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Role of dopamine D3 receptors in methamphetamine-induced behavioural sensitization and the characterization of dopamine receptors (D1R–D5R) gene expression in the brain

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Abstract

Introduction: *As a central nervous system stimulant, methamphetamine (METH) can cause lasting changes after being abused, including possible changes of gene expression in the brain. The dopamine (DA) system plays a fundamental role in METH-induced behavioural changes, but the expression levels of various subtypes of DA receptors, especially the dopamine D3 receptor (D3R), remains unclear.*

Material and methods: *We explored the effect of the D3R on METH-induced behavioural sensitization by comparing D3R knockout (D3R-/-) mice with wild type (WT) mice. The quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression levels of the five DA receptor (D1R, D2R, D3R, D4R, and D5R) genes in four brain regions: the prefrontal cortex (PFc), nucleus accumbens (NAc), caudate-putamen (CPu), and hippocampus (Hip).*

Results: *The behavioural test results revealed that METH could induce behavioural sensitization both in WT and D3R-/- mice. Moreover, in D3R-/- mice, the increase in movement distance induced by methamphetamine was significantly less than that of wild-type mice. The response of the five DA receptors to METH exposure varies in different brain regions. To be more specific, METH increased the expression of the D3R gene in most brain regions of WT mice, decreased D1R and D2R gene expression both in the NAc and CPu of WT mice and in CPu of D3R-/- mice.*

Conclusions: *These results suggested that D3R may play a positive regulatory role in the locomotor effects of METH, and five DA receptors, especially D1R, D2R, and D3R, may concurrently participate in the adaptive changes and the regulation of METH-induced behavioural sensitization.*

Key words: *methamphetamine, behavioural sensitization, dopamine receptor, qRT-PCR, mice, drug addiction, knockout, gene expression, brain region, adaptive changes.*

Introduction

Drug addiction is a chronic relapsing brain disease characterized by persistent, and compulsive drug seeking and taking. If the previous addict is exposed to drugs again, even after a long period of withdrawal, it is very likely to relapse [11,24,47]. Methamphetamine (METH), a substance similar to

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amphetamine, is one of the most addictive psychoactive drugs abused [1,10]. METH addiction has serious consequences for personal health and public safety [33]. The "World Drug Report 2020" [39] showed that more than a quarter of a billion people worldwide use drugs at present, and the METH market shows signs of continuous expansion. According to the "China Drug Situation Report 2019" [38], METH remains the most abused drug in China.

It is well known that METH addiction has a strong reward effect [10], which is closely related to the dopamine (DA) system [40]. The system consists of DA neurons originating in the midbrain and projecting into multiple brain regions of the prefrontal cortex (PFc), nucleus accumbens (NAc), caudate-putamen (CPu), and hippocampus (Hip) [24]. METH can stimulate neurons to release large amounts of DA, which binds to the DA receptors and triggers many molecular, physiological, and behavioural changes.

Dopamine receptors include two distinct families, D1-like (D1R and D5R) and D2-like (D2R, D3R, and D4R), which were initially distinguished based on their opposite influence on adenylyl cyclase [3,8,22,31,45]. Different subtypes of DA receptors have substantially different expression levels in neuronal populations of the brain, thereby playing fundamentally different roles in the development of drug addiction and relapse [48]. Previous studies have shown that D1R and D2R genes are widely expressed in the brain [5,7,23]. D1R is involved in regulating locomotor activity and cognition [7,18]. It is reported that mice lacking D1R-containing neurons exhibit weaker behavioural sensitization induced by METH compared to intact mice [19]. D2R is closely associated with drug-seeking and relapse behaviour [4], indicating that it has a specific role in addictive behaviours. Additionally, pharmacological studies also demonstrated that D3R might play an important role in mediating drug-induced reward and positive reinforcing effects [16]. D3R has been proposed as a therapeutic target for Parkinson's disease and schizophrenia [33]. Compared with other receptors, D4R and D5R are distributed at a slightly lower level in the brain. D4R has been reported to be associated with increased motor sensitivity caused by substance abuse such as methylphenidate and cocaine [20,21], and D4R gene expression may also be involved in diseases of the DA system, such as attention deficit/hyperactivity disorder (ADHD) [2]. D5R is a functionally important target site for the indirect actions of cocaine, and it was found that the response of rats to cocaine was dramatically attenuated by D5R knockout [12].

