

BEHAVIOURAL, BIOCHEMICAL AND NEUROCYTOARCHITECTURAL IMPACT OF MDMA IN MALE ADOLESCENT MICE

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ABSTRACT

Neurotoxicity of 3, 4-Methylenedioxymethamphetamine (MDMA, ecstasy) is still controversially discussed. MDMA is an amphetamine derivative that has gained significant popularity in recent years and has become the recreational drug of choice for many young, adolescents and adults. This study sought to investigate that MDMA causes a behavioral, biochemical and neurocytoarchitectural change in male adolescent mice. Fifteen adolescent male mice were used for this study. They were randomly divided into 3 groups, of five mice each, control group received normal saline (Ctrl), 5 mice received 10 mg/kg of MDMA, s.c (LoD) and another 5 mice received 20 mg/kg of MDMA, s.c (HiD). The animals were subjected to behavioural tests to check their motor activity. Biochemical analysis to test for the level of catalase and monoamine oxidase activity was also done. The motor activity of animals treated with 10 mg/kg and 20 mg/kg of MDMA was significantly ($p < 0.05$) lower when compared with the control. Likewise, there was a reduction in the catalase and monoamine oxidase activity in the treated groups. In the cerebellum, cellular distortion was seen in the treated group. It could be concluded that MDMA affects the motor activity of the treated animals and thus cause an alteration in the neurocytoarchitecture, catalase and monoamine oxidase activities of the animals.

Keywords: MDMA, Motor function, Hippocampus, Cerebellum, Biochemical.

INTRODUCTION

Neurotoxicity of 3, 4-Methylenedioxymethamphetamine (MDMA, ecstasy) is still controversially discussed. MDMA is an amphetamine derivative that has gained significant popularity in recent years and has become the recreational drug of choice for many young, adolescents and adults (Bankson and Cunningham, 2001). Like amphetamine, MDMA elicits hyperactivity when administered to rodents. Unlike amphetamine, which has effects mediated by the release of dopamine (DA) from nerve terminals, MDMA-induced hyperactivity is

thought to be dependent upon the release of 5-hydroxytryptamine (5-HT, serotonin) (Bankson and Cunningham, 2001). The positively rated effects of MDMA consumption include euphoria, arousal, enhanced mood, and increased sociability, a general sense of well-being, decreased anxiety and heightened perceptions as a hallmark of the MDMA experience (Morgan, 2000; Parott, 2001). Some common adverse reactions are nausea, headache, tachycardia, bruxism, and trismus (Meyer, 2013). Lowering of mood is an aftereffect that is sometimes

Submitted 16th June 2016, revised on 12th January 2017. Published online 20th January 2017. To cite: Oloruntola B.J, Adeniyi P.A, Ishola A.O, Ogedengbe O.O, Olatunji B.P, Olabiyi A.A, Shallie P.D. 2017. Behavioural, biochemical and neurocytoarchitectural impact of MDMA in male adolescent mice. *Anatomy Journal of Africa*. 6: 884 - 895.

reported from 2 to 5 days after a session of ecstasy use (Meyer, 2013).

The United Nations estimated that global ecstasy use affects some nine million people of the population between 15 and 64 years. There are more than three million ecstasy users in Europe accounting for 36% of ecstasy users worldwide. The annual prevalence rate of 'ecstasy' use is estimated at 0.9% of the population age 15-64 in the west and central Europe, exceeding the level reported from North America (0.8%) (UNODC, 2007).

In the European Union (EU), the European monitoring center on drugs and drugs addiction estimated that more than one million adults take ecstasy every month. In the United Kingdom (UK) alone, it is estimated that every weekend, 500,000 young people consume the drug (EMCDDA, 2007). Traditionally, population surveys have shown that after cannabis, amphetamines are the most commonly used illegal substances (Johnston *et al.*, 2008). The aim of this work is to access the behavioral, biochemical and neurocytoarchitectural changes in the hippocampus and cerebellum of adolescent mice after the induction of MDMA.

MATERIALS AND METHODS

Experimental animals

Fifteen (15) adolescent male mice were used for this study. The animals were obtained from the animal holding facility of Afe Babalola University, Ado-Ekiti, Nigeria. The animals were allowed to acclimatize for 5 days and had free access to standard pellet (purchased from the ABUAD feed mill, Ado-Ekiti) and clean water.

Experimental design

The mice were randomly divided into 3 groups, of 5 mice each in a standard laboratory environment with controls humidity, pressure, and temperature. The treatment protocol was in accordance with the ethical requirement of the Animal Use and Care Committee of Afe Babalola University, Ado-Ekiti, Nigeria. Control group received normal saline (Ctrl), low dose received 10 mg/kg, s.c MDMA (LoD) and high dose received 20 mg/kg, s.c MDMA (HiD). The mice were treated for 21 days.

