Dual effects of 3, 4-methylenedioxymethamphetamine (ecstasy) on survival and apoptosis of primary hippocampal neurons

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Abstract

BACKGROUND: 3, 4-methylenedioxymethamphetamine (MDMA, also known as "ecstasy") has been shown to exhibit neurotoxic effects on the hippocampus. However, exposure to sub-lethal insults of MDMA has been reported to result in neuroprotection.

OBJECTIVE: To investigate the effects of MDMA on hippocampal neuronal viability, caspase-3 activity, and mRNA expression of the N-methyl-D-aspartate (NMDA) receptor 2B (NR2B) subunit.

DESIGN, TIME AND SETTING: A cytological, in vitro experiment was performed at the Department of Anatomy, School of Medicine, and Department of Toxicology-Pharmacology, Faculty of Pharmacy, Tehran University of Medical Sciences in 2008.

MATERIALS: MDMA was extracted from ecstasy tablets, which were kindly supplied by the Pharmacology-Toxicology Department, Faculty of Pharmacy, Tehran University of Medical Sciences, Iran.

METHODS: Hippocampal neurons were isolated from Wistar rats at gestational day 18. Following primary culture, hippocampal neuronal viability was detected by MTT assay. Varying concentrations of MDMA (100–5 000 µmol/L) were used to determine lethal concentration 50 (LC50), which was around 1500 µmol/L. Five concentrations of MDMA below 1500 µmol/L (100, 200, 400, 800, and 1050 µmol/L) were used for the remaining experiments. After 24 hours of MDMA treatment, NR2B mRNA expression was detected by RT-PCR, and caspase-3 relative activity was determined by colorimetric assay.

MAIN OUTCOME MEASURES: Hippocampal neuronal viability, caspase-3 activity, and NR2B mRNA expression.

RESULTS: MDMA-induced neurotoxicity in hippocampal neuronal cultures was dose-dependent. In high concentrations (1000–5000 µmol/L) of MDMA, neuronal viability was decreased. However, with a 500 µmol/L dose of MDMA, neuronal viability was significantly increased (P < 0.01). Low concentrations of MDMA (200 and 400 µmol/L) significantly decreased caspase-3 activity (P < 0.01), whereas high concentrations of MDMA significantly increased caspase-3 activity (P < 0.01). NR2B subunit mRNA expression was not significantly altered after 100–1 050 µmol/L MDMA exposure.

CONCLUSION: MDMA exhibits dual effects on hippocampal neuronal viability and caspase-3 activity. These effects are independent from NR2B subunit expression levels.

Key Words: 3, 4-methylenedioxymethamphetamine; ecstasy; apoptosis; N-methyl-D-aspartate; neuronal culture
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INTRODUCTION

3,4-methylenedioxymethamphetamine (MDMA) or ecstasy is one of the most abused drugs, partly because of the psychoactive effects(1). This phenomenon is mainly attributed to binding of MDMA to the 5-hydroxytryptamine (5-HT) reuptake transporter, which results in 5-HT release and 5-HT reuptake inhibition(2). MDMA induces emotional excitability hallucination, and long-term neuropsychiatric...
behaviors, such as depression and psychosis[5], which are due to reduction of 5-HT2A receptor density[4]. In humans, inhibition of the 5-HT receptor results in attenuation of MDMA effects[5]. However, little is known about the pharmacology and molecular mechanisms of these events in the brain[6,8]. Previous studies have shown the neurotoxic effects of high-dose MDMA, which leads to neuronal apoptosis, reduced neuronal viability, and mitochondrial damage[9]. The neurotoxic mechanisms of MDMA, however, remain poorly understood. Glutamate, an excitatory amino acid, is involved in long-lasting adaptive changes that occur in response to drug exposure, and inhibition of the receptors leads to protection against methamphetamine neurotoxicity[10]. N-methyl-D-aspartate (NMDA) is an ionotropic glutamate receptor that plays a critical role in neurotoxicity, apoptosis, and neuronal plasticity, such as memory formation in the hippocampus[11-12]. This receptor acts via receptor-gated channels and is permeable to Ca2+[13]. Functional NMDA receptors are heteromers and are composed of NR1, NR2 (A–D), and NR3 subunits[13]. NR2B-containing receptors play a role in protecting neurons from apoptosis in immature systems[14], but it is not clear whether NR2B has a special functional relationship with apoptosis in the hippocampal formation[14].