It has been reported that DA receptors are involved in the regulation of drug addiction. Current studies mostly focus on receptor agonists, antagonists, or protein levels [30,32,37,44], but the expression of DA receptor genes (D1R–D5R) in drug addiction in different brain regions remains largely unknown. In this study, an animal model of drug addiction was established in WT and $D3R^{-/-}$ mice to explore the role of D3R in sensitization, and the quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression of five DA receptor genes in different brain regions of the PFc, NAc, CPu, and Hip, so that the effect of DA receptors (D1R–D5R) on METH addiction can be evaluated.

Material and methods

Animals

Twenty-four adult male $D3R^{-/-}$ mice and 24 adult male wild type (WT) mice (8 weeks, 20-25 g) were used in the current study. The $D3R^{-/-}$ mice were generated originally by Xu *et al*. [46]. Homozygous mutants and WT littermates were produced *via* crossing D3R heterozygous mutant mice and then genotyped by polymerase chain reaction (PCR). Four oligonucleotide primers were used in PCR with genomic DNA isolated from the WT and $D3R^{-/-}$ mice tails. The primer sequences for PCR genotyping are 5'-AGCAAGGCGAGATGACAGGA-3', 5'-CAAGATGGATTGCACGCAGG-3', 5'-GCTCACCACTAGGTAGTTG-3', and 5'-ACCTCTGAGCCAGATAAGC-3'. $D3R^{-/-}$ mice develop normally, and the gene knockout does not affect the expression of other DA receptor subtypes, which have been described in previous reports [46].

All mice were housed in cages (four per cage) with a 12-h light/dark cycle (lights on at 7 a.m.) with food and water ad libitum at all times. The temperature of the animal feeding room was 21-24°C, and the humidity was 50%. All behavioural tests were performed during the light phase. All the animal practice was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978), and the Use Committee of Shanxi Medical University approved the experiments. All efforts were made to minimize the number of animals used and their suffering.

Drugs

METH hydrochloride (purity > 99.9%), purchased from the National Institutes for Food and Drug Control (NIFDC) (Beijing, PR China), was dissolved in 0.9% saline. The volume of intraperitoneal (i.p.) injection was 10 ml/kg, and the dosage used in this experiment was 2.0 mg/kg.

Behavioural sensitization and open field locomotor activity

The experimental scheme of behavioural sensitization was shown in Figure 1. The mice were allowed to adapt to the new environment for one week in the laboratory before the experiment. All mice were injected with saline once a day for two consecutive days (days 1-2). WT mice and $D3R^{-/-}$ mice then were randomly divided into four groups of 12: WT SA, WT METH, $D3R^{-/-}$ SA, and $D3R^{-/-}$ METH. The mice were intraperitoneally (i.p.) injected with METH (WT METH group, $D3R^{-/-}$ METH group, 2.0 mg/kg) or saline (WT SA group, $D3R^{-/-}$ SA group) once a day for five consecutive days (days 3-7). Neither injection nor treatment was given for two days (days 8-9). This procedure was repeated once (days 10-16). On day 17, the mice were injected with a challenge dose of either METH (2.0 mg/kg, i.p.) or saline. After injection, the horizontal locomotor activity was performed in an open field apparatus (40 cm × 40 cm × 40 cm) and a smart video tracking system (version 2.5; Panlab Technology for Bioresearch, Barcelona, Spain) was used to record the locomotor activity for 60 min.

Brain tissue collection

After completing the behavioural test, the mice were immediately euthanized with cervical dislocation. Referring to the map of the mouse brain tissue [41], we rapidly separated the PFc, NAc, CPu, and Hip brain tissues on an ice-cold plate and promptly stored them at –80°C.