Drug

The drug was requested by P.A. Adeniyi from the National Drug Law Enforcement Agency (NDLEA) head office for the research. The drug was delivered to him with a letter with ref no. **NDLEA/DP/3/VOL.1**. The total of 14.64 mg of MDMA was used for the study.

Behavioral analysis

The behavioral tests were carried out in a closed area with proper illumination and sound control

behavioral room of Afe Babalola University holding.

All behavioral test performance was captured using a digital video recorder and were later analyzed. The following tests were carried out;

- **Rotarod test:** rotarod test were done to assay for motor coordination (Deacon, 2013). The animals were placed on a rotarod as described by Perrine and Takesue (1968) to determine the latency of fall (LOF) and passive rotation (PR). The maximum duration allowed was 5 minutes.
- **Open field test (OFT):** This was done to test for the locomotive activities of the mice. OFT was made of white wood with dimensions, 100cm wide, 100cm long, and 50cm high and was marked with dark lines dividing the floor into 16 uniform square and a center square. A high definition video recording system (SONY) was placed in a position to capture all corners and sides of the open field area (OFA) in order to record animal movement and behavior (Ajonijebu *et al.*, 2014). For the actual test, the animals were allowed to freely move about for 5 minutes following which analysis was done for the total locomotive activity of

the mice using the following equation (Adeniyi and Olatunji, 2015).

Total locomotive activity (TLA) = sum of line crossed + number of rearing

Animal sacrifice

After administration, two (2) mice from each group were sacrificed by cervical dislocation on day 21 of the treatment and their brains were perfused with 10% formalsaline and transferred quickly to a specimen bottle containing 10% formalsaline (Baron, 1986; Adeniyi, 2010). Thereafter, the hippocampus at bregma - 1.82mm using stereotaxic atlas (Franklin and Paxinos, 2007) and cerebellum was excised to process for histological analysis. Also, the other three (3) mice from each group were sacrificed by cervical dislocation and their brain were harvested and transferred into 30% sucrose in a frozen environment in order to preserve the chemicals for biochemical analysis.

Histological analysis

After the tissue samples were fixed 10% formalsaline, they were dehydrated, embedded

in paraffin wax and sectioned with a rotary microtome at 5 μ m. They were then stained in cresyl echt violet stain and PTAH (phosphotungstic acid hematoxylin) stain to demonstrate for the glial fibers, nissil substance in neurons and of astrocyte respectively.

Routine H&E (hematoxylin and eosin) stain were also been used to give a general overview of the tissues.

Biochemical analysis

The biochemical assay was done to ascertain the catalase activity in the tissue samples as well as the monoamine oxidase (MAO) activity.

Statistical analysis

The data used were generated from the recorded videos manually. A number of the perpendicular lines crossed was used to determine the locomotive activity. The data was plotted in one-way ANOVA (GraphPad Prism Version 6.0) with Tukey posthoc test. Significance was set at * $P < 0.05$ and results were expressed as means \pm standard error of the mean (SEM).

RESULTS

Gross morphological observations

There were no changes in the skin color and the color of their eyes was normal compared to the control groups throughout the administration period. During the course of administration, it was observed that HiD animals were relatively more alert to their environment and showed restless when compared with the control and LoD animals. Similarly, comparing the LoD animals showed more agility and sensitivity than the control. After the 1st week of administration, an increase in body weight was observed in all groups throughout the experiment but no significant difference ($p > 0.05$) in weight change was observed throughout (see fig. 1).

Motor Activity

Rotarod test: a significant decrease in LOF was observed in LoD animals ($P < 0.05$) when compared with the control. Also, decrease in LOF was observed in HiD animals when compared with control. HiD animals showed greater LOF when compared with LoD animals but no significant difference ($p > 0.05$) was observed.

Open field test (OFT): LoD animals show significantly low locomotor activity compared to HiD animals and control group ($p < 0.05$). No significant difference was observed between the control group and HiD animals ($P > 0.05$).

Biochemical parameters

Catalase

LoD animals show significant reduction of catalase activity compared to the control ($p < 0.01$). No significant difference was observed between control and HiD animals. High dose treated animals had high catalase activity than the low dose treated group but no significant difference between the two groups.

Monoamine oxidase (MAO) activity:

Monoamine oxidase activity of LoD animals is significantly lower than the control group ($P < 0.01$). HiD animals show low MAO activity than the control group and higher activity than LoD animals but no significant difference was observed ($P > 0.05$).