The present study analyzed the effects of low to high doses of MDMA on neuronal viability, caspase-3 activity, and NR2B subunit gene expression in hippocampal neuronal cultures to determine the influence on hippocampal neurons.

MATERIALS AND METHODS

Design
A cytological, in vitro study.

Time and setting
Performed at the Department of Anatomy, School of Medicine, and Department of Toxicology-Pharmacology, Faculty of Pharmacy, Tehran University of Medical Sciences in 2008.

Materials
Reagents and instruments are listed as follows:

<table>
<thead>
<tr>
<th>Reagent and instrument</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffers, culture plates, and other cell culture materials (except media), rabbit anti-microtubule-associated protein-2 (MAP2) polyclonal antibody, Hoechst 33342, Mowiol 40-88 (324590), caspase-3 colorimetric assay kit</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>Neurobasal medium and supplement B27, FITC-goat anti-rabbit antibody</td>
<td>Invitrogen, Germany</td>
</tr>
<tr>
<td>Polymerase chain reaction reagents</td>
<td>Fermentase, Canada</td>
</tr>
<tr>
<td>TriPure Isolation Reagent</td>
<td>Roche Applied Science, Germany</td>
</tr>
<tr>
<td>Anthos2020 microplate reader</td>
<td>Biochrom, UK</td>
</tr>
<tr>
<td>Fluorescence microscope</td>
<td>Nikon, Japan</td>
</tr>
</tbody>
</table>

Animals: 16 female, pregnant, Wistar rats were obtained from the Razi Vaccine and Serum Research Institute in Karaj, Iran. The rats were housed in a temperature-controlled room (22 ± 2) °C and maintained on a 12-hour light/dark cycle with free access to food and water. All procedures were performed in accordance with institutional guidelines for animal care and use.

Methods

MDMA extraction
MDMA was extracted from ecstasy tablets, which were kindly supplied by the Pharmacology-Toxicology Department, Faculty of Pharmacy, Tehran University of Medical Sciences, Iran. The following process was performed to extract pure MDMA. Tablets were pulverized by mortar and pestle and dissolved in methanol/ammonium (100: 1.25, pH 10). MDMA was separated from other chemical fractions using the silica column chromatography method and then dried from methanol/ammonium solution using a vacuum evaporator without heat. The final product – the base form of MDMA – was again dissolved in chloroform and converted to the crystal form of MDMA by HCl gas. Component purity was measured by melting temperature and high-performance liquid chromatography (HPLC) technique[15].

