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Research Article

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The Effects of MDMA on Brain: An in Vivo Study in Rats

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ABSTRACT

Background and objective : 3, 4-Methylenedioxymethamphetamine (MDMA) is a powerful central nervous system stimulant that acts by increasing the activity of several neurotransmitter systems. This study aimed to investigate the effect of different doses of MDMA on the level of serotonin in the brain measured from the cerebrospinal fluid (CSF) and their effects on the neurons. Materials and methods : Twenty-six adult male rats were divided into 4 groups. The control group (n=7) received intraperitoneal (IP) injections of 1 mL/gm normal saline twice daily for one week. The MDMA treated groups (n= 6/7) were further subdivided into 3 groups, each receiving 5, 10 or 15mg/kg MDMA intraperitoneal (IP) injections twice daily for one week ; respectively. 24 hours after the last injection of the rats, the CSF was collected. The brains were dissected and processed for paraffin sections and stained by cresyl etch violet stain. Histology, morphometry and statistical analysis were done. Results : The administration of different doses of MDMA significantly increased the level of serotonin in comparison with the control group. The morphometry study indicated that the number of intact neurons in the hippocampus significantly decreased following the administration of different doses of MDMA comparing to the control group. The results also indicated that there was a strong negative correlation between the serotonin levels and the number of intact neurons. Conclusion : MDMA administration even at a low dose had degenerative effects on the neurons in the hippocampus as evidenced by morphometric study, and the intake should be avoided.

Key words: *Serotonin, Cerebrospinal Fluid, MDMA, Cresyl Echt Violet, Neuronal Damage.*

INTRODUCTION

3, 4-Methylenedioxymethamphetamine (MDMA) was discovered in 1914 by a biochemist Anton Kollisch, who worked for German pharmaceutical company Merck. In the mid-1980s, following a short period of experimental use of MDMA as an adjunct in psychotherapy, the drug became a common recreational drug of abuse [1-3]. The drug has some resemblance to hallucinogen mescaline [4]. The psychological effects of MDMA last for 3 to 5 hours per administration, and the user feels relaxed, happy, empathic and close to others [5].

The administration of MDMA on various experimental animals has shown to induce a selective damage to serotonergic axon terminals, which led to long-term depletion of serotonin neurotransmitter (5-HT). It also caused an enhanced and widespread secretion of serotonin in the brain [5-7]. Another similar study by Capela and colleagues (2009), also found the serotonergic system as the main target of MDMA [8]. Furthermore, high dose of

MDMA administration also showed a persistent neurochemical disruption that particularly affected the serotonergic nerve terminals in rats and nonhuman primates [9, 10].

MDMA affected the brain areas rich in serotonergic pathways [6]. MDMA administration led to serotonin transporter (SERT) depletion in certain areas in the rat brain that included midbrain, hippocampus, hypothalamus, thalamus, caudate putamen and also the frontal cortex [11]. Hippocampus is one of the most important areas in the brain, forming part of the limbic system that contains abundant serotonin receptors [12]. This area is responsible for memory processing, controlling emotions, judgment, affection, and motivation [13]. Besides that, serotonin also plays a role in controlling various psychiatric functions such as anxiety and depression [14]. The disruption in serotonin secretion can lead to an altered and dysfunctional memory and learning [15-18].

In conjunction to that, axonal degeneration to the serotonergic terminal has been observed after MDMA exposure due to massive efflux of the serotonin in the synaptic cleft [9, 19]. The MDMA has a great affinity with the presynaptic serotonin transporter [20]. Its administration leads to an alteration in the serotonin reuptake transporter, which leads to an increased release of serotonin into the synapse [21]. The massive increase in the level of serotonin in the central nervous system may cause serotonin syndrome or serotonin toxicity [22, 23]. Serotonin syndrome is most likely to occur within 24 hours after administration of a serotonergic substance [24]. The administration of MDMA regardless of the number of administration can cause long-lasting neurochemical and histological changes, which can be used as markers of serotonergic function in the rats' brains [25].

Cerebrospinal fluid (CSF) is a colorless body fluid present in the brain and spine [26]. The CSF is secreted by cells lining the choroid plexuses of the brain ventricles [27]. It circulates via the interconnected system that comprises of four ventricles, central canal of spinal cord and subarachnoid space [28]. The CSF provides a basic mechanical and immunological protection to the brain [28]. The CSF also functions to shuttle waste products from the central nervous system into the blood [29]. Besides that, CSF also has a crucial role in homeostasis as well as in hormonal and signaling functions [30].