Histological observations

Hippocampus:

The LoD possess more granular cells than the control group in terms of density, although relatively they are similar. HiD is not densely packed due to its invasion into the molecular layer. The granular cells are not in clustering orientation. The cells in HiD are quite scattered and display lesser denseness in orientation when compared with LoD which is relatively more packed. In LoD and the control group, their fibers were not so picked by the stain, but the LoD seems to appear more densely packed with more glial cells in a dense orientation than the control group. Comparing the control group and the HiD, the difference in both groups is quite significant with HiD appearing more densely packed than the control group. In comparing the treated groups, the LoD appearing similar to the control group making it relatively less dense than the HiD in terms of glial fibers and glial cells.

Cerebellum:

Comparing the control group and low dose treated group (LoD), the cells in LoD appears more densely packed than the control group and are also the cells are more deeply stained. Also in the high dose treated group (HiD), the cells appeared freely packed, but their cytoarchitecture appears similar to the control group. The Purkinje cells in LoD and HiD are majorly distorted with LoD cells shape appeared like it going through necrosis. Unlike the treated groups, the control group is normally shaped (oval) with slight vacuolation. LoD group appears to have lesser stained neuronal cells when compared with the control group. In terms of density and sparseness of neuronal cell, the control group appeared to be denser than HiD. Overall, both LoD and HiD show similarities in terms of cellular density and arrangement in the tissue. Comparing the control and the treated groups, the shape of the Purkinje cells in the control group are oval with no sign of distortion but in the LoD and HiD major distortion of the cell shape from an oval shape to spherical shape was been observed as well as vacuolation. Comparing the HiD and LoD, HiD appeared to be more prominently distorted. LoD treated group appeared to possess more cells than the control group but the glial fibers are not easily identified but in the control group, the fibers are clearly visible. Like the LoD, HiD to have almost no signs of glial fibers but still dominate in the density of glial cells when compared with the control group. LoD possesses more glial cells than HiD in terms of density. Observing at X100, the control group has more visible glial cells and fibers when compared with the treated groups due to lesser density in the glial cells and fibers. Comparing HiD and LoD, HiD appears to have more densely packed glial cells and fibers making it not quite visible.

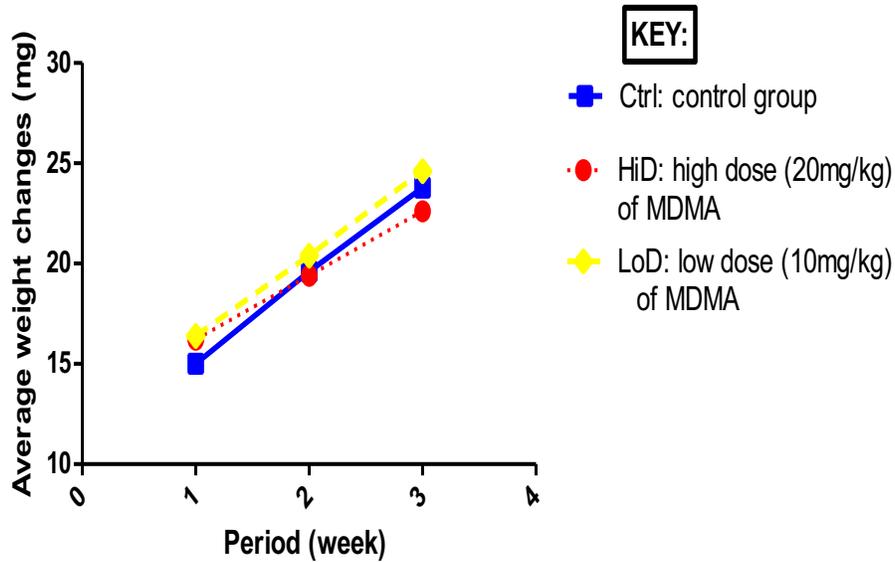


Figure 1: Line graph showing average body weight change of the experimental animals for 3 weeks. There is no significant difference ($P > 0.05$) in the weight change of the animals throughout the 3 weeks of administration.

