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3,4-methylenedioxyamphetamine upregulates p75 neurotrophin receptor protein expression in the rat brain★

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Abstract

The p75 neurotrophin receptor, which is a member of the tumor necrosis factor receptor superfamily, facilitates apoptosis during development and following central nervous system injury. Previous studies have shown that programmed cell death is likely involved in the neurotoxic effects of 3, 4-methylenedioxy-N-methylamphetamine (MDMA), because MDMA induces apoptosis of immortalized neurons through regulation of proteins belonging to the Bcl-2 family. In the present study, intraperitoneal injection of different doses of MDMA (20, 50, and 100 mg/kg) induced significant behavioral changes, such as increased excitability, increased activity, and irritability in rats. Moreover, changes exhibited dose-dependent adaptation. Following MDMA injection in rat brain tissue, the number of apoptotic cells dose-dependently increased and p75 neurotrophin receptor expression significantly increased in the prefrontal cortex, cerebellum, and hippocampus. These findings confirmed that MDMA induced neuronal apoptosis, and results suggested that this effect was related by upregulated protein expression of the p75 neurotrophin receptor.

Key Words: 3, 4-methylenedioxyamphetamine; apoptosis; cytotoxicity; neural regeneration; p75 neurotrophin receptor

Abbreviations: MDMA, 3, 4-methylenedioxy-N-methylamphetamine; $p75^{NTR}$, p75 neurotrophin receptor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling

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INTRODUCTION

3,4-methylenedioxy-N-methylamphetamine (MDMA) is an amphetamine derivative with hallucinogenic effects and is widely used as a recreational drug. Increasing evidence has confirmed brain toxicity with use[1-3]. MDMA is neurotoxic for serotonergic and dopaminergic neurons and results in decreased 5-hydroxytryptamine transporters[2-3]. Non-human primate studies[4-6] have shown that MDMA-induced changes in 5-hydroxytryptamine terminal markers persist for > 1 year, similar to changes observed in some human MDMA users. Moreover, a stimulatory effect of MDMA on cortical and hippocampal acetylcholine release has also been documented[7-9]. The influence of MDMA on the brain and behavior has aroused great concern. [^{18}F]fluorodeoxyglucose positron emission tomography reveals metabolic increases in cortical and limbic regions in human volunteers, who abused cocaine and were exposed to drug-related stimuli[10]. Positron emission tomography showed that the majority of drug abusers (particularly those taking a new drug for the first time) exhibit brain imaging abnormalities in the frontal lobe, temporal lobe, thalamus, cerebellum, and hippocampus.

The present study analyzed effects in the prefrontal cortex, hippocampus, and the cerebellum. In addition, an *in vitro* animal model was utilized to analyze MDMA-induced neurotoxicity and apoptosis, behavioral changes, and p75 neurotrophin receptor ($p75^{NTR}$) protein expression in three different brain regions (prefrontal cortex, cerebellum, and hippocampus) to determine the relationship between $p75^{NTR}$ and MDMA-induced neurotoxicity and apoptosis.

RESULTS

Quantitative analysis of experimental animals

A total of 20 male Wistar rats were equally randomized into control, low-, middle-, and high-dose MDMA groups. Three MDMA groups were injected with 20, 50, and 100 mg/kg[11], respectively, while the control group was injected with an equal volume of normal saline. The rats were sacrificed 24 hours after the final drug injection, and brain tissues were harvested for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay and western blot analysis. All 20 rats were included in the

final analysis.

Effects of MDMA on cell apoptosis in various rat brain regions

TUNEL assay results showed that compared to the control group, the number of apoptotic cells significantly increased in brain regions treated with high-, middle-, and low-dose MDMA groups ($P < 0.05$), and the number of cells increased in a dose-dependent manner. In addition, the number of apoptotic cells was greater in high- and middle-dose MDMA groups compared with the low-dose MDMA group ($P < 0.05$; Table 1).