The CSF contains more information regarding the central nervous system as it is physically in contact with the brain compared to the blood [31, 32]. The CSF also provides central serotonergic parameters in the study of the serotonin level and its main metabolites [5]. The levels of the serotonin (5-HT) and its main metabolite (5-HIAA) in the CSF reflect the concentration released during neuronal activity in the brains [4]. Besides that, the results from in vivo brain microdialysis research showed that serotonin concentrations in the extracellular fluid such as cerebrospinal fluid is a suitable indicator reflecting the neuronal activity of the serotonergic system [33]. Furthermore, the serotonin level measured from cerebrospinal fluid also provides a better index to determine a functionally active serotonergic system [34, 35].

At present, there are limited data available regarding the histological changes in the brain after administration of MDMA and their correlation with neurotransmitter activity in the brain. Therefore, the present study aimed to assess the level of serotonin concentration in the CSF of the rats after administration of different doses of MDMA, and correlate the level of serotonin concentration with the neuronal damage.

MATERIALS AND METHODS

Drug and treatment protocol

3, 4-Methylenedioxymethamphetamine (MDMA) hydrochloride was purchased from Lipomed, A.G. (Arlesheim, Switzerland). The MDMA was dissolved in saline (0.9% NaCl) to give a volume of 1 mL/kg. The drug was injected via intraperitoneal (IP) injections to the rats twice daily at 9:00 a.m. and 5:00 p.m. for one week.

Experimental animals

Twenty-four adult Sprague–Dawley male rats weighing 200- 250 g each were used in the study. The animals were purchased from Eprovider Sdn.Bhd based in Kota Bharu, Kelantan Malaysia. The rats were kept in cages consisted of three individuals per cage, under the stainless steel cover in the animal house, Faculty of Medicine University Sultan Zainal Abidin (UniSZA). They were kept for one week for acclimatization and reduction of post-travel stress. Following that, the animals were further kept under a controlled condition (12 h light :12 h dark cycle, the room temperature of 22±2 °C, and the relative humidity of 55±5 %) with free access to food and water. The experiment was approved by the Animal Experimentation Ethics Committee of UniSZA (ID : UAPREC/16/002).

Experimental protocol

The rats were randomly divided into four groups as follows : i. (Control group): It consisted of 7 adult male rats, receiving intraperitoneal (IP) injections of 1 ml/gm normal saline twice daily for one week. ii. (Treated group 1):

This group consisted of 6 rats, receiving 5 mg/kg MDMA (low dose). iii. (Treated group 2): This group consisted of 7 rats, receiving 10 mg/kg MDMA (moderate dose); iv. (Treated group 3): This group consisted of 6 rats, receiving 15 mg/kg MDMA (high dose) via intraperitoneal (IP). The drug was administered to all the groups according to the treatment protocol.

All the animals were maintained up to 24 hrs after the last dose of drug administration during which the CSF collection was carried out.

Cerebrospinal fluid collection

Following 24 hours of the last injection of either saline or MDMA according to the study protocol, the CSF was collected from the Cisterna magna of the rats. The rats were anesthetized prior to the collection of CSF using ketamine with the dose of 0.15 ml/100gm body weight. The fur on the neck region of the rats was removed. The head position of the rats was then maintained downward at approximately 45º on the stabilizer block. A swab soaked with 70% of ethanol was rubbed over the depressible surface of the rhomb between the occipital protuberance and the spine. A 23G butterfly needle (B|Braun Venofix) connected to 1 ml tuberculin syringe (TERUMO) was inserted horizontally and centrally into the cisterna magna of the rat. A gentle aspiration was performed to allow the CSF to flow through the needle, and get drawn slowly into the syringe. (a Modified method by Nirogi and colleagues (2009) [36].