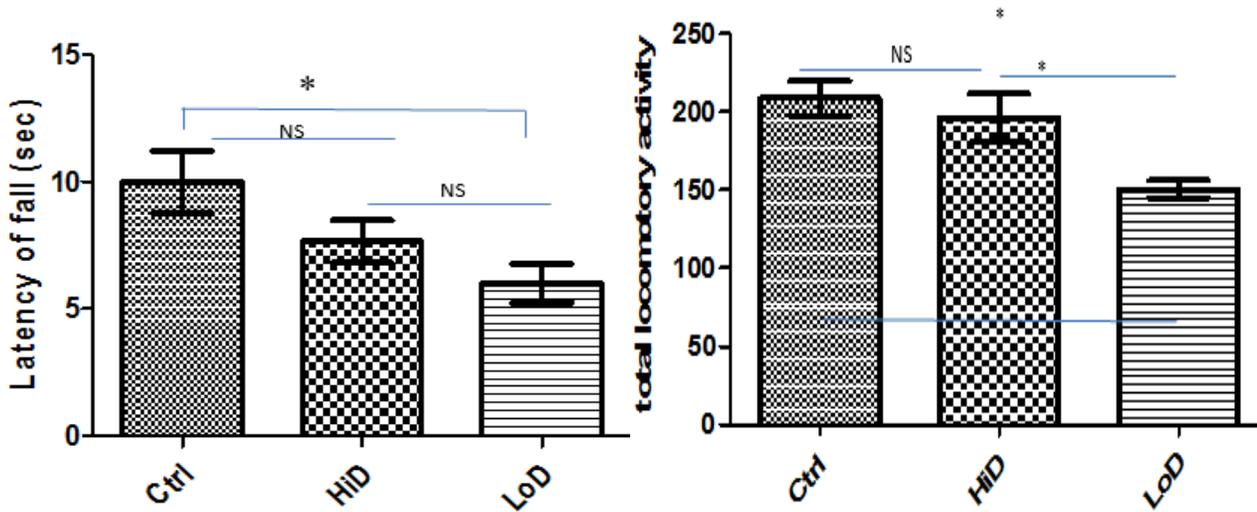


Figure 2: Graph showing the motor activity of the experimental animals. Latency of fall (LOF) of animals treated with 10mg/kg of MDMA is significantly lower than the control group ($*p < 0.05$) (left image). The locomotor activity of the experimental animals are shown in the right image. Animals with 10mg/kg of MDMA is significantly lower than the high dose treated animals and the control group ($*p < 0.05$).

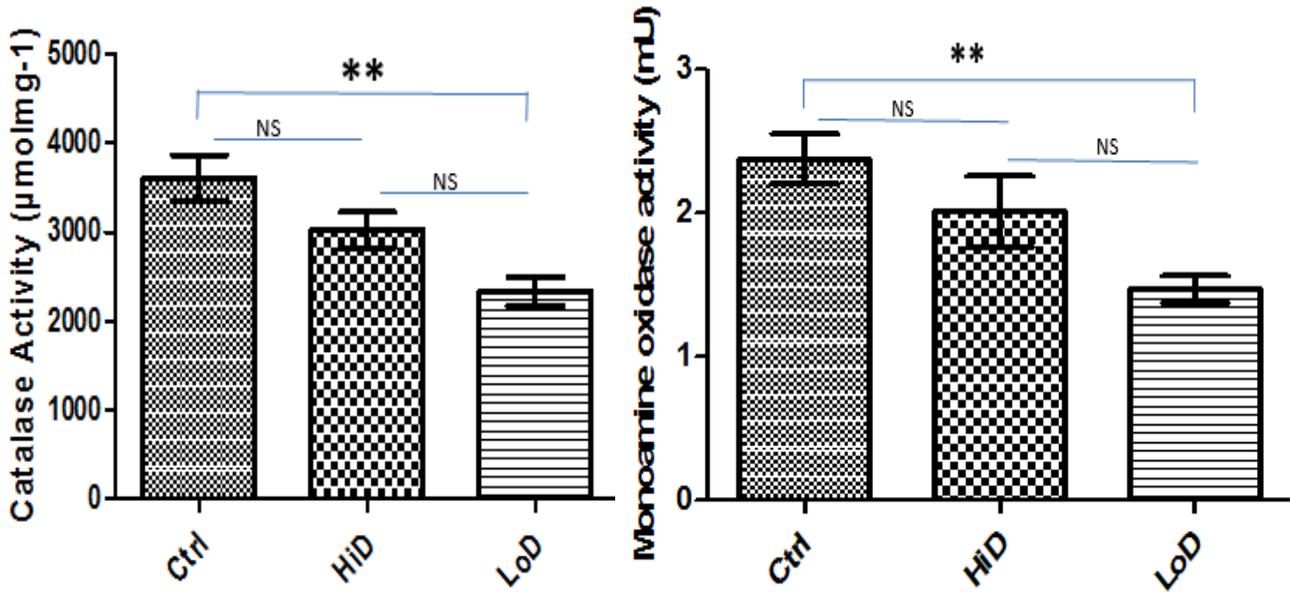


Figure 3: Graph showing the biochemical assays (catalase (L) and monoamine oxidase (R) activity) of experimental animals. Animals treated with 10mg/kg of MDMA is significantly lower than the control group (** $p < 0.01$). Monoamine oxidase (MAO) activity of animals treated with 10mg of MDMA is significantly lower than the control group (** $p < 0.01$).

