positive signals of BOLD in the prefrontal cortex, SHR showed a significantly larger volume of activated prefrontal cortex than WKY, indicating that there might be more neuronal activities or increased glucose metabolism in PFC of SHR in the stressful environment. The PFC of SHR might be hyper-responsive to stressful stimulation, just as SHR are hyper-adrenergic to stress stimuli with consequently higher levels of plasma corticosterone [33]. Normal brain development requires the coordinated maturation of many processes occurring in a temporally and regionally dependent manner. Stress is the most important stimulus to demonstrate coordinated maturation of the brain's anatomical parts (volume loss of different brain areas, such as hippocampus and PFC) from its functional parts (hypothalamo-pituitary-adrenal or HPA function) [34,35]. Stress exposure, just like amphetamine administration, can induce excessive catecholamine release [36]. Therefore, stress could trigger the potential dysfunction in the brain. In this study, SHR showed a dysfunctional stress axis similar to reports on ADHD children [37]. A more important finding in our research was dysfunction in PFC, which is one of the most sensitive brain areas to stress exposure [36]. We believe this is the first demonstration of PFC dysfunction in SHR using *in vivo* imaging.

In order to identify the potential causes of dysfunction in PFC of SHR, we studied the effects of D-AMP on behavioral and neurobiological changes. The results of this part of the experiment are

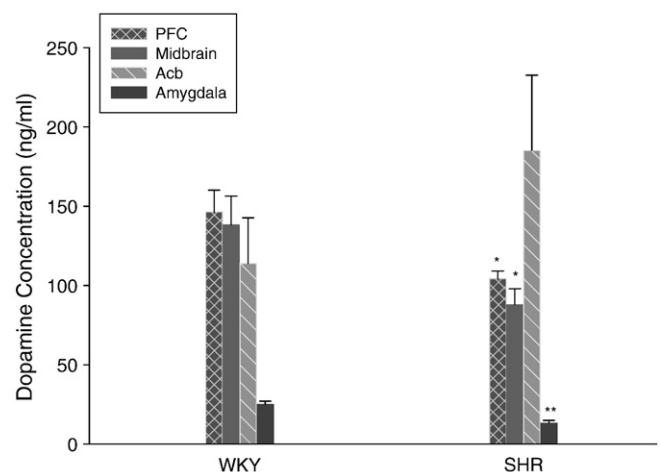


Fig. 6. Differences of dopamine concentration in the prefrontal cortex (PFC), midbrain, nucleus accumbens (NAc) and amygdala. This is the baseline expression of dopamine concentration measured by using ELISA in the different brain areas between SHR and WKY. There were significantly lower dopamine concentrations (ng/ml) in prefrontal cortex, midbrain and amygdala in SHR compared to WKY. All the data we obtained were from the cubic formulae ($y = -0.729 + 10.817x - 13.688x^2 + 6.435x^3$). This cubic curve model provided a good fit of the data obtained from the positive control and standard. Significant differences of dopamine concentration between SHR and WKY are indicated by * $p < 0.05$, ** $p < 0.01$.

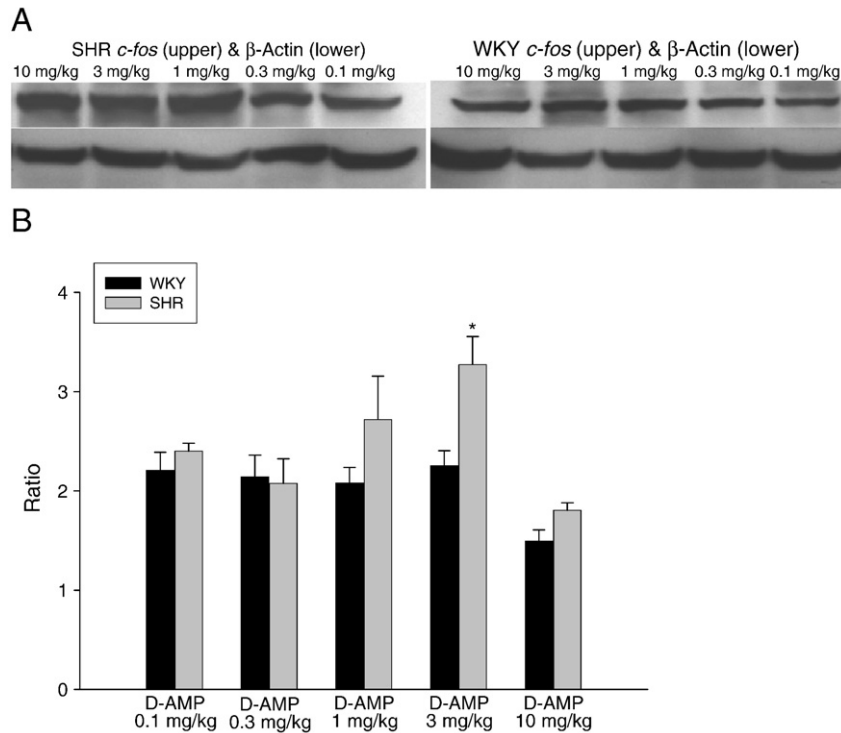


Fig. 7. (A) Western blotting plot of c-fos protein and β -Actin in the prefrontal cortex of SHR and WKY after an acute systemic amphetamine treatment. SHR and WKY rats ($n=5$ /group/strain) were killed 24 h after the last drug injection. Then we measured c-fos protein expression using Western blotting. The left side showed c-fos protein and β -Actin in the prefrontal cortex of SHR. The right side showed those of WKY. The upper plot showed c-fos protein expression and the lower plot showed β -Actin. (B) Relative density of c-fos protein expression in the prefrontal cortex after the systemic amphetamine treatment. * $p < 0.05$, WKY vs. SHR. (A) Different dopamine-related gene expression profiles normalized with β -Actin when WKY received amphetamine compared to SHR received amphetamine. (B) Different dopamine-related gene expression profiles normalized with β -Actin. (Left columns showed WKY with saline administration compared to WKY received amphetamine. Right columns showed SHR with saline administration compared to SHR received amphetamine).

summarized as follows: (1) An acute D-AMP treatment could decrease the locomotor activities in SHR receiving the systemic dosage of D-AMP. The differences in locomotor activity after systemic administration of D-AMP between SHR and WKY disappeared. (2) The defect of inhibition ability in SHR under 85-dB prepulse stimulus disappeared after administration of 1 mg/kg and 3 mg/kg amphetamine. (3) The repeated 3 mg/kg D-AMP treatment starting from the second day increased the locomotor activities in both SHR and WKY. (4) SHR exhibited increased c-fos synthesis after 3 mg/kg D-AMP treatment in the prefrontal cortex. (5) There was no difference in expression of dopamine-related gene between SHR and WKY after repeated treatment of 3 mg/kg D-AMP, although D1R, D2R and D3R gene expression increased in WKY, and D1R gene expression was increased in SHR after D-AMP treatment.

Clinical research has revealed that the responses of normal and ADHD children to psychostimulants are qualitatively similar and include reductions in activity level and impulsivity, as well as enhancement of attention-related processes [38]. In this experiment, both SHR and WKY rats exhibited reductions in locomotor activities after a single exposure to D-AMP at the lower dosage level. At the moderately low dose (1 and 3 mg/kg), SHR manifested hypoactivities compared to the saline-injected group. Following the higher dose (10 mg/kg) of D-AMP, intensive stereotypical behavior could be observed in SHR, including gnawing, sniffing, licking, and rhythmic head movements [39]. It seemed that SHR were more sensitive to D-AMP than WKY. We chose the moderately low doses of 1 and 3 mg/kg and the moderately higher dose of 5 mg/kg D-AMP to treat the rats during the prepulse inhibition task (PPI) in order to avoid influences of the stereotypical behaviors resulting from the higher dose of 10 mg/kg. In the PPI study, SHR showed impaired inhibition at the higher prepulse inhibition level (85 dB) after saline injection. Moreover, the defect of inhibitory ability in SHR under 85 dB prepulse

stimulus disappeared after administration of 1 mg/kg and 3 mg/kg amphetamine. But the inhibition ability in SHR compared to WKY was downregulated at the higher prepulse stimulus (85 dB) after injection of 5 mg/kg amphetamine. In humans, disruption of PPI has been reported in ADHD children, but the lower dose of methylphenidate may have increased inhibition in ADHD children when compared to the children who did not receive medicine [40]. In rodents, amphetamine may disrupt PPI and startle reactivity in a dose-specific manner [41,42]. In our study, there was no disruption of inhibition in SHR after administration of 3 mg/kg amphetamine.

We used changes in the expression of the nuclear protein, c-fos, as a substitutional measure of neural activation in order to assess SHR vs. WKY differences in the cortical neural response to amphetamine. We measured the protein level of c-fos in PFC to identify any functional alterations in PFC after systemic administration of D-AMP. Our study showed that there was increased c-fos expression in the prefrontal cortex of SHR compared to WKY after administration of 3 mg/kg amphetamine. Also the gene level of c-fos was higher in SHR than WKY after amphetamine injection. Other authors found no differences in the basal level expression of c-fos between SHR and WKY in cortex, striatum, and nucleus accumbens [43]. Therefore, we deduced that an increased neuronal activity in PFC may contribute to the lower locomotor activity and proper PPI responses in SHR after an acute injection of 3 mg/kg D-AMP.

In clinics, psychostimulants are prescribed for at least 7 days [44]. Therefore, we tried to determine the neurobiological changes after repeated administration of amphetamine at a moderate dose (3 mg/kg). Both SHR and WKY showed obvious hyperactivity in an open-field box after the second injection of amphetamine and the reaction was sustained up to the last day of drug administration. This behavioral sensitization has been demonstrated for many amphetamine-induced behaviors [45], including locomotion, stereotypical behavior, and

disruption of selective attention [46]. We have no documentary information about how such behavioral differences mentioned above using amphetamine can be translated into differential neural activity in cortico-striatal neural circuits, which are the open-field test and PPI-regulatory circuitry [47]. A recent study of methylphenidate (Ritalin), one of the popular prescribed drugs for ADHD children, chronically administered to normal juvenile rats resulted in short-term effects on the rat mPFC, striatum, hippocampus, and hypothalamus. Meanwhile, Ritalin reduced the rats' anxiety-like behaviors [48].

As dopamine receptors play an important role in regulation of immediate early genes (IEGs) expression, such as *c-fos*, we explored the responses of dopamine-related genes in PFC after repeated

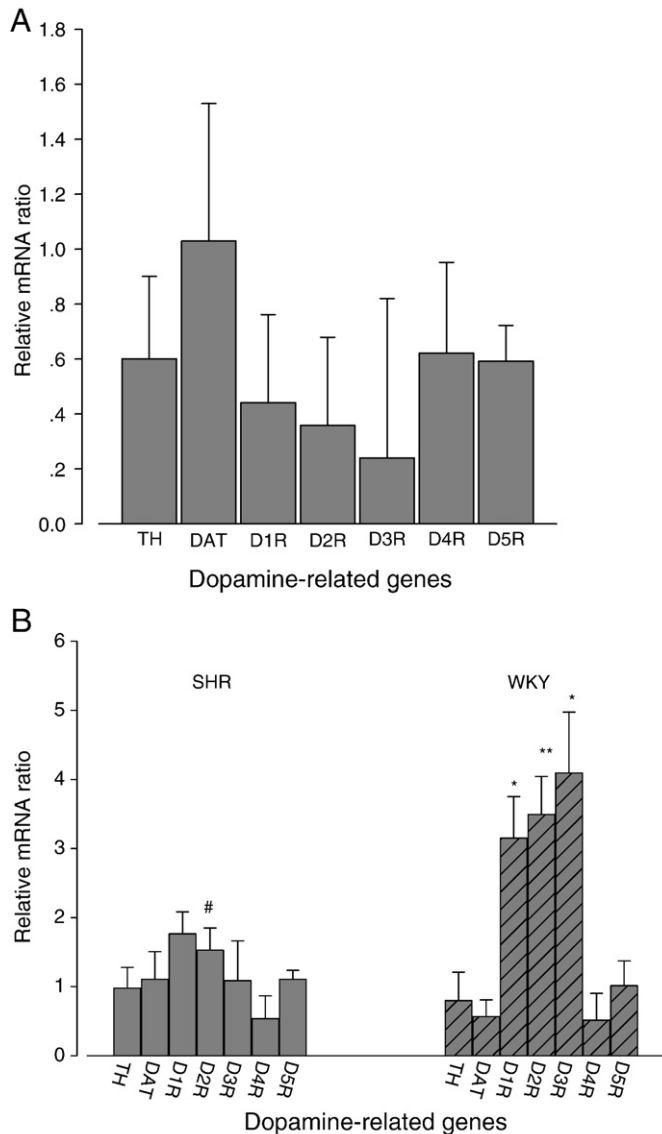


Fig. 8. Relative expression of dopamine-related genes in the prefrontal cortex after repeated amphetamine treatments using quantity real-time PCR. The $\Delta\Delta CT$ method was used to determine the relative gene quantity (Fold induction = $2^{-[\Delta\Delta CT]}$). (A) Gene expression level changes, data of SHR received amphetamine vs. WKY received amphetamine. (B) Left columns showed gene expression level changes, data of WKY used saline compared to WKY received amphetamine. Right columns showed gene expression level changes, data of SHR used saline compared to SHR received amphetamine. WKY D-AMP treatment vs. saline treatment, significant differences are indicated by * $p < 0.05$, ** $p < 0.01$. SHR D-AMP treatment vs. saline treatment, significant differences are indicated by # $p < 0.05$. Tyrosine hydroxylase – TH; Dopamine transporter – DAT; Dopamine receptor 1 – DRD1; Dopamine receptor 2 – DRD2; Dopamine receptor 3 – DRD3; Dopamine receptor 4 – DRD4; Dopamine receptor 5 – DRD5.

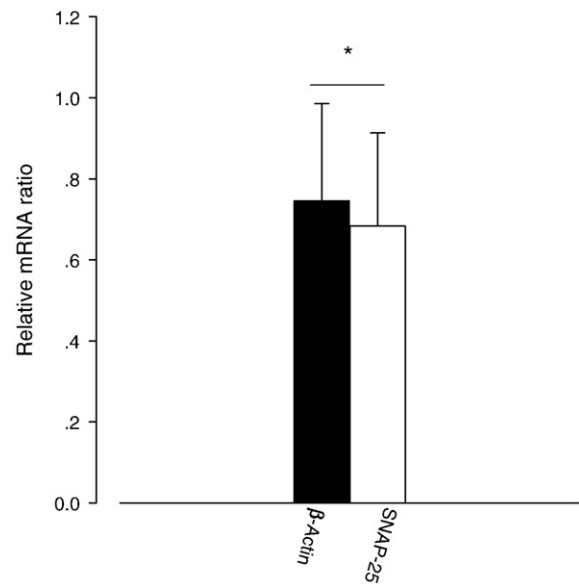


Fig. 9. Relative basal expression of SNAP-25 gene in the prefrontal cortex between WKY and SHR measured using quantity real-time PCR. WKY vs. SHR, significant differences are indicated by * $p < 0.05$.

amphetamine administrations. The results showed that dopamine 1 receptor increased after amphetamine injection in the PFC of both SHR and WKY. Dopamine 2 and 3 receptor gene expression also increased in WKY. A recent study provided evidence that the effects of a low dose of D-AMP in increasing locomotor activity are mediated by D1-receptors [49]. D1-like receptors are positively coupled to adenylyl cyclase, which activates cAMP and induces increases in the locomotor activity. Besides, D1-like receptor agonist produces a pronounced increase in *c-fos* expression in the cortical area in SHR [43]. These results may induce the hyperactivity in SHR, and higher level of *c-fos* protein expression in PFC after repeated or acute injection of D-AMP. D2/D3 receptor stimulation after D-AMP exposure may play a neuroprotective role in the cortex. The stimulation of D2 presynaptic autoreceptors might exert neuroprotective effects by a negative feedback mechanism, reducing the release of dopamine for oxidation by monoamine oxidase [50]. SHR did not show an increase of D2 and D3 receptors after repeated administrations of amphetamine, indicating that there is a potential neuronal damage through deficit of D2-like receptors protective functions in the PFC of the SHR [51]. However, the exact mechanism of behavioral and neurobiological alterations after the repeated administrations of amphetamine is still unclear.

The most compelling evidence that dopaminergic mechanisms were involved in ADHD is by pharmacological challenge in both animals and humans. In our study, there are less dopamine-related gene responses to D-AMP in SHR and WKY. Therefore, the question remains whether any other central factors that regulate the process of dopaminergic neurotransmission are involved in the origins of ADHD. In this study, we speculated that “synaptogenesis hypotheses” might be involved in ADHD [52].

A large number of proteins have been identified at nerve terminals involved in the cycling of synaptic vesicles. Studies have revealed that the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) core complex, consisting of synaptobrevin VAMP (vesicle-associated membrane protein – a synaptic vesicle-associated protein), syntaxin, and SNAP-25, plays essential roles in synaptic vesicle exocytosis [53]. The SNARE complex interacts with calcium channels that provide calcium for triggering exocytosis in the central nervous system [54]. Disruption of this interaction alters the Ca^{2+} -dependence of neurotransmitter release at the neuromuscular junctions [55]. SNAP-25 not only plays a key role in the presynaptic

regulation of synaptogenesis [52], but is also involved in the regulation of neuronal excitability via controlling calcium responsiveness to depolarization [56]. Besides, SNAP-25 is expressed in axons and nerve terminals and is essential for neurotransmitter storage and release [57,58]. SNAP-25 is therefore a multifunctional protein that participates in exocytotic function both at the mechanistic and regulatory levels. Above all, we speculated that a reduced release of dopamine in PFC might be caused by an impaired vesicular storage and/or a reduced release of dopamine from neurons in SHR [59]. This may explain our findings of the defects of dopamine storage and/or release in mesocortical and mesolimbic dopamine pathways in SHR. This finding might also be explained by the compensation of SNAP-25 for the continually increased dopamine secretion in the presynaptic area. In further studies, it would be interesting to demonstrate the relationship between SNAP-25 and the expression of dopamine-related genes. Also, the relationship between the agonists and antagonists of SNAP-25 and the dopamine system should be explored.

Acknowledgements

We would like to thank the Department of Diagnostic Radiology and Organ Imaging for their valuable assistance. Also we thank Prof. Yeung of the Department of Surgery for the use of the startle box to do the prepulse inhibition task.

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