Serotonin analysis

The serotonin neurotransmitter level was quantified from the CSF collected from the cisterna magna of the rats. Serotonin ELISA test kit (DLD EA602/96) was used to quantify the serotonin level. Firstly, for the preparation of samples (acylation), 10 µl of standard 1-6, 10 µl of controls 1-2 and 50 µl of CSF were pipetted into the respective wells of the reaction plate. Next, 25 µl of acylation buffer, 200 µl of equalizing reagent, 25 µl of acylation reagent were pipetted into the wells, and the mixture was properly mixed immediately. The mixture was incubated for 30 minutes at room temperature (approximately 25ºC) on an orbital shaker. An amount of 20 µl of each acylation samples was pipetted into respective wells on the coated microtiter strips. Following that, 100 µl of antiserum was pipetted into all the wells. The plate was then covered using adhesive foil, and incubated for 30 minutes at room temperature on an orbital shaker. After completing the incubation, the contents of the well were aspirated and the wells were washed thoroughly using 250 µl wash buffer, and this step was repeated for three to four times.

The residual liquid in the wells was removed by gently tapping the inverted plate onto a clean absorbent paper. Later, 100 µl enzyme conjugate was pipetted into all the wells, and the enzyme conjugate was incubated for 15 minutes at room temperature on an orbital shaker. The same washing steps were repeated to wash the remaining unbounded enzyme conjugate. Subsequently, 100 µl of the substrate was then pipetted into all the wells, and was incubated for 20 minutes at room temperature on an orbital shaker. Finally, the 100 µl stop solution was pipetted into all the wells. The test was read using a microplate photometer at the optical density of 450nm (reference wavelength between 570 and 650 nm). The test was done in triplicate.

Histological study

The skulls of the rats were dissected, and the brains were harvested after the rats underwent the transcardial perfusion procedure. The brain tissues were then immediately immersed in 10% neutral buffered formalin, and were fixed for 24 hrs. The tissues were dehydrated in ascending concentrations of alcohol, and cleared in xylene after which they were embedded in paraffin. Serial coronal sections of 4 µm in thickness were later prepared and subjected to Cresyl Etch Violet staining to demonstrate the neuronal cells. In this study, the intact neurons having clearly defined nuclear and cytoplasmic membrane with prominent nucleoli were counted.

Morphometric study

A morphometric study was performed to determine the number of intact neurons in the control and treated groups. Two main areas were selected for the study : CA1 and CA3 subfields of the hippocampus. The intact pyramidal cells were counted by using image software (Cell count) from five random counting frames (50 × 50 µm), and the average count was calculated.

Statistical analysis

Serotonin concentrations from the CSF and the number of intact neurons were analyzed by using Graph Pad Prism version 7.00 for Windows software (Graph Pad Software Inc., San Diego, CA, USA). Analysis of variance (ANOVA) and Student t-test were used to compare the groups. Pearson's correlation test was used for correlations between groups. A 95% confidence interval was recognized as a significant result.

RESULTS

Serotonin levels in rat CSF after MDMA administration

ANOVA analysis indicated that all MDMA groups (5, 10,15mg/kg) showed a significant increase in serotonin levels as compared to the control group ($p < 0.005$) after 7 days of intraperitoneal injections twice daily. However, there was an insignificant increase in the level of serotonin between these MDMA groups (Figure 1).

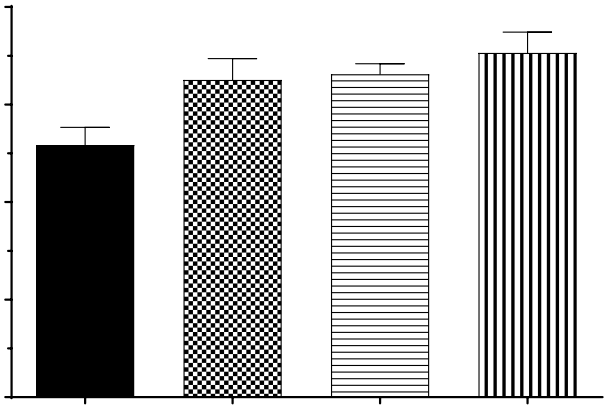

Figure 1: The effects of MDMA treatments on serotonin level in CSF over 24 hours after the last injection.

The results showed a significant increase of serotonin level in response to MDMA administration ($^{c}p < 0.05$, when compared to the control group).