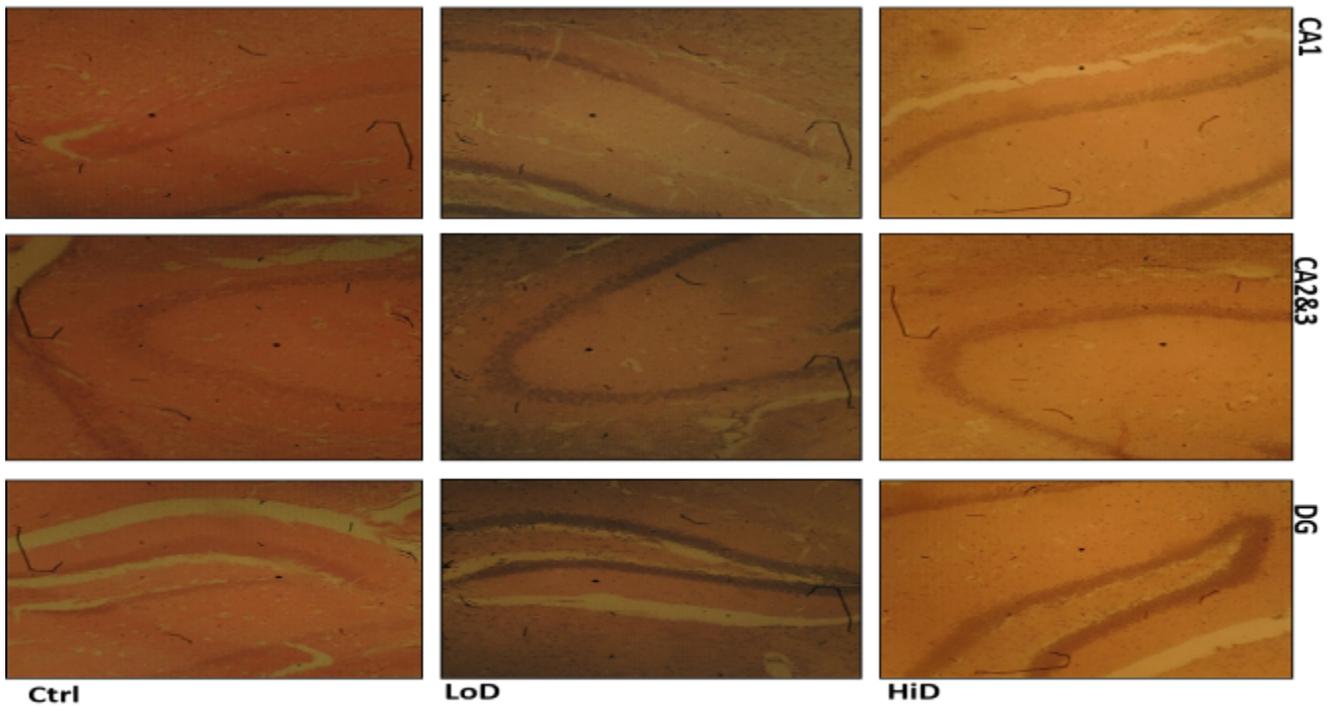


Figure 4: Histological slides showing the CA1, 2 and 3 and Dentate gyrus (DG) region of the hippocampus (X10, H&E). GL (granular layer), ML (molecular layer)