RNA isolation

Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions, and its concentration was determined by the Infinite M200 PRO (Tecan, Switzerland). After that, 1 µg of total RNA was taken and reverse-transcribed into cDNA with a HiFiScript gDNA Removal cDNA Synthesis Kit (Cwbiotech, China) according to the manufacturer's directions.

qRT-PCR analysis

qRT-PCR was conducted to determine the expression levels of five DA receptor genes in different brain regions. The cDNA was amplified by Applied Biosystems QuantStudio 5 (Thermo Fisher Scientific, USA) and M5 HiPer SYBR Premix (Beijing Mei5 Biotechnology Co., Ltd., China) under the following conditions: 95°C for 30 s; 95°C for 15 s, 56°C for 15 s, and 72°C for 30 s. The number of cycles was carried out 40 times. A set of custom sequence-specific primers, as shown in Table I, was used for amplification. The qRT-PCR reactions were performed in triplicate. GAPDH served as the internal reference for normalization, and the $2^{-\Delta\Delta Ct}$ method was adopted to calculate the relative expression levels of each gene [34].

Statistical analysis

The movement distance of mice within 60 min after treatment was used for behavioural sensitization analysis. All data were presented as mean

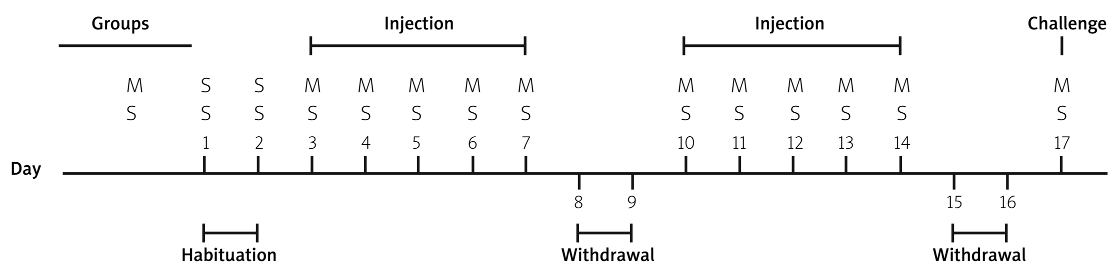

Fig. 1. Behavioural sensitization procedure. Day 1-2: Mice were injected i.p. with saline (10 ml/kg) once a day. Horizontal locomotor activity of mice was recorded and measured 60 min after injection. Day 3-7, day 10-14, and day 17: METH (2.0 mg/kg) or saline was injected once a day in the METH group and the SA group, and their horizontal locomotor activity was measured 60 min after injection. Days 8, 9, 15, and 16: mice were not given any treatment.

Table I. Primer sequences used in qRT-PCR

Gene	Gene bank	Forward (5'-3')	Reverse (5'-3')	Size
D1R	NM_001291801.1	CTCTAAAGCAAGGGCATTTGG	GGCCTCTTCCTGGTCAATCT	130 bp
D2R	NM_010077.3	GACACCACTCAAGGGCAACT	ATCCATTCTCCGCCTGTTCA	103 bp
D3R	NM_007877.2	CAACTACCTAGTGGTGAGCCT	GCAAATGCGGCTGAAATTCC	115 bp
D4R	NM_007878.3	CATCAGCGTGGACAGGTTC	CATCATTGAGGCCACACACC	140 bp
D5R	NM_013503.3	GGCACAGAAGAGAGTTCCCT	TGCAAGTCACAGAACAAGCC	105 bp
GAPDH	NM_001289726.1	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	123 bp

± SEM and analysed with SPSS software (version 25; IBM Corp., Armonk, NY, USA). Repeated-measures ANOVA was used to analyse the effects of METH, D3R, and time on behavioural sensitization, and a post hoc multiple comparison test was performed to analyse the differences of the same group at different time points and the differences of different groups at the same time point. The effects of METH and genotypes on gene expression within each brain region were compared using factorial ANOVA with Tukey's post hoc analyses to determine significant differences between groups. $P < 0.05$ was the statistical significance standard.