Histology, morphometric and statistical results of the number of intact neurons in CA1 and CA3 subfields of the hippocampus

The findings of this study showed that MDMA caused a dose-dependent decrease in the number of intact neurons in CA 1 and CA 3 subfields of the hippocampus of the rat brain, in comparison to the saline control group. The mean values ± S.D of the intact number of neurons in the CA1 subfield were 197.6 ± 31.46, 123.8±115, 107.4 ±7.091 and 77.5±14.9 for saline control, 5mg/kg MDMA, 10mg/kg MDMA and 15mg/kg MDMA for treated groups; respectively. The mean values ± SD of the intact number of neurons in the CA3 subfield were 112.6 ± 11.4, 78.5±6.9, 61.3 ±4.9, 47.2±6.2 for saline control, 5mg/kg, 10mg/kg and 15mg/kg MDMA for treated groups; respectively. A statistically significant difference was noted between the number of the intact pyramidal cells in MDMA treated groups compared to the control saline group ($p<0.001$). All sections from the MDMA treated groups showed a decrease in the number of intact neurons. The mean values ± S.D of the number of intact neurons in the CA1 and CA3 subfield are simplified in table 1 and 2 to show a comparison between each treatment group. Figure 3 and 5 show the mean value ± SD of the decreasing intact neurons after the MDMA administration in CA 1 and CA 3 subfields; respectively.

In the control group, the CA1 and CA 3 subfields of the hippocampus exhibited a higher number of intact neurons having clearly defined cell bodies and nuclei (CA 1, Figure 2A ; CA 3, Figure 5 A).

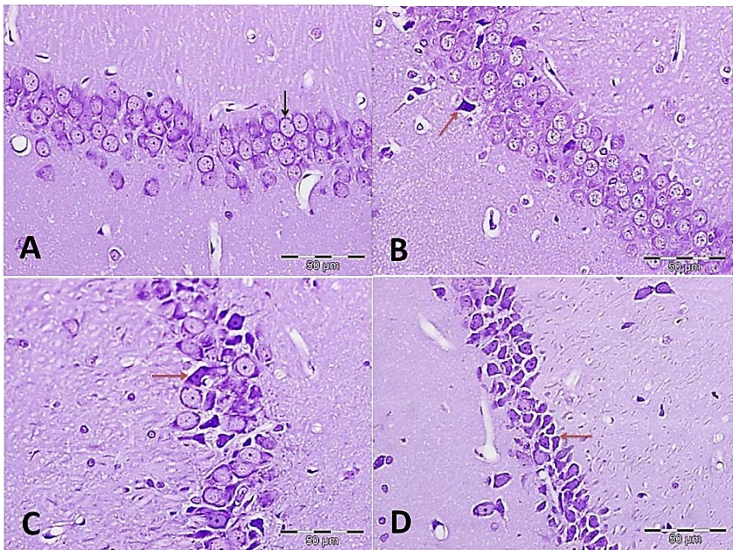

Figure 2: Photomicrographs of a coronal section of the rat brain in the CA1 subfield of the hippocampus from the control group (**Fig. 2a**) and treated groups (**Figs. 2c, 2d and 2e**).

Fig. 2a shows mostly intact pyramidal cells with well-defined nuclear and cytoplasmic membrane with prominent nucleoli (black arrow). Fig. 2b, 2c and 2d show the presence of degenerated pyramidal cells characterized by poorly defined nucleus and shrunken cell cytoplasm which is dark in colour. The nucleoli are not visible (red arrows). There was an increasing number of degenerated pyramidal cells seen when the dose of MDMA was increased. Hence, the number of intact pyramidal cells is reduced per counted area (Cresyl Echt Violet ×40).

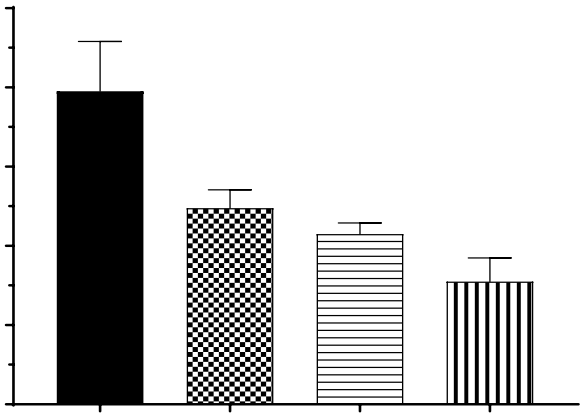

Figure 3: A histogram showing mean values ± S.D (standard deviation) of the number of intact neurons in CA1 subfield of hippocampus from the control group and treated groups with 5, 10 and 15 mg/kg MDMA. (n = 6 or 7). ($^{a}p < 0.001$, when compared to the control group)

Table 1: The summary of the comparative Mean ± SD of the number of intact neurons at CA 1 subfield of hippocampus for each dosage of MDMA treatments.

