Original Article

The Effect of MDMA-Induced Anxiety on Neuronal Apoptosis in Adult Male Rats' Hippocampus

(MDMA / anxiety / apoptosis / plus-maze / hippocampus / rats)

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Abstract. Ecstasy or MDMA as a psychoactive drug and hallucinogen is considered one of the most commonly used drugs in the world. This psychotropic substance is discussed both as sexually stimulating and reducing fear and anxiety. Amphetamines also destroy neurons in some brain areas. The aim of this study was to investigate the effects of MDMA on anxiety and apoptosis of hippocampal neurons. Forty-two male Wistar rats of mean weight 200–220 g were used and distributed into six groups [control, control-saline, and experimental groups (1.25, 2.5, 5, 10 mg/kg)]. Rats in experimental groups received MDMA at different doses for seven days by intraperitoneal injection and the control-saline group received saline (1 ml/kg); anxiety was then investigated by plus-maze test. Forty-eight hours after behavioural testing brains were taken from animals and fixed, and after tissue processing, slices were stained with TUNEL kit for apoptotic cells. The area densities of apoptotic neurons were measured throughout the hippocampus and compared in all groups ($P < 0.05$). Physiological studies showed that 1.25 mg/kg and 2.5 mg/kg doses caused anti-anxiety behaviour and 5 and 10 mg/kg doses of MDMA caused anxiety-like behaviour. Moreover, our histological study showed that ecstasy increased apoptotic cell numbers and the highest increase was observed with the 10 mg/kg dose of MDMA. We concluded that MDMA can cause different responses of anxiety-like behaviour in different doses. This phenomenon causes a different ratio of apoptosis in hippocampal formation. Reduction of anxiety-like behaviour induced by the 2.5 mg/kg dose of MDMA can control apoptosis.

Introduction

Anxiety is one of the most common mental disorders affecting many people in different populations. Anxiety is an uncomfortable feeling evoked by an unknown danger (Wang et al., 2007). Different parts of the brain such as amygdala, hypothalamus, hippocampus and septum are involved in mediating anxiety (Cho et al., 2007, 2008; Wang et al., 2007).

Hippocampus as a part of the limbic system is involved in the pathophysiology of affective disorders, fear and anxiety behaviour (Kjelstrup et al., 2002; McHugh et al., 2004; Wang et al., 2007; Cho et al., 2008). Hippocampal cholinergic input from the septum and the middle part of Broca diagonal band (Rosene and Van Hoesen, 1987) plays an important role in physiological changes during memory formation and learning (Stephan, 1983) and anxiety-related behaviours (Knowles, 1992).

The elevated plus-maze is a widely used behavioural paradigm in the experimental anxiety research (Rodgers and Cole, 1994). During a typical plus-maze test fear-motivated avoidance behaviour is measured (Handley and McBlane, 1993). Naturally, the animals due to their fear of open spaces spend most of the time in the closed arms of the maze (Treit, 1985). Anxiogenic drugs can increase this natural aversion toward the open arms, whereas anxiolytic drugs can decrease it (Pellow et al., 1985).

MDMA (3, 4-methylenedioxymethamphetamine or ecstasy) as an amphetamine derivative is a popular drug of abuse (Fantegrossi et al., 2008) and it can increase the levels of free radicals, which are involved in the neurotoxic effects (Green et al., 2003). Anxiolytic and anxiogenic-like behavioural outcomes have been extensively studied in MDMA-treated animals using a variety of anxiety models (Palenicek et al., 2005). The effects of MDMA vary according to the dose and the frequency and duration of use (Kalant, 2001). Although MDMA has been shown to reduce humans' anxiety in the labora-

Received October 2, 2013. Accepted March 12, 2014.

The project was financially supported from research affair of Golestan University of Medical Sciences.

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Abbreviations: MDMA – 3, 4-methylenedioxymethamphetamine or ecstasy.

tory, it may also cause an increase of anxiety (Cole and Sumnall, 2003). There is some evidence for deficits in serotonergic biochemical markers in human ecstasy abusers (Capela et al., 2009; Riezzo et al., 2010).