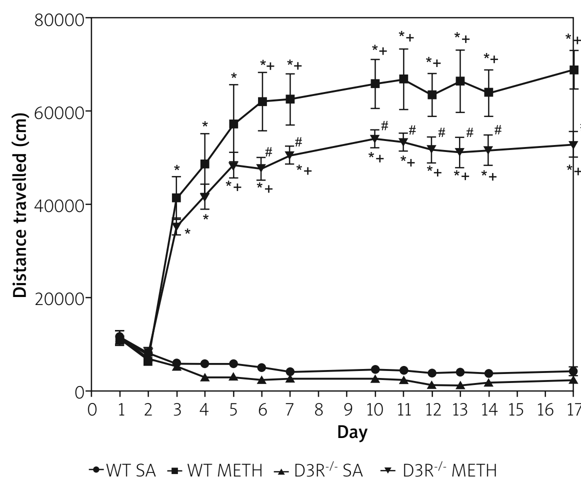

Fig. 2. Effect of D3R on METH-induced behavioural sensitization. The locomotor activity of mice in four groups was recorded for 60 min after injection of METH (2.0 mg/kg) or saline. Data are presented as mean ±SEM. $N = 12$ animals per group. $P < 0.05$ was considered as being statistically significant. WT METH vs. WT SA, $D3R^{-/-}$ METH vs. $D3R^{-/-}$ SA, $^{*}p < 0.05$; $D3R^{-/-}$ METH vs. WT METH, $^{\#}p < 0.05$; compared with day 3, $^{+}p < 0.05$.

Results

Effect of D3R on METH-induced behavioural sensitization

To investigate the role of D3R in METH-induced behavioural sensitization, $D3R^{-/-}$ and WT mice were divided into four groups, WT injected with saline (WT SA), D3R KO injected with saline ($D3R^{-/-}$ SA), WT injected with METH (WT METH), and D3R KO injected with METH ($D3R^{-/-}$ METH). The results of repeated-measures ANOVA showed that time ($F_{12,492} = 59.454$, $p < 0.001$), METH treatment ($F_{1,41} = 466.265$, $p < 0.001$), and D3R ($F_{1,41} = 7.838$, $p < 0.01$) all had significant effects on behavioural sensitization.

There was no significant difference in basal spontaneous locomotor activity after saline injections between the $D3R^{-/-}$ and WT mice. In addition, repeated saline treatments did not induce appreciable changes in locomotor activity in WT and $D3R^{-/-}$ mice ($n = 12$ mice each, Fig. 2). After repeated treatment with METH, both groups of mice with different genotypes showed behavioural sensitization (compared with day three, $^{+}p < 0.05$, Fig. 2); METH-treated $D3R^{-/-}$ and WT mice showed a significant increase in locomotor activity, compared with the corresponding saline group (WT METH vs. WT SA, $^{*}p < 0.05$; $D3R^{-/-}$ METH vs. $D3R^{-/-}$ SA, $^{*}p < 0.05$; Fig. 2). It is noteworthy that $D3R^{-/-}$ mice showed a significant decrease in response to repeated and intermittent injection of METH ($D3R^{-/-}$ METH vs. WT METH, $^{\#}p < 0.05$, Fig. 2).

These results suggested that repeated and intermittent METH (2.0 mg/kg) administration could induce behavioural sensitization in WT and $D3R^{-/-}$ mice. D3R was not apparently involved in regulating basal spontaneous locomotor activity. However, $D3R^{-/-}$ mice showed a reduced response to METH, suggesting that D3R may play a positive regulatory role in the psychomotor effects of METH.