Rat behavioral changes

Control rats exhibited no significant behavioral changes. However, at 5–7 minutes post-MDMA injection, the rats exhibited behavioral changes, which were maintained for > 30 minutes. Low-dose MDMA group rats exhibited increased activities, repetitive stereotyped action, and exploratory sniffing; middle-dose MDMA group rats exhibited accelerated breathing, vertical tail positioning, piloerection, irritability, increased physical activities, and leaping out of the cage in response to tiny stimulations; high-dose MDMA group rats exhibited shortness of breath, decreased limb muscle strength, double-leg dragging, unsteady gait, inability to stand, piloerection response, decreased physical activity, and a rapid anesthesia state. However, behaviors in the low-dose MDMA group rats remained unchanged in response to continuous injection over the next days and were characterized by adaptive changes.

Table 1 Changes in the number of apoptotic cells in different brain regions of rats treated with MDMA

Group	Frontal cortex	Hippocampus	Striatum
Control	3.70±0.84[a]	1.60±1.10[a]	2.50±0.84[a]
MDMA			
Low-dose (20 mg/kg)	28.00±0.84[b]	28.90±1.48[b]	18.50±1.14[b]
Middle-dose (50 mg/kg)	38.40±1.82[ab]	30.40±2.41[ab]	22.20±1.30[ab]
High-dose (100 mg/kg)	44.80±1.92[ab]	42.20±2.59[ab]	38.40±1.67[ab]

[a]$P < 0.05$, *vs.* low-dose MDMA group; [b]$P < 0.05$, *vs.* control group. Results are represented by the number of apoptotic cells in a 250-fold field of view using light microscopy.

Data are expressed as mean ± SD from five rats in each group (two-sample *t*-test). MDMA: 3,4-methylenedioxy-N-methylamphetamine.

$p75^{NTR}$ protein expression in the prefrontal cortex, cerebellum, and hippocampus of rats following MDMA injection (Figure 1)