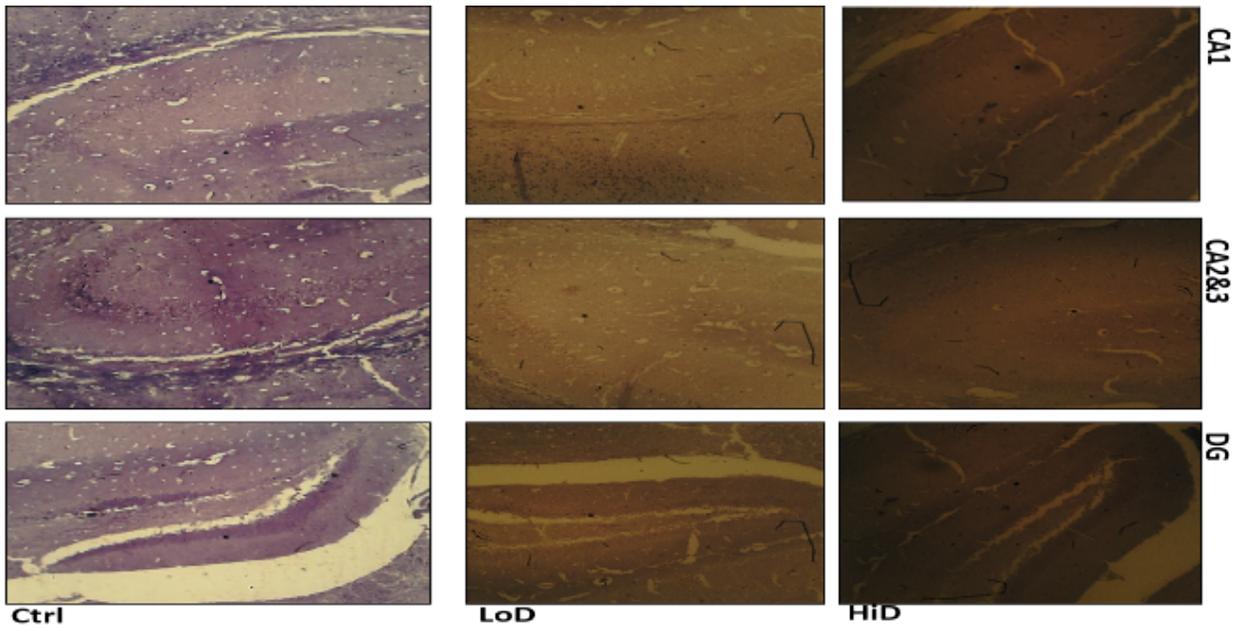


Figure 5: Histological slides showing the CA1, 2 and 3 and Dentate gyrus (DG) region of the hippocampus (X10, PTAH). GL (granular layer), ML (molecular layer)

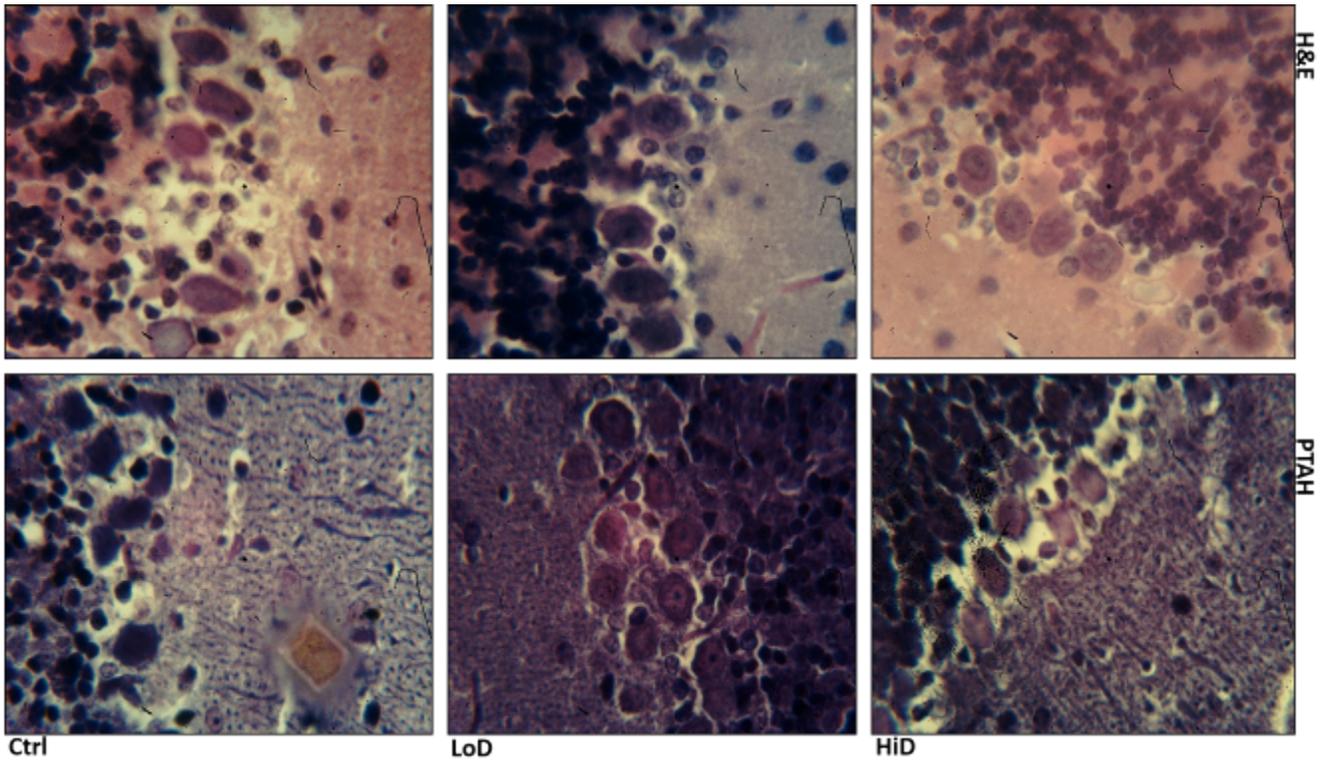


Figure 6: Histological slides showing the granular layer, Purkinje cell layer and molecular layer of the cerebellum (X100, H&E and PTAH).