Hippocampal neuronal culture
Primary neuronal cultures were obtained from 18–19 day embryos. Briefly, pregnant female rats were anesthetized and killed by cervical dislocation and subjected to caesarean section to obtain fetal brains. A previously described culture method[16] was used with some modifications. Brains were removed from the skull and collected in Hank’s balanced salt solution on ice. The meninges were removed from cerebral hemispheres and the hippocampi were dissected, minced into small pieces, and digested with 0.25% trypsin for 20 minutes at 37 °C. Fetal bovine serum was used to inactivate the trypsin. Finally, the cells were centrifuged for 5 minutes at 900 r/min, resuspended in neurobasal medium supplemented with 1% B27 and 500 μM L-glutamine plus antibiotics, and plated in poly-D-lysine hydrobromide (100 μg/mL)-coated plates. After 1 day, the culture medium was replaced by the same medium containing 5 μmol/L Ara-C for 24 hours. The cultures were maintained at 37 °C temperature and 5% CO2 and cultured for 5 additional days prior to treatment. Because the neuronal cultures were serum-free and Ara-C kills all dividing cells, glia and microglia were virtually absent in the cultures. Neuronal purity was assessed by incubation with rabbit anti-MAP2 polyclonal antibody (1: 300 dilution) overnight at 4 °C, followed by FITC-labeled goat anti-rabbit antibody (1: 1000 dilution) for 1 hour at room temperature, Hoechst 33342 counterstaining (1: 10 000 dilution) for 10 minutes, and coverslipping in Mowiol 40-88. A density of 2.5 × 105 cells/well was used in 24-well plates.
Hippocampal neuronal viability
The (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) assay was used to evaluate the reduction-oxidation status of living cells and mitochondrial activity due to formation of formazan\[17\]. A density of 1 × 10^4 cells/well in 96-well plates was used for the MTT assay. Several MDMA concentrations (100–5 000 µmol/L) were used to determine lethal concentration 50 (LC50), which was subsequently determined to be 1 500 µmol/L. Neurons which were not exposed to MDMA served as the controls. For the remaining experiments, five concentrations below 1 500 µmol/L were used (100–1 050 µmol/L). Briefly, neurons were incubated in medium containing 500 µg/mL MTT for 3 hours at 37 °C. MTT-containing medium was removed by plate inversion and 100 µL DMSO was added to each well to dissolve the formazan crystals. The plates were read using an Anthos2020 microplate reader at a wavelength of 570 nm and a reference of 690 nm.

NR2B mRNA determination by reverse transcription-polymerase chain reaction (RT-PCR)
Total RNA was isolated from neurons in 6-well plates at a density of 8 × 10^5 cells/well, which were treated for 24 hours with MDMA, using TriPure isolation reagent according to manufacturer’s protocol. RNA (1 µg) from each sample was reverse-transcribed into cDNA using Moloney Murine Leukemia Virus reverse transcriptase. cDNAs were polymerase chain reaction amplified, according to a standard protocol, using 35 cycles (94 °C for 30 seconds, 57 °C for 30 seconds, and 72 °C for 40 seconds) for NR2B primers and 30 cycles (94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 40 seconds) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. Primers are as follows:

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Product size (bp)</th>
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</thead>
<tbody>
<tr>
<td>NR2B Forward</td>
<td>5' ACT TCT CAC CCC CTC TCC GCT 3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5' GTT CTT CCA CTC CAC CAT CTC CA 3'</td>
</tr>
<tr>
<td>GAPDH Forward</td>
<td>5' TAC CAG GGC TGC CTC TCT CTC 3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5' TGG AAG ATG GTG ATG GTT TT 3'</td>
</tr>
</tbody>
</table>

Polymerase chain reaction products were resolved on a 1% agarose gel and visualized by ethidium bromide staining. The band density of polymerase chain reaction products was measured using band densitometer software (UVItec Limited, UVIband, Cambridge, UK). β-actin was used as an internal standard.

Caspase-3 activity assay
Neurons (8 × 10^5 cells/well in 6-well plates) were treated with MDMA for 24 hours and were assessed for caspase-3 activity assay. The assay was performed according to previously described methods\[18\] using the caspase-3 colorimetric assay kit. Briefly, after replacing cell culture medium with caspase lysis buffer, the cell lysates were incubated at 37 °C with Ac-DEVD-pNA colorimetric substrate. The amount of P-nitroanilide was continuously monitored over a 60-minute period through the use of a plate reader. Absorbance was measured at 405 nm, normalized to absorbance of control groups, and expressed as percent of control. Each experiment was run in triplicate.

Main outcome measures
Primary cultured hippocampal neuronal viability, caspase-3 activity, and NR2B mRNA expression.

Data analysis
Data were analyzed using SPSS software (version 11.0, Chicago, IL, USA). One-way analysis of variance was used to determine overall significance. Differences between control and experimental groups were assessed with post-hoc Bonferroni comparison, with significant differences represented as \( P < 0.01 \) and \( P < 0.05 \) (versus control cultures).

RESULTS
Neuronal purity
Assessment of neuronal purity was performed using an antibody specific to the neuronal marker MAP2, followed by nuclear counterstaining with Hoechst 33342 dye. Approximately 90% of the cells were MAP2-positive (Figure 1).