Effect of METH on the expression of D1R–D5R genes in WT and D3R$^{-/-}$ mice

To further investigate the role of D3R in METH-induced behavioural sensitization and to determine whether other receptors are involved, we detected the D1R–D5R gene expression levels in reward-related brain regions, including the PFc, NAc, CPu, and Hip. The results of statistical analysis showed that, although D1R gene expression was not significantly altered in the PFc and Hip of WT and D3R$^{-/-}$ mice, METH treatment significantly decreased the expression of the D1R gene in the NAc of WT mice (WT METH vs. WT SA, $^{*}p < 0.05$, Fig. 3A) and the CPu of WT and D3R$^{-/-}$ mice (WT METH vs. WT SA, $^{*}p < 0.05$; D3R$^{-/-}$ METH vs. D3R$^{-/-}$ SA, $^{*}p < 0.05$; Fig. 3A). The expression of D2R gene was similar to that of the D1R, D2R gene expression also remarkably decreased in the NAc of WT mice (WT METH vs. WT SA, $^{*}p < 0.05$, Fig. 3B) and in the CPu of WT and D3R$^{-/-}$ mice (WT METH vs. WT SA, $^{*}p < 0.05$; D3R$^{-/-}$ METH vs. D3R$^{-/-}$ SA, $^{*}p < 0.05$; Fig. 3B). This study focused on the function of the D3R gene, which was not detected in D3R$^{-/-}$ mice confirming that D3R was successfully knocked out. In WT mice, D3R gene expression was significantly increased in the PFc, NAc, and Hip after the METH injection (WT METH vs. WT SA, $^{*}p < 0.05$; Fig. 3C). Although METH did not significantly change D4R and D5R gene expression in the four brain regions of WT mice, it significantly increased the gene expression of D4R in the PFc and NAc (D3R$^{-/-}$ METH vs. D3R$^{-/-}$ SA, $^{*}p < 0.05$; Fig. 3D) and decreased the gene expression of D5R in the CPu and Hip of D3R$^{-/-}$ mice (D3R$^{-/-}$ METH vs. D3R$^{-/-}$ SA, $^{*}p < 0.05$; Fig. 3E). Compared with WT mice, D3R$^{-/-}$ mice showed a significant decrease in the expression of D5R in the CPu and Hip (D3R$^{-/-}$ METH vs. WT METH, $^{\#}p < 0.05$; Fig. 3E).

These results suggested that D3R may play a positive regulatory role in the locomotor effects of METH, and five DA receptors, especially D1R, D2R, and D3R, may concurrently participate in the regulation of METH-induced behavioural sensitization.

Discussion

Behavioural sensitization, a behaviour paradigm of drug addiction, refers to a progressive increase in locomotor activity following intermittent repeated administration of the drug such as METH [35]. Previous studies have found that D3R plays a vital role in reconsolidating cocaine-induced conditioned place preference and the locomotor activity changes induced by addictive drugs in mice [9,48].

In the present study, we found that METH can increase locomotor activity and induce persistent behavioural sensitization in WT and D3R$^{-/-}$ mice. Moreover, compared with WT mice, D3R$^{-/-}$ mice showed a significantly lower intensity in response to METH stimulation (Fig. 2), suggesting that D3R may play a positive regulatory role in the psychomotor effects of METH. Li *et al*. found that the absence of D3R inhibited acute morphine-induced rapid motor activity and chronic morphine-induced behavioural sensitization [28]. Leriche *et al*. reported that hyperactivity produced by a low dose of MK-801 is dependent upon D3R stimulation [26]. Le Foll *et al*. also concluded that blocking D3R prevents nicotine from inducing locomotor activity and sensitization [25,33]. The above findings are supporting our novel findings that D3R could regulate repetitive stimulus-induced locomotor activity and contribute to the development of drug-induced behaviour.

D3R, distributed in the limbic system, is one of the key targets in the study of the mechanism of drug addiction [15,16,25,37]. Given the vital role of D3R in regulating METH-induced behavioural sensitization, it is necessary to further identify the changes of D3R gene expression levels in relevant brain regions. The present study showed that, in WT mice, the expression level of the D3R gene increased in the PFc and Hip, and especially in the NAc (Fig. 3C), which was consistent with the more active behaviour in the WT mice than in D3R$^{-/-}$ mice (Fig. 2). The NAc is believed to be the core nucleus in the brain involved in modulating the reinforcement and reward effects of addictive drugs [15]. In the present study, D3R was highly and specifically expressed in the NAc (Fig. 3C), further suggesting that D3R is closely related to drug addiction. In other words, the present study revealed that, in the expression level factor of the D3R gene, D3R may play a positive regulatory role in the locomotor effects of METH. Heidbreder *et al*. also summarized that the gene expression level of D3R significantly increased in the NAc in the studies of cocaine self-administration, nicotine-induced behavioural sensitization, and chronic morphine exposure [16]. These results suggested that inhibition of D3R may help prevent drug addiction.