DISCUSSION

Gross morphology and general observations

In the present study, no morphological changes were observed (skin and eyes color) in the treated animals when compared with the control. Addict or drug dependent people usually don't exhibit changes in the skin color as well as eyes color in the short term. Although there were changes in the average body weight, but there is no significance difference between the treated groups when compared with control group at 95% confidence interval ($p > 0.05$) (see figure 1). During the period of administration, some behavioural changes were observed in the animals treated with different doses of MDMA (10 mg/kg and 20 mg/kg s.c) when compared with those in the control group; the treated animals were relatively more alert to their environment (an indication of an increase in anxiety level) and showed restlessness when compared with the control group.

Monoamine oxidase (MAO) activity

MAO is a family of enzymes that catalyze the oxidation of mono amines (Tipton *et al.*, 2004; Edmondson *et al.*, 2004). They are found bound to the outer membrane of mitochondria in most cell types in the body. MAO helps in catalyzing the breakdown of dopamine to dihydroxy phenylacetic acid (DHPA) which is a direct precursor of dopamine. Biochemical analysis of the experiment animals in this study showed a significance reduction in the monoamine oxidase (MAO) activities (see figure 5) when compared with the control ($P < 0.01$); this result agreed with findings of Efthimia *et al.*, (1994) MDMA had been reported to act as MAO activity inhibitor by preventing the conversion of dopamine in the synaptic cleft into DHPA (Efthimia *et al.*, 1994). A significant reduction in MAO activity will result in a reduction in DHPA leading to high level of dopamine in the system. The accumulation/retention of dopamine in the system will result in hyperexcitation and if this

continues for a longer period would result in excitotoxicity, glial cell activation (indicated by the prominent glial fibers), gliosis and neuronal death (See figure 15).

Catalase activity

Free radicals are atoms or groups of atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules (Orchin *et al.*, 2005). Once formed these highly reactive radicals can start a chain of reaction. These free radicals are very reactive and they can react with most of the micro-molecules in the system even with those that normally doesn't react in their natural state such as DNA. Their chief danger comes from the damage they can do when they react with important cellular components such as DNA, or the cell membrane; this in cells dysfunction and eventually can result in cell death (Conte *et al.*, 1996). To prevent free radical damage the body has a defense system of antioxidants, free radicals scavengers, e.g. catalase. Catalases are antioxidants that catalyze the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor (Zámocký and Koller, 1999; Chelikani *et al.*, 2004). This protein is localized to peroxisomes in most eukaryotic cells (del Rêo *et al.*, 1992). Catalase is an unusual enzyme since, although hydrogen peroxide is its only substrate, it follows a ping-pong mechanism. Here, its cofactor is oxidized by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to the second molecule of the substrate (Hiner *et al.*, 2002). The result from this study reveal that MDMA significantly decreases ($P < 0.01$) the level of catalase activity in the brain of the MDMA-treated groups when compared with the control (see figure 4). As a result of the reduction in the level of catalase activity in the brain of the treated animals this could lead to an increase in oxidative stress as reported by Brioukhanov *et al.* (2006). Increase in the free radicals in the brain has been reported to result in the decrease in density of

dopaminergic receptors in the brain (Tiago *et al.*, 2004) and this will result in having more dopamine in the system for longer period of time which in turn results in hyper-excitation, cell death, glial activation, and gliosis (Björklund and Dunnett, 2007).

Neurobehaviour

MDMA is a psychoactive drug that acts on the different neurotransmitters (dopamine and serotonin) to have its effect. It is shown that MDMA induces serotonin syndrome in rodent (Isbister *et al.*, 2007). Most users of MDMA abuse it with other drugs. A symptom of serotonin syndrome in the rodent is a jerky movement of the limbs this has been attributed to excess serotonin and dopamine at the synapse (Ener *et al.*, 2003). This study shows that animals treated with MDMA had lower motor function compared with the control (Fig. 2 and 3). Other studies have reported the similar observation that MDMA affects motor coordination and memory function (Callaghan *et al.*, 2006; Li *et al.*, 2006; Capela *et al.*, 2009). Altered motor coordination may be due to the effect of the serotonin syndrome as the animal may not be able to balance well on the rotarod bar, although animal given the higher dose (20 mg/kg, s.c) still perform more than the lower dose (10 mg/kg, s.c). The explanation for this looks tricky but epigenetic modification of the receptors at a higher dose may be a cause. It has been reported that alcohol which is another psychoactive agent has a different effect on dopamine release at acute and chronic state (Boileau *et al.*, 2003). In this study, it was observed that the total locomotive activity of animals treated with 10 mg/kg of MDMA was significantly ($P < 0.05$) lower than the control group. No significant difference was observed between the control group and the high dose treated animals ($p > 0.05$).