		Number of intact neurons
	Control	197.6 ± 31.46
MDMA	5 mg/kg	123.8 ± 11.5 [a]
	10 mg/kg	107.4± 7.091 [a,c]
	15 mg/kg	77.5 ± 14.9 [a,b,d]

Note: [a] $p < 0.001$ when compared to control
[b] $p < 0.001$ when compared to 5 mg/kg
[c] $p < 0.001$ when compared to 15 mg/kg
[d] $p < 0.05$ when compared to 10 mg/kg

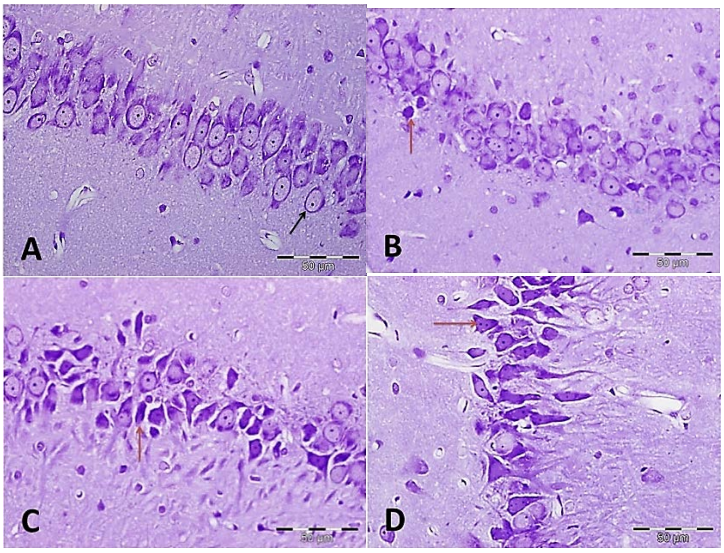

Figure 4: Photomicrographs of a coronal section of the rat treatment, the CA3 subfield of the hippocampus from the control group (Figs. 4a) and treated groups (Figs. 4c, 4d and 4e).

Fig. 3a shows mostly intact pyramidal cells with well-defined nuclear and cytoplasmic membrane with prominent nucleoli (black arrow). Fig. 3b, 3c and 3d show the presence of degenerated pyramidal cells characterized by poorly defined nucleus and shrunken cell cytoplasm which is dark in colour. The nucleoli were not visible (red arrows). There was an increasing number of degenerated pyramidal cells seen when the dose of MDMA was increased. Hence, the number of intact pyramidal cells was reduced per counted area (Cresyl Echt Violet ×40).

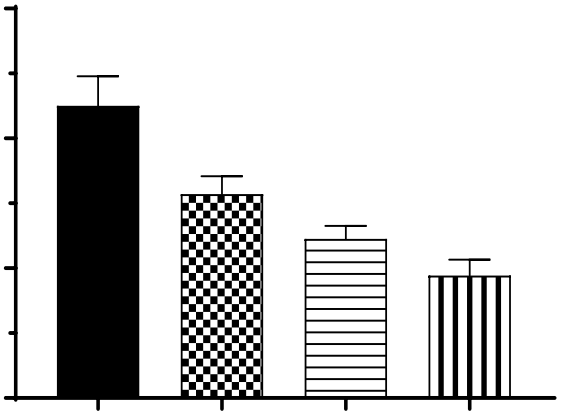

Figure 5: A histogram showing mean values ± S.D (standard deviation) of the number of intact neurons in CA3 subfield of hippocampus from the control group and treated groups with 5, 10 and 15 mg/kg MDMA. (n = 6 or 7). ($^{a}p < 0.001$, when compared to the control group)

Table 2: The summary of the comparative Mean ± SD of the number of intact neurons at CA 3 subfield of hippocampus for each dosage of MDMA treatments.