Apoptosis is one of the most characterized types of cell death. The main molecular pathways of apoptosis are: one extrinsic signal involving activation of death receptors in the cell membrane, and another intrinsic stimulus involving mitochondrial membrane permeabilization. These pathways may activate cysteine aspartic proteases, activated by proteolysis, named caspases (Kroemer and Martin, 2005)**.**

The main cells in the hippocampus are pyramidal neurons, and in dentate gyrus these are granular neurons (Jahanshahi et al., 2006); therefore, the aim of this study was to determine various effects of MDMA on anxiety and the effects of anxiety on apoptosis of neurons in the rats' hippocampus.

Material and Methods

Animals

Forty-two Wistar male rats weighing 200 ± 20 g (Pasteur Institute, Tehran, Iran) were used. Animals were housed in plastic cages, under standardized lighting conditions (12 h light / 12 h dark cycle), a constant temperature (22–24 °C), food and tap water available and libitum, except during behavioural tests.

For a week, the rats were allowed to adapt to laboratory conditions before starting the experiment, and the tests were performed at a certain time of day. This experiment was carried out in accordance with the guiding principles for care and use of laboratory animals approved by the Golestan University of Medical Sciences.

Drug administration

Animal were randomly allocated to six groups (N = 7); one control group without injection and behavioural test, a control-saline group receiving normal saline (1 ml/kg) and behavioural test, and four experimental groups (N = 7) receiving MDMA (1.25, 2.5, 5, 10 mg/kg) intraperitoneal injections and behavioural test. MDMA (Sigma Laboratories, Sigma-Aldrich, St. Louis, MO) was diluted in physiological saline to appropriate doses for injections (for seven consecutive days).

Apparatus and behavioural test

The plus-maze apparatus consists of two open arms and two closed arms, with two pairs of identical platforms which emerge from a central platform (5 × 5 cm), positioned opposite each other. The apparatus was elevated 50 cm above the floor. Animals were tested in the maze in randomized order. The test was initiated by placing the rat on the central platform of the maze, facing one of the open arms, and letting it move freely. Each session lasted 5 min; all tests were carried out under dim red lighting between the second and seventh hour of the dark phase. After each test, the maze was thoroughly cleaned. Behavioural analysis was performed by a trained experimenter who was unaware of treatment of the groups (Jahanshahi et al., 2013a).

A number of classical parameters were collected during the session, including: (1) open arm duration: the total amount of time the rat spent in the open arms; (2) closed arm duration: the total amount of time the rat spent in the closed arms; (3) central platform duration: the total amount of time the rat spent in the central platform; (4) open arm frequency: the frequency of rat entry with all four paws into the open, unprotected arms; and (5) closed arm frequency: the frequency of rat entry with all four paws into the closed, protected arms.

Histology

Forty-eight hours after the behavioural testing session, rats were anaesthetized with chloroform and after perfusion, their brains were removed from the skull and stored in paraformaldehyde (4%) for two weeks, and then, the brains were placed in tissue processor apparatus for tissue procedures. Following this session, samples of the brain were embedded in paraffin and kept in a refrigerator. Then the brains were sliced coronally into 6-µm sections (from bregma -2.5 mm to -4.5 mm of the hippocampal formation) with a rotary microtome (MK 1110) and for 10 s with 50 µm interval were stained with TUNEL assay in accordance with routine laboratory procedures (Jahanshahi et al., 2013b). Photographs of each section were produced using an Olympus BX 51 microscope (Olympus, Tokyo, Japan) and DP 12 digital camera (Olympus) under 400× magnification for the pyramidal layer of CA1, CA3 and granular layer of DG areas (Fig. 1). To measure the apoptotic cells, the images were transferred to the computer and the cells were counted with image G software.

