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RESEARCH ARTICLE

Neural and behavioural changes in male periadolescent mice after prolonged nicotine-MDMA treatment

Philip A. Adeniyi[1,4] · Azeez O. Ishola[2] · Babafemi J. Laoye[3] · Babawale P. Olatunji[3] · Oluwamolakun O. Bankole[3] · Philemon D. Shallie[4] · Olalekan M. Ogundele[2,3]

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Abstract The interaction between MDMA and Nicotine affects multiple brain centres and neurotransmitter systems (serotonin, dopamine and glutamate) involved in motor coordination and cognition. In this study, we have elucidated the effect of prolonged (10 days) MDMA, Nicotine and a combined Nicotine-MDMA treatment on motor-cognitive neural functions. In addition, we have shown the correlation between the observed behavioural change and neural structural changes induced by these treatments in BALB/c mice. We observed that MDMA (2 mg/Kg body weight; subcutaneous) induced a decline in motor function, while Nicotine (2 mg/Kg body weight; subcutaneous) improved motor function in male periadolescent mice. In combined treatment, Nicotine reduced the motor function decline observed in MDMA treatment, thus no significant change in motor function for the combined treatment versus the control. Nicotine or MDMA treatment reduced memory function and altered hippocampal structure. Similarly, a combined Nicotine-MDMA treatment reduced memory function when compared with the control. Ultimately, the metabolic and structural changes in these neural systems were seen to vary for the various forms of treatment. It is noteworthy to mention that a combined treatment increased the rate of lipid peroxidation in brain tissue.

Keywords MDMA · Nicotine · Motor cortex · Striatum · Hippocampus · Behaviour

Introduction

MDMA, also known as “molly” or “ecstasy”, is the most widely used synthetic drug among adolescent humans which represents a major percentage of illicit drug users (NIH-NIDA 2013; Azagba et al. 2014). It holds central and peripheral nervous system effects such as increased heart rate, empathy towards others, blur vision, increased libido and suppression of hunger (Teixeira-Gomes et al. 2014; Kwack et al. 2014; Downey and Loftis 2014; Kiyatkin et al. 2014, 2015; Fulceri et al. 2011). Most of these effects have been attributed to the ability of MDMA to modulate neurotransmitter activities, specifically dopaminergic (DA) (Izco et al. 2007; Palfreyman et al. 1993), serotoninergic (5-HT) (Lizarraga et al. 2014; Homberg et al. 2007) and glutamergic signalling in multiple brain centres involved in motor coordination, anxiety and cognitive functions (Meamar et al. 2012; Anneken et al. 2013; Sarkar and Schmued 2010). MDMA is known to facilitate the release of serotonin (5-HT), followed by inhibition of its re-uptake in synaptic terminals (Lizarraga et al. 2014).Similarly, it blocks dopaminergic re-uptake in dopaminergic (DA) neurons; but to a lesser extent when compared to its effect at 5-HT terminals (Seger 2010; Rouine et al. 2014; Roger-Sánchez et al. 2013). Despite the effect of MDMA on multiple

✉ Olalekan M. Ogundele
ola.ogundele@abuad.edu.ng

1 Cell Biology and Neurotoxicity Unit, Department of Anatomy, College of Medicine and Health Sciences, Afe Babalola University, Ado-Ekiti, Ekiti State, Nigeria

2 Neural Systems Unit, Department of Anatomy, College of Medicine and Health Sciences, Afe Babalola University, Ado-Ekiti, Ekiti State, Nigeria

3 Neurobiology Division, Department of Biological Sciences, College of Sciences, Afe Babalola University, Ado-Ekiti, Ekiti State, Nigeria

4 Department of Anatomy, Obafemi Awolowo College of Health Sciences, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria

neurotransmitter systems, most of its action is known to be mediated through its effect on the serotoninergic (5-HT) system (Rouine et al. 2014; Roger-Sánchez et al. 2013). Furthermore, MDMA facilitates the oxidative deamination of monoamines (5-HT and Dopamine) leading to the production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and auto-oxidation (Karuppagounder et al. 2014; Fornai et al. 2002). As a result of these effects, MDMA causes cellular oxidative stress and lipid peroxidation.