		Number of intact neurons
	Control	112.6 ± 11.4
MDMA	5 mg/kg	78.5 ± 6.9 [a]
	10 mg/kg	61.3± 4.9 [a,c]
	15 mg/kg	47.2 ± 6.2 [a,b, d]

Note: [a] $p < 0.001$ when compared to control
[b] $p < 0.001$ when compared to 5 mg/kg
[c] $p < 0.001$ when compared to 15 mg/kg
[d] $p < 0.05$ when compared to 10 mg/kg

Correlation of serotonin levels in CSF with the number of intact neurons after different doses of MDMA administration

Based on the results of this study, there was a trend seen in the serotonin levels comparing to the number of intact neurons, in which the increasing serotonin level was correlated with decreasing number of intact neurons in both CA1 and CA3 subfields of the hippocampus after the administration of different doses of MDMA. From the statistical analysis (Graph Pad PRISM), the correlation was significant and strong in CA1 (r = -0.879) and CA3 (r = -0.905) ($p < 0.001$) (Figure 5 and 6).

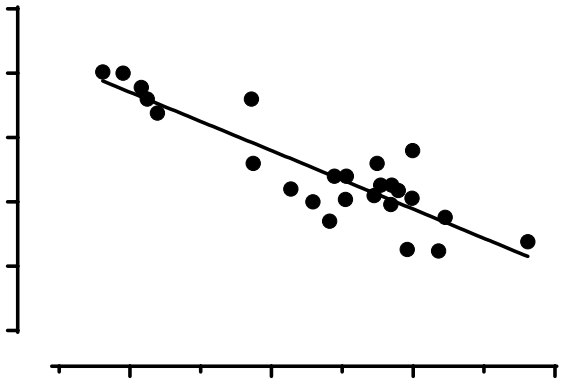

Figure 6: The correlation between serotonin level in CSF and the number of intact pyramidal cells in the CA1 subfield of the hippocampus (n=26).

The graph in fig. 6 shows the inverse correlation between the number of intact pyramidal cells in CA1 subfield of the hippocampus and the serotonin level in the CSF of the rats (slope = -0.909 ± 0.103, r = -0.879, $p < 0.001$).

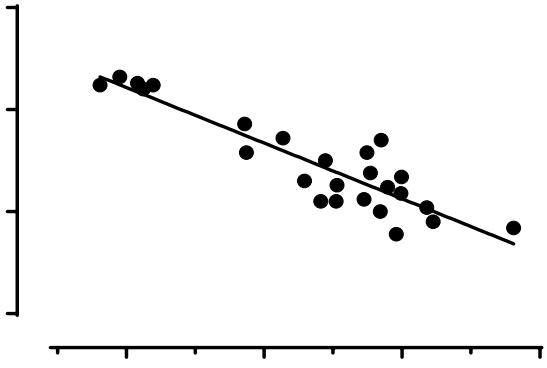

Figure 7: The correlation between serotonin level in CSF and the number of intact pyramidal cells in the CA3 subfield of the hippocampus (n=26).

The graph in fig.7 indicates that there is an inverse correlation between the number of intact pyramidal cells in CA3 subfield of the hippocampus and the serotonin level in the CSF of the rats (slope = -0.545 ± 0.053, r = -0.905, $p < 0.001$).

Table 3: Coefficient correlation (r) and the p-value of the number of intact neurons for CA 1 and CA 3 subfields of the hippocampus.

Subfield	Correlation of coefficient (r)	p value
CA 1	-0.879	<0.001
CA 3	-0.905	<0.001

DISCUSSIONS

In the present study, the effects of different doses of MDMA administration on the serotonin levels in the CSF of the rats and the number of intact neurons, which reflected the severity of neuronal damage induced by MDMA were observed. The results demonstrated that the intraperitoneal administration of different doses of MDMA caused a significant increase in serotonin level as compared to the control group ($p<0.001$). Serotonin levels in the CSF reflected the concentration released during the neuronal activity in the brain [4]. The MDMA acted by binding on the presynaptic serotonin transporter (SERT), subsequently enhanced the net release of serotonin into the synapse, and inhibited its reuptake [37-39].

The high concentration of serotonin in the synapse improved mood and caused sensory alteration. However, chronic administration of MDMA caused a massive efflux of serotonin that consequently resulted in neuronal damage especially on the serotonergic terminals [40]. Besides that, the rapid and active release of serotonin into the synapse after the MDMA administration later caused long-term serotonin depletion [41].

The current study's findings were consistent with a human study done by McCann and colleagues (1999), in which the administration of MDMA showed an increasing level of serotonin in the brain [42]. In their study, they found that the levels of serotonin in the CSF reflected the index of global brain release. In addition, they also claimed that MRI or proton magnetic resonance spectroscopy would be useful to estimate the number of intact neurons after the neurodegenerative effects following MDMA administration, in different regions of brain.