As the most widely distributed DA receptor, D1R plays a vital role in learning, memory, and locomotor activity [7] and is implicated in some neuropsychi-

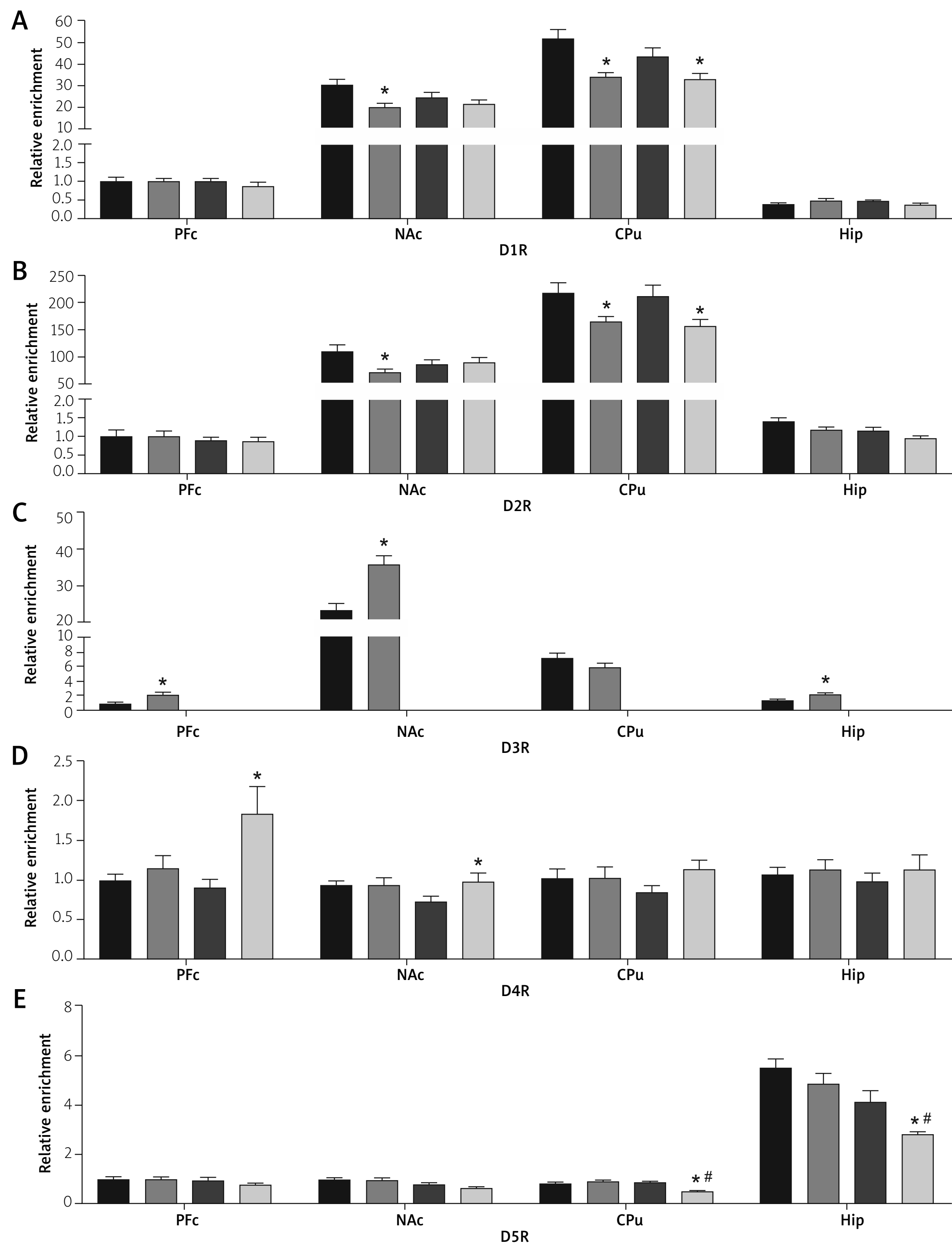

Fig. 3. Effect of METH on the DA receptors genes (D1R–D5R) in WT and D3R$^{-/-}$ mice. qRT-PCR was used to determine the expression changes of five DA receptors – D1R (**A**), D2R (**B**), D3R (**C**), D4R (**D**), and D5R (**E**) – in four brain regions in WT and D3R$^{-/-}$ mice treated with METH. $2^{-\Delta\Delta Ct}$ method was used to calculate all five dopamine receptors' expression levels relative to those in PFc of WT mice treated with saline. GAPDH was used as the internal control. The data are presented as means ±SEM. *N* = 8-12 animals per group. *P* < 0.05 was considered statistically significant. WT METH vs. WT SA, D3R$^{-/-}$ METH vs. D3R$^{-/-}$ SA, $^{*}p < 0.05$; D3R$^{-/-}$ METH vs. WT METH, $^{\#}p < 0.05$.