Data analysis

Data were analysed using SPSS software (version 11.0, Chicago, IL). One-way analysis of variance was used to determine the overall significance. Differences between control and experimental groups were assessed

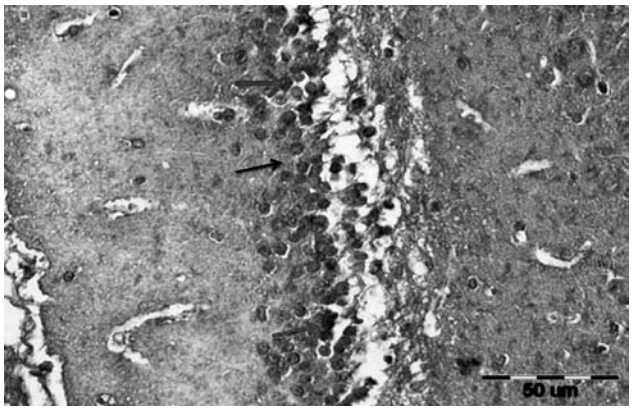

Fig. 1. TUNEL assay technique for apoptotic cells in the DG area of hippocampus. Red arrow points to an apoptotic cell and blue arrow to normal neurons.

Table 1. Mean and SD of apoptotic neuron numbers in the CA1 area of hippocampus

Groups – CA1	Mean	Std. deviation	P value
Control	30.88	21.651	
Control-saline	33.38	8.808	0.911
1.25 mg/kg MDMA	49.38	17.629	0.013 *
2.5 mg/kg MDMA	37.56	9.612	0.825
5 mg/kg MDMA	53.37	20.438	0.001 *
10 mg/kg MDMA	61.25	8.258	0.000 *

All groups compared to the control (* means significant)

Table 2. Mean and SD of apoptotic neuron numbers in the CA3 area of hippocampus

Groups – CA3	Mean	Std. deviation	P value
Control	27.25	18.013	
Control-saline	33.31	10.084	0.822
1.25 mg/kg MDMA	41.50	12.754	0.054
2.5 mg/kg MDMA	31.38	8.484	0.960
5 mg/kg MDMA	60.06	19.406	0.000 *
10 mg/kg MDMA	59.19	11.560	0.000 *

All groups compared to the control (* means significant)

with post-hoc Tukey test comparison, with significant differences represented as $P < 0.05$.

Results

Behavioural results

Our findings showed that the highest increase of open arm entries as an index for decrease of anxiety was observed in the 2.5 mg/kg group compared to the others, and the difference between this group and the control-saline group was significant ($P < 0.05$). The highest percentage of remaining in the open arms was also observed in the 2.5 mg/kg group.

Also, the least delay on entering the open arm as an index for decrease of anxiety was observed in the 2.5 mg/kg group in comparison with the other groups. Rats treated with high doses of MDMA showed elevated levels of anxiety-like behaviour.

The findings suggest that the locomotor activity index in the 1.25 and 2.5 mg/kg experimental groups compared to the control-saline group showed significant differences ($P < 0.05$). In summary, the effective dose for reduction of anxiety was 2.5 mg/kg.

Histological results

In the CA1 region of hippocampus the number of apoptotic cells was increased in all groups compared to the control. However, there were no significant differences in the number of apoptotic cells between the control and control-saline groups. Also, there were no significant differences in the number of apoptotic cells between the 2.5 mg/kg experimental group and control group ($P < 0.05$). Nevertheless, the differences between the other three experimental groups and the control group were significant (Table 1).

Table 2 shows the mean and SD of the number of apoptotic cells in the CA3 region of hippocampus in all groups. In the CA3 region of hippocampus the number of apoptotic cells was increased in all groups compared to control. As we show, the least change compared to the control group was in the 2.5 mg/kg group.

The number of apoptotic cells in the DG area of hippocampus is shown in Table 3. There were no significant differences in the number of apoptotic cells between the 2.5 mg/kg experimental group and control group ($P < 0.05$). Nevertheless, the differences between the other three experimental groups and the control group were significant (Table 3).