According to Sprague and colleagues (2003), MDMA is one of the psychostimulant drugs that causes a disturbance in brain functions and neurotoxicity [43]. Hippocampus is one of the regions in the brain that contains abundant serotonin receptors [44, 45]. The region controls the memory and learning process. Hence, chronic administration of MDMA may cause an alteration in the memory and learning process of the users.

From the results of this study, the number of intact neurons in CA1 and CA3 subfields of the hippocampus was significantly decreased after the administration of 5mg/kg of MDMA. This indicated that MDMA caused a deleterious effect even at low doses. Brain tissue is very susceptible to damage due to brain microenvironmental changes. MDMA administration can alter the brain microenvironment by increasing its metabolite that leads to the production of reactive oxygen species (ROS) and protein-bound quinones, inducing the oxidative stress and depleting the intracellular glutathione [46]. The neurons are susceptible to the unfavorable changes in their environment, as their antioxidant mechanisms have limited capacity to counteract the free radicals [47, 48]. Other than that, the MDMA was also found to bind and activate the pre and post-synaptic serotonin receptors [49] thus enhancing the intracellular pathways such as adenylate cyclase, $Na2+$ or $Ca2+$ ligand gate that consequently affected the expression of several genes including Bcl-2 family [50]. The MDMA affected the Bcl-2 gene by downregulating its expression [51]. The Bcl-2 protein is responsible for apoptosis that occurs after amphetamine administration [46, 52]. The Bcl-2 protein inhibited the release of cytochrome C from mitochondria, ultimately blocked the caspase activation leading to apoptosis [53].

In the current study, it was postulated that the increasing level of serotonin in the synapse causes neuronal damage in the hippocampus via excitotoxicity mechanism. Excitotoxicity occurs through a series of mechanisms that enhance the glutamate release, and subsequently causes an increase in the intracellular calcium levels that further cause activation of calcium-dependent proteolytic enzymes, free radicals formation, and activate the apoptotic pathways that contribute to the neuronal damage or cell death [54-56]. According to Preedy (2016), excitotoxicity is a neurotoxic process enhanced by the overstimulation of NMDA and AMPA glutamate receptors on the postsynaptic neurons. This causes an influx of $Ca+$ ions that activate both the apoptotic signalling, and induce necrotic cell death.

The MDMA triggers the increase in the level of glutamate in the hippocampus, partly mediated through a non-neuronal mechanism [57]. According to Mark and colleagues (2004), the MDMA exerts neurotoxicity effect by increasing the glutamate release [58]. The increase in the extracellular glutamate levels in the hippocampus is the result of an increase in serotonin level after MDMA exposure via activation of 5HT2 receptors [59]. After the activation of the 5HT2 receptors, cyclooxygenase-2 (COX-2) activity is enhanced which consequently affect the prostaglandins release and later results in an increase in the glutamate level released into the synapse [56, 60]. According to a study by Sanzgiri, Araque, & Haydon (1999) [61], the prostaglandins, specifically prostaglandin E2 (PGE2), was activated by EP1 receptors which caused the release of glutamate within hippocampus region causing excitotoxicity [62, 63].

Therefore, the MDMA administration causes overstimulation of the NMDA receptor [64]. The activation of NMDA receptors consequently enhances the glutamate actions in mediating the decrease in the number of parvalbumin (PV)

interneurons [65]. The increase in cyclooxygenase activity is also contributed to the decrease in the number of PV interneurons due to GABA toxicity [60] following a massive efflux of serotonin after MDMA administration [56].
With these findings, the researchers would like to suggest a further study with an additional exploration of the rats' behavior. Murnane et al., (2012), already performed a study comparing the behavioural changes in the neurotransmitter levels in the brain tissue after MDMA administration [66]. However, their limitation was in the demonstration of the histopathological changes in the brain to compare with the behavioural changes and neurotransmitter levels. The researchers believe that many more comparative histopathological studies can be done using the CSF as a tool for measuring the neurotransmitter.

CONCLUSION

The present study provided evidence that chronic MDMA intake exerts a deleterious effect on the brain structure in the CA1 and CA3 subfields of the hippocampus-mediated by an increase in the serotonin level. The damage was seen even at the low dose of chronic MDMA administration. Further explorative studies would be advocated for better understanding and comparison.

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Conflict of Interest

The authors have declared that there was no conflict of interest existing.

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