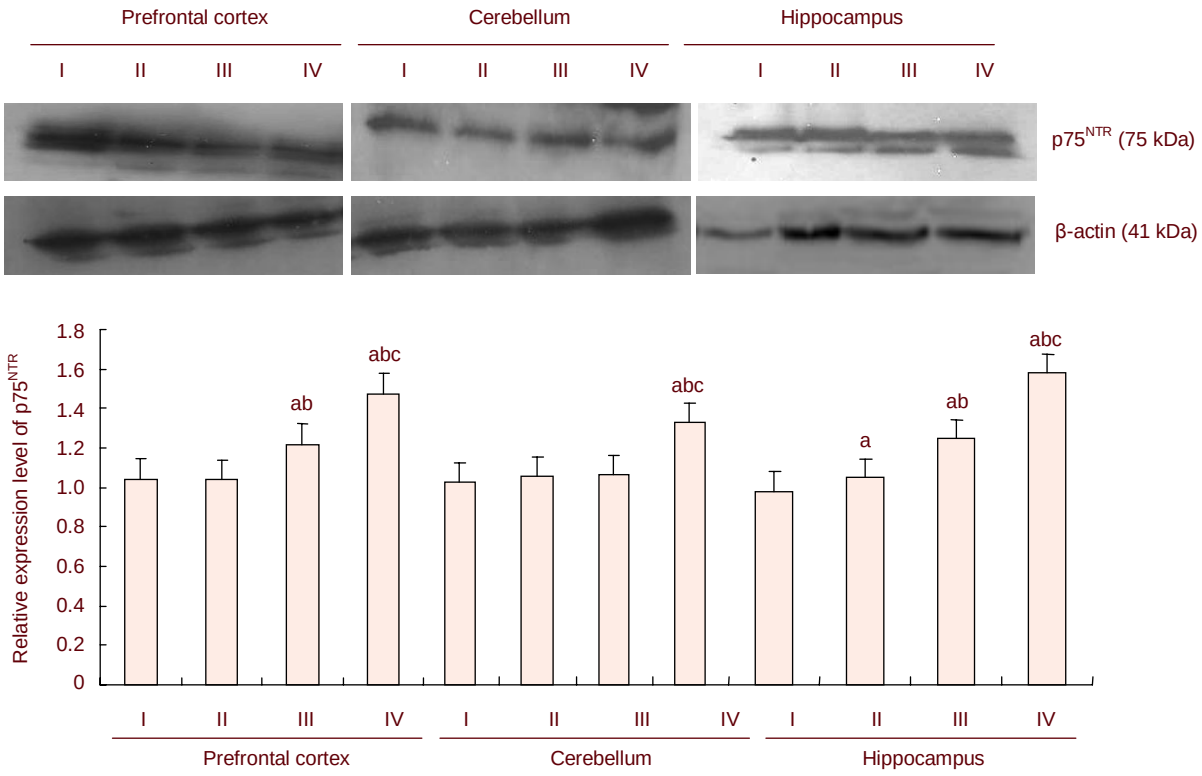


Figure 1 p75 neurotrophin receptor ($p75^{NTR}$) protein expression in the prefrontal cortex, cerebellum, and hippocampus of rats following 3,4-methylenedioxy-N-methylamphetamine injection (MDMA).

[a]$P < 0.05$ *vs.* control group; [b]$P < 0.05$ *vs.* low-dose MDMA group; [c]$P < 0.05$ *vs.* middle-dose MDMA group (one-way analysis of variance). Data are shown as the mean ± SD using the gel imaging system.

I–IV: Control, low-, middle- and high-dose MDMA groups, respectively. Relative amount was expressed as absorbance ratio of target protein to β-actin.

Western blot analyses revealed that MDMA induced significantly increased $p75^{NTR}$ protein expression in different brain regions of rats ($P < 0.05$). In the prefrontal cortex, the middle- and high-dose MDMA groups exhibited a dose-dependent increase. In the cerebellum, $p75^{NTR}$ expression significantly increased in the high-dose MDMA group. In the hippocampus, $p75^{NTR}$ expression dose-dependently increased in the low-, middle-, and high-dose MDMA groups (Figure 1).

DISCUSSION

During pathogenic mechanisms of MDMA, various genetic and epigenetic alterations accumulate to facilitate cell transformation, further resulting in neuronal toxicity. Results from the present study confirmed that apoptosis was included in the MDMA-induced neurotoxicity effects. However, the specific mechanisms of MDMA-induced apoptosis remain poorly understood. Therefore, it is vital to identify the apoptosis-related factors related to MDMA, which may provide a better understanding of the pathogenic mechanisms of MDMA to help develop novel targets for therapy.

Upon activation, $p75^{NTR}$ initiates apoptosis through a series of protein interaction interfaces within the cytoplasm. Previous *in vivo* and *in vitro* experiments have demonstrated that increased $p75^{NTR}$ expression could lead to neuronal death. For example, *in vitro* analyses have shown that $p75^{NTR}$ induces cell death in hippocampal cells[12-13], Schwann cells[14-16], and neuroblastoma cells[17]. Results have also shown that injury, such as Purkinje neuronal axotomy, results in significant re-expression of $p75^{NTR}$ in injured neurons[18]. In the present study, results suggested that $p75^{NTR}$ protein expression was involved in MDMA-induced neurotoxicity and apoptosis. Specifically, $p75^{NTR}$ protein was expressed differently in the three brain regions. Compared with the normal control group, $p75^{NTR}$ protein expression significantly increased in a dose-dependent manner in various brain regions following injection of different MDMA doses. In addition, significant differences existed in the hippocampus between groups. Specifically, there was no statistical difference in $p75^{NTR}$ protein expression in the prefrontal cortex between the middle- and high-dose groups, as well as in the cerebellum between low- and middle-dose groups. These results suggested that the prefrontal cortex was tolerant to a certain range of MDMA doses, and the cerebellum was not sensitive to small doses of MDMA.

Therefore, it is possible that an increased dose of MDMA results in more significant neurotoxicity. As described above, when the injection dose was maintained at a minimal dose (20 mg/kg MDMA), behavioral changes were similar to the first injection. However, the duration of behavioral changes became short, and some rats were quiet within 30 minutes of observation. However, in the high-dose MDMA group, behaviors remained unchanged compared to the first injection. Despite continuous MDMA injections, behavioral changes were not obvious, which suggested drug tolerance. Notably, different doses of MDMA resulted in increased $p75^{NTR}$ expression, suggesting that the hippocampus was highly sensitive to MDMA. In conclusion, results showed that MDMA induced increased $p75^{NTR}$ protein expression in different brain regions, suggesting a role in MDMA-induced apoptosis.