Hippocampal neuronal metabolism and viability
MDMA-induced neurotoxicity in hippocampal neuronal cultures was dose-dependent. At high concentrations (1 000–5 000 µmol/L), cell viability decreased. However, 500 µmol/L MDMA resulted in increased cell viability (Figure 2).

Effects of MDMA on caspase-3 enzyme activity of hippocampal neurons
Exposure of hippocampal primary neurons to 100 µmol/L MDMA did not alter active caspase-3 expression compared with the control groups. However, 200 and 400 µmol/L MDMA decreased caspase-3 activity to 86% and 78% of control value, respectively, whereas higher concentrations (800 and 1 050 µmol/L) increased caspase-3 activity to 115% and 125% of control value, respectively (Figure 3).

Effects of MDMA on NR2B mRNA expression
mRNA expression of the NR2B subunit was not significantly altered following exposure to 100, 200, 400,
DISCUSSION

In the present study, treatment of hippocampal primary cultures with low MDMA concentrations resulted in increased cell viability. A previous study using fetal mesencephalic cells reported similar increased cell survival in MDMA-treated cultures, compared with control culture\(^\text{[19]}\). That study used MDMA concentrations (0.165, 0.825, and 1.65 µg/mL) that were relatively low, as well as 96-hour incubation. The present results demonstrated that low-concentration MDMA exposure enhanced hippocampal neuronal survival, suggesting that MDMA could provide protection against a wide range of toxic insults. A previous result reported no cell death following 50 and 100 µmol/L MDMA exposure to organotypic rat hippocampal cultures, and the authors assumed that removal of presynaptic serotonin transporters from the raphe nuclei could prevent apoptosis induction\(^\text{[20]}\). A recent study focused on MDMA protection introduced the concept of preconditioning-induced neuroprotection\(^\text{[21]}\).

In the present study, low MDMA concentrations reduced caspase-3 activity, which resulted in increased cell survival. This was in agreement with the cell viability test results. Caspase-3 is a key element in many apoptotic pathways. Therefore, reduced caspase-3 expression could lead to neuronal survival. The lack of cell death following exposure to low concentrations of MDMA in the present study was consistent with previous \textit{in vivo}\(^\text{[22]}\) and \textit{in vitro}\(^\text{[23]}\) studies that demonstrated the lack of neurotoxicity and cell death following MDMA treatment. It is noteworthy that low MDMA concentrations could be used to study neuroprotective reagents, although much controversy surrounds this conjecture, because there is concern about MDMA metabolite toxicity\(^\text{[24-25]}\).

In the present study, higher concentrations of MDMA (over 1 000 µmol/L) decreased neuronal viability and increased caspase-3 activity, which is consistent with previous results\(^\text{[9]}\) showing the same effect with 3 000–5 000 µmol/L MDMA concentrations. Neuronal apoptosis could be due to overstimulation of neurons induced by MDMA, which leads to activation of apoptotic pathways. Capela \textit{et al.}\(^\text{[26]}\) reported increased Ca\(^{2+}\) levels as a result of NMDA-receptor activation, which led to the production of damaging free radicals and ultimately apoptosis. This NMDA action has been reported by other studies\(^\text{[12, 27-28]}\), although the present results demonstrate that high MDMA doses increases neuronal apoptosis, but does not result in significant changes to the NR2B subunit. This suggests that the mechanisms of action could be through other subunits, or by phosphorylation of this subunit.
The present results do not demonstrate a significant association between MDMA exposure and NMDA receptor NR2B subunit mRNA expression. The NR2B subunit has been shown to play a role in memory and learning\(^\text{[29]}\). However, behavioral and molecular studies have shown that a neurotoxin regimen of MDMA does not result in disrupted NR2B protein levels\(^\text{[30-31]}\). It should be considered that the role of MDMA in neuronal viability or apoptosis might not be NMDA receptor-dependent. In conclusion, the extent of MDMA neuroprotection or neurotoxicity remains poorly understood, and future studies are needed to clarify the possible role of this drug as a neuroprotective in neurodegenerative disease.

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REFERENCES


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