Aside the effects of MDMA on neurotransmitter re-uptake and induced oxidative stress, studies have shown that MDMA can act as a partial agonist for nicotinic acetylcholine receptors (α7 nAChRs) at neuromuscular junctions (Garcia-Ratés et al. 2010; Chipana et al. 2008; Ciudad-Roberts et al. 2014; Nuutinen et al. 2005); thus generating effects similar to the actions of nicotine at nicotinic neuromuscular junctions (Lettfuss et al. 2013; Pubill et al. 2013; Escubedo et al. 2009). In addition, use of nicotine analogues up regulates the expression of nicotinic receptors; thus, MDMA (a partial agonist) and nicotine (agonist) combined use can facilitate over expression of nicotinic receptors causing neurotoxicity through increased receptor sensitivity (Ciudad-Roberts et al. 2014; Pubill et al. 2013).

Similar to MDMA, nicotine also acts centrally through potentiation of cholinergic receptors, alteration of 5-HT and dopaminergic neurotransmission (Matsuura and Urushihata 2014; Guy and Fletcher 2014; Wang et al. 2014). As a result of nicotinic modulation of dopaminergic D_2 receptor stimulation, nicotine increases dopaminergic activity in the nigrostraitum; thus facilitating motor activities in the central nervous system (Wang et al. 2014; Cosgrove et al. 2014). Nicotine also potentiates α7 nAChRs at the nicotinic neuromuscular junctions to increase motor function peripherally (Kiss et al. 2014; Vu et al. 2014; Kiguchi et al. 2012). Although dopaminergic stimulation accounts for the central increase in motor function seen in nicotine use, it is also responsible for the brain reward effects; which, in part, causes addiction to nicotine after chronic use (Wang et al. 2014; Wing et al. 2014).

In this study we examined the effect of Nicotine, MDMA and a combined Nicotine + MDMA treatment on motor-cognitive function of periadolescent mice. This was aimed at showing the effect of early life (periadolescent) exposure on the behaviour of these animals during the final postnatal development of the motor-cognitive neural systems. In addition, we have shown the correlation between the observed behavioural changes and cellular changes observed in the corticostriatum and hippocampus.

Materials and methods

Materials BALB/c breeder Mice were obtained from the animal holding facility of Afe Babalola University, Nigeria. Nicotine salt was a generous gift from Dr. Anna DeLuca of the University of Cagliari, Italy. MDMA crystals was obtained from the Nigerian Drug Law Enforcement Agency (NDLEA), Lagos, Nigeria. All enzyme assay kits (ALP, SOD, MDA and GPx), Formalin, sucrose, sodium chloride and picric acid were procured from Sigma-Aldrich, Germany.

Animal preparation $N = 20$ (P.21) periadolescent male BALB/c mice were bred in the animal holding facility of the Afe Babalola University, Nigeria. The animals were housed under standard laboratory conditions of alternating light/dark and were fed ad libitum. Animals were randomly divided into four groups of $n = 5$ animals and labelled as appropriate. All protocols were in accordance with the IACUC animal use guidelines and were approved by the Animal Use Ethical Committee of the Afe Babalola University, Nigeria.

Treatment Male periadolescent mice (P.21) were treated for a period of 10 days with the vehicle, Nicotine, MDMA and Nicotine + MDMA. The control group received normal saline (subcutaneous). MDMA was administered subcutaneously to a set of $n = 5$ animals (2 mg/Kg body weight) at two days interval. The Nicotine treated group received 2 mg/kg BW of Nicotine daily while the Nicotine-MDMA group was treated with Nicotine (2 mg/Kg BW; s.c.) daily and MDMA (2 mg/Kg; s.c.) at 2 days interval. The total duration of treatment for all groups was 10 days.

Body weight measurement The body weight of all animals in each group was taken daily to determine the average weight/group/day for 10 days. The change in body weight for the control and treatment groups was determined in linear regression analysis to determine the *P value* and level of significance at *95 % Confidence Interval* (GraphPad Prism Version 5).

Behavioural tests Behavioural studies commenced on the last treatment day (P.31) and extended till the day after the last treatment (P.32). The animals were moved to the behavioural testing room 24 h prior to the commencement of tests (acclimatization) (Fig. 1a).

Rotarod test This was done to measure motor activity as a function of latency of fall (LOF) on the treadmill. Each animal was placed on the rotating bar of the Rotarod following which the speed was gradually increased from 3 to 35 rpm over a period of 3 min. The duration spent on the Rotarod (before fall) was measured to determine the LOF. In this study, each animal was tested in three consecutive trials (T_1, T_2 and T_3) with an inter-trial time of 30 min. Subsequently, we determined the average LOF/animal/group and compared the treatment groups with the control.