However, the study had some limitations. Following MDMA injection, the behavioral changes were not quantified. The present results did not confirm a correlation between increased p75 protein expression and behavioral changes in rats. However, signaling cascades activated by $p75^{NTR}$, which further result in apoptosis, remain poorly understood, although the signaling events that link $p75^{NTR}$ activation to apoptosis are beginning to emerge. $p75^{NTR}$-dependent apoptosis is associated with increased Rac and Jun kinase (JNK) activity, as well as caspase activation[19-22]. Hennigan *et al* [23] showed that $p75^{NTR}$ expression in the dentate gyrus significantly increases in rats with long-range memory and cognitive dysfunction as a result of lipopolysaccharide-induced hippocampal damage. In addition, the JNK pathway becomes activated, suggesting that the JNK-p53-Bax pathway plays an important role in $p75^{NTR}$-mediated apoptosis. Results from the present study supported the hypothesis that MDMA-induced neurotoxicity and apoptosis were related to the p75 pathway, which initiated apoptosis. This apoptosis pathway could provide a better understanding in future studies of cellular mechanisms of MDMA-regulating neuronal apoptosis. The development of novel therapeutic strategies is needed to slow or halt MDMA-induced neural injury.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The experiment was conducted in the West China Second University Hospital, Sichuan University, West China Institutes of Women and Children's Health, China, from November 2010 to November 2011.

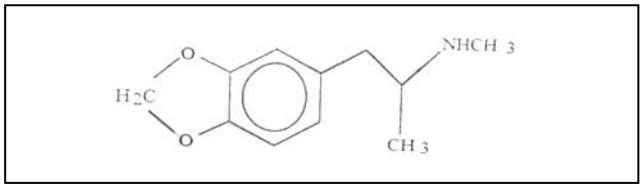
Materials

Animals

A total of 20 male Wistar rats, aged 2–2.5 months and weighing 120 ± 2 g, were purchased from the Experimental Animal Center of Sichuan University (License No. 046). The rats were group-housed in polypropylene cages at 21–22°C with 50% humidity in a 12-hour light/dark cycle and were allowed free access to water and food during the entire process. All rats were allowed a 3-day acclimation period prior to treatment. The experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[24].

Drugs

MDMA was generously donated by China Pharmaceutical and Biological Products, a reference substance, and the purity was 99%. The chemical structure of MDMA was as follows:



Methods

Establishment of a sub-acute MDMA poisoning model

The 20 rats were assigned to four groups, respectively, and were intraperitoneally injected with 20, 50, and 100 mg/kg MDMA[11], as well as normal saline, at identical time points for 7 consecutive days. The rats were observed for 30 minutes after daily drug injections. All rats were sacrificed immediately by decapitation, and intact brain tissues were harvested on ice. The brain tissues were separated along the sagittal plane. Frozen brain sections from one hemisphere were used in the TUNEL assay to analyze apoptosis; the remaining hemisphere of brain tissues was utilized for western blot analysis of $p75^{NTR}$ expression in three different brain regions (frontal cortex, cerebellum, and hippocampus).

TUNEL assay in the prefrontal cortex, hippocampus, and corpus striatum

Cellular apoptosis was examined using TUNEL staining according to the TUNEL detection kit (Boehringer Mannheim, Mannheim, Germany). Briefly, frozen sections were fixed in 4% paraformaldehyde at 4°C for 20 minutes, washed twice in 0.01 M phosphate-buffered saline (PBS), incubated in 3% hydrogen peroxide solution in the dark at room temperature for 20 minutes, washed with 0.01 M PBS, incubated with 30 μL terminal transferase enzyme working solution TdT (Roche Diagnostics, Indianapolis, IN, USA) for 1 hour at 37°C, washed three times with 0.01 M PBS, mixed with 50 μL avidin-alkaline phosphatase (Roche Diagnostics; Indianapolis, IN) for 30 minutes at 37°C, washed three times with 0.01 M PBS, colorized with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (1:50 dilution of substrate buffer) in the dark, washed with running water, and blocked in buffered glycerin. The negative control was treated with PBS, not TdT, and the positive control utilized lymphoma specimens. The positive and blank controls were used in each experiment. Results were processed using Nikon ECLIPSE Ti-U microscopy (Nikon, Tokyo, Japan) and data were analyzed using image analysis system Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA).