Table 3. Mean and SD of apoptotic neuron numbers in the DG area of hippocampus

Groups – DG	Mean	Std. deviation	P value
Control	49.62	19.765	
Control-saline	53.88	14.674	0.913
1.25 mg/kg MDMA	75.12	27.633	0.007 *
2.5 mg/kg MDMA	67.06	20.032	0.152
5 mg/kg MDMA	90.87	21.260	0.000 *
10 mg/kg MDMA	108.37	14.818	0.000 *

All groups compared to the control (* means significant)

Discussion

The elevated plus-maze test uses the natural aversion of rodents to heights and open spaces. Therefore, the animals prefer to spend time in the closed arms rather than in the open arms of a maze. The present study showed that taking low doses (1.25 and 2.5 mg/kg) of MDMA in five days had an anxiolytic effect and taking high doses (5 and 10 mg/kg) of MDMA had an anxiogenic effect. Our behavioural finding was similar to others (Bhattacharya et al., 1998; Lin, et al., 1999; Navarro and Maldonado, 2002; Ferraz-de-Paula et al., 2011).

Our data also showed that the relationship between MDMA doses and increase of apoptotic cells in all areas of the hippocampus is not linear. On the other hand, when the rats displayed less anxiety (2.5 mg/kg dose of MDMA), the number of apoptotic cells was lower than with the other doses. Gurtman et al. (2002) examined the long-term behavioural and neurotoxic effects of MDMA in rats. They showed that four and six weeks after the drug administration the rats previously treated with MDMA showed elevated levels of anxiety-like behaviour in the emergence and social interaction tests, respectively. The authors compared their results with human studies and they suggested that exposure to high doses of MDMA may predispose to long-term psychological problems such as anxiety and depression (Gurtman et al., 2002).

Sometimes MDMA not only has acute and dose-dependent effects in the elevated plus maze, but can also have relatively long lasting effects on the behaviour. It seems that these latter effects depend (A) on the kind of the test paradigm used, and (B) on subject-dependent factors, such as individual levels of anxiety (Ho et al., 2004). Hernandez-Rabaza et al. (2006) showed that the administration of MDMA damages the survival rate of neural precursors in adult hippocampal dentate gyrus cells, but not the proliferation rate of progenitor cells. In another study, cell proliferation in the dentate gyrus was suppressed by chronic administration of MDMA (Cho et al., 2007). In contrast, MDMA did not cause cell death in organotypic hippocampal cultures (Sveen et al., 2004).

Hernandez-Rabaza et al. (2006) found that administration of MDMA has deleterious effects on adult neurogenesis by impairing the short-term survival of vulnerable neural precursors. Also, Cho et al. (2007) showed that chronic exposure to MDMA reduces cell proliferation in the adult dentate gyrus in a dose-dependent manner in male and female mice.

Wang et al. (2009) studied the neuron apoptosis induced by i.p. MDMA and the expression of apoptosis-related factors in the rat brain. Neuron apoptosis was measured by the TUNEL assay similarly to our study. These authors showed that MDMA could induce neuron apoptosis and expression of apoptosis-related factors such as caspase 3 and cytochrome c (Cyt c) in the rat brain (Wang et al., 2009).

Azami et al. (2009) also showed that MDMA exhibits dual effects on hippocampal neuronal viability and caspase 3 activity.

The mechanism of MDMA effect on cell apoptosis was shown by Riezzo et al. in 2010. They suggested that injection of a single dose of MDMA in rats caused an increase in oxidative stress and subsequent apoptosis (TUNEL assay) in the striatum, hippocampus, and frontal cortex (Riezzo et al., 2010). Also chromatin condensation, DNA fragmentation, and a significant increase in caspase 3 activity and Cyt c are markers of apoptosis (Jiménez et al., 2004).

Conclusion

We concluded that MDMA in different doses can cause different responses of anxiety-like behaviour. This phenomenon causes different ratios of apoptosis in the hippocampal formation. Reduction of anxiety-like behaviour induced by the 2.5 mg/kg dose of MDMA can control apoptosis in all areas of the hippocampus. On the other hand, if we can control the anxiety, we can probably also inhibit neuron apoptosis.

Acknowledgment

The authors would like to thank the Neuroscience Research Centre for behavioural and histological experiments.

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