Neurohistology

The cerebellum plays an important role in motor function. Muscular input through mossy fibers project to the cerebellum, synapsing of the granular cells which later project to the Purkinje

cells (Loewenstein *et al.*, 2005). Purkinje neurons are the inhibitory neurons in the cerebellum which helps in modulating the cerebellum firing. This finding shows that animals given 10mg/kg (s.c) of MDMA have more compacted granular cell layer than the control and the high dose (20mg/kg, s.c) (see fig. 13). Most input into the cerebellum enters into the granular cell layer to make a synapse before later relating to the Purkinje cells for modulation. Observations of the Purkinje cells indicate that animals given 10 mg/kg BW of MDMA have distorted Purkinje cells than the control, the effect of high dose been higher as there are lesser Purkinje cells. Since Purkinje cell is the only inhibitory neuron in the cerebellum, fewer Purkinje cells will lead to lesser inhibition of cerebellar output (Yartsey *et al.*, 2009). This correlate with the behavioral result as animals given MDMA has low motor coordination (fig. 2 and 3). Lesser Purkinje cells lead to disinhibition of the cerebellar output thereby increasing the crude motor movement (Loewenstein *et al.*, 2005), this makes fine movement coordination impossible.

The Glial cells and fibers are more stained in the treated group with higher dose showing more stain (see fig.14). Increase in glial cells especially the microglia is a sign of inflammation (Dissing-Olesen *et al.*, 2007). Since MDMA inhibits MAO activity leading to excess dopamine at the synapse (Efthimia *et al.*, 1994). Astrocyte has been shown to have receptors for neurotransmitters and help in mopping up excess neurotransmitters from the synapse (Figley and Stroman, 2011) and thereby by the result in their activation if prolonged. This explains why more microglia are generated to clear off dead neuron, in the same way, more the astrocyte is generated take up the excess neurotransmitters at the synapse. This may be a reason for increase in the No. of cells/unit area in the treated animals when compared with the control (see fig.9)

Research has shown that the hippocampus and the cerebellum are connected polysynaptically

(Yu *et al.*, 1989). This connection has been attributed to the function of the place cells in the hippocampus, the place cells are responsible for familiarizing with previous locations and areas. Studies show that the place cell depends on cues from the cerebellum (Rochefort *et al.*, 2011) and report of experience of patients with cerebellar lesion shows that they have cognitive deficit especial matching space with time (Schmahmann, 1998). The present work shows that more granular cells were seen in the treated group (see fig. 11), the hippocampal-cerebellar connection involves the projection from CA1 and CA3 to lobule VI of the cerebellar cortex (Yu *et al.*, 1989). Although, the cerebellum have been hypothesized to involve in higher cognitive function (Timmann and Daum, 2007). This study shows that MDMA increases the cell count in all areas of the hippocampus (CA1, CA2, 3 and DG) (fig. 6 – 8). Adeniyi *et al.* (2015) show that low dose (2 mg/kg, s.c) of MDMA reduced cell count in all areas of the hippocampus after a short period of time. This is not consistent with the present result which shows that 10 mg/kg and 20 mg/kg of MDMA increase the cell count in all regions of the hippocampus for 21 days. Although memory test was not done in this study, Adeniyi *et al.* (2015), shows that MDMA did not affect spatial memory at the dose used.

We hypothesized that MDMA at 10 mg/kg and 20mg/kg in mice may affect spatial memory since hippocampus spatial memory processing depends on cues from the cerebellum as reported (Tomlinson *et al.*, 2014).

In conclusion, Administration of MDMA (in short term) have no significant effect on the gross morphology of the animal. MDMA causes the excess release of neurotransmitters at the synapse as well as alteration in some biochemical metabolite involve in the metabolism of these neurotransmitters. The motor activities of animals treated with MDMA are affected. MDMA affect the cytoarchitecture of the brain cerebellum and the hippocampus bringing above cell death in the cerebellum as well as the proliferation of more glial cells in the hippocampus. The effects of the MDMA are dose depended.

Acknowledgment

We are grateful to the Nigerian Drug Law Enforcement Agency (NDLEA) for giving us the MDMA crystal. Also, we acknowledge Mr. Olawale Oso of the Department of Chemical Sciences, Afe Babalola University who worked with us in the preparation reagents.

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