Western blot for p75 protein expression detection

To prepare total protein extract, three different brain regions (prefrontal cortex, cerebellum, and hippocampus) were immediately removed from the skull, and all brain tissues were stored at −80°C for 24 hours. Frozen brain tissues were thawed and homogenized in RIPA lysis buffer comprised of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and a cocktail of protease inhibitors (Roche Diagnositics, Tokyo, Japan) with freshly added 1% phenylmethane sulfonyl fluoride and 1% propidium iodide, followed by centrifugation at 12 000 r/min for 20 minutes at 4°C. Homogenates were cleared by centrifugation, and supernatant was transferred to new tubes. Protein concentrations were measured using a microplate reader (wavelength 595 nm) and the Bradford assay kit (Bio-Rad, Hercules, CA, USA)[25]. Samples were boiled in gel-loading buffer and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% for $p75^{NTR}$). Total protein (40 μg) was loaded into each lane. An electricity transfer device (100 V for 45 minutes with two films, followed by 90 V for 30 minutes with one film) was used. The gel protein was transferred onto nitrocellulose polyvinyldi-fluoride membranes (Bio-Rad Laboratories, Hercules, PA, USA), and the blotted nitrocellulose membranes were blocked in freshly prepared Tris-buffered saline containing 5% non-fat dry milk (Tris-buffered saline with 0.05% Tween) for 30 minutes at room temperature under constant agitation. Membranes were then incubated with primary polyclonal rabbit anti-$p75^{NTR}$ (1:250 dissolved in 2% non-fat milk) overnight while shaking at 4°C or mouse anti-β-actin as the internal control (1:2 500) at room temperature for 1 hour. Subsequently, the membranes were washed three times with Tris-buffered saline/0.05% Tween-20 for 10 minutes each while shaking, followed by incubation with biotinylated secondary antibodies (goat anti-rabbit $p75^{NTR}$ or goat anti-mouse β-actin) for 1 hour, followed by washing, and incubation with streptavidin-conjugated peroxidase for 10−15 minutes. The specific reaction was visualized using a freshly prepared enhanced chemiluminescence detection system (Minipore, Billerica, MA, USA), and the antibodies were stripped by incubating the membranes at 50°C for 30 minutes in stripping buffer comprised of 62.5 mM Tris-HCl, pH 6.7, 2% sodium dodecyl sulphate, and 100 mM 2-mercaptoethanol for 1 minute, followed by exposure to Kodak X-OMAT films (Kodak, Chengdu, Sichuan, China). The membranes incubated with $p75^{NTR}$ were re-probed with mouse anti-β-actin. Images were scanned and gel bands were analyzed using gel-scanning integrated optical density software (Scion Imaging 4.0.2). The absorbance value of gel bands was determined, and protein expression was represented by the ratio of absorbance value/β-actin using the gel imaging system.

Statistical analysis

Data were expressed as mean ± SD and were analyzed using the SPSS 17.0 (SPSS, Chicago, IL, USA) and Prism 3.0 (GraphPad Prism, San Diego, USA). Apoptotic cells in different brain regions were analyzed using the

two-sample *t*-test, and comparisons of p75 absorbance values in the three brain areas were performed using analysis of variance. Statistical significance was represented by $P < 0.05$.

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Author contributions: Chaomin Wang wrote the manuscript and provided and integrated the data. Zugui Peng, Weihong Kuang, Hanyu Zheng and Jiang Long were responsible for data analysis and statistics processing; Xue Wang was in charge of funding and was responsible for the conception and design of the study, as well as providing technical and information support.

Conflicts of interest: None declared.

Ethical approval: The experimental project was approved by the Ethics Committee of West China Hospital, China.

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