atric disorders [29]. D1R agonist has been reported to enhance amphetamine-induced behavioural sensitization [18]. In the present study, METH treatment decreased the levels of D1R gene expression in the NAc of WT mice and the CPu of WT and D3R$^{-/-}$ mice (Fig. 3A). We speculated that the reduction in D1R expression is most likely an adaptation to the repeated METH administration, which causes a substantial rise in synaptic dopamine (an unpublished paper of ours revealed that the level of DA significantly increases in the NAc in METH-induced conditioned place preference in mice). A reduction in D1R gene expression may be how post-synaptic neurons adapt to the onslaught of METH-induced DA release and persistent D1R activation. In addition, previous studies found that D2R was also involved in drug addiction. Volkow *et al*. [43] reported that the changes in D2R expression levels were observed in abusers of METH, cocaine, heroin, and alcohol even months after withdrawal. Imaging studies have shown that a common abnormality among cocaine and heroin abusers is the lower-than-normal level of availability of D2R [50]. D2R knockout mice exhibited increased sensitivity to the locomotor effects and the rewarding properties of cocaine [43,50] and showed enhanced reactivity to cocaine-paired cues [42,43]. Our present study showed that METH treatment decreased the levels of D2R gene expression in the NAc of WT mice and the CPu of WT and D3R$^{-/-}$ mice (Fig. 3B), which is consistent with the results of our previous study on the effects of ketamine [6]. Therefore, our findings indicated that D1R and D2R play essential roles in METH-induced behavioural sensitization in the NAc and CPu.

D4R dysregulation has been shown to be associated with a variety of behaviours, such as novelty-seeking and ADHD, and D4R gene expression plays an important and distinct role in cocaine-seeking behaviour in mice [2]. This means that D4R gene expression experiences adaptive changes in drug addiction. In this study, we found that the levels of D4R and D5R expression did not change significantly in WT mice after the administration of METH. In D3R$^{-/-}$ mice, D4R gene expression levels increased substantially in the PFc and NAc (Fig. 3D), and D5R gene expression levels decreased in the CPu and Hip after the administration of METH (Fig. 3E). It was speculated that, in the absence of D3R, the gene expression of the two subtypes of D4R and D5R is more likely to undergo adaptive changes.

In addition, it should be noted that the role of D3R in the regulation of drug-induced locomotor activity may not be entirely consistent with some previous studies. Our results revealed that there was no significant difference in the amount of locomotor activity induced by saline between the two groups of WT and D3R$^{-/-}$ mice, suggesting that D3R does not participate in the regulation of basal spontaneous locomotor activity in mice. It was also reported that no difference was observed in amphetamine-induced locomotor activity between D3R$^{-/-}$ mice and WT mice [13]. Nonetheless, other studies reported that D3R$^{-/-}$ mice even exhibited higher amphetamine- or cocaine-induced locomotor activity than WT mice [17,27]. The discrepancy might be attributed to the differences in drug type, drug dosage, injection frequency, and withdrawal time. Besides, the mice used in our study differ from the mice used by Song *et al*. in genetic backgrounds and specific gene fragments knocked out, resulting in the difference in compensatory adaptation [27]. The strain of mice in our study was a crossbreed between 129/Sv and C57BL/6, and changes took place in both the first exon and the second exon of the D3R gene in these D3R$^{-/-}$ mice. However, the mice used by Song *et al*. were bred from a single strain of C57BL/6, and changes occurred only in the second exon of the D3R gene in their D3R$^{-/-}$ mice [36].

In conclusion, our present study indicated that D3R might play a positive regulatory role in the locomotor effects of METH and five DA receptors, especially D1R, D2R, and D3R, which may concurrently participate in the regulation of METH-induced behavioural sensitization.

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Shanxi Medical University. No patient was involved. The experimental protocol was approved by the ethics committee of Shanxi Medical University.

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Disclosure

The authors report no conflict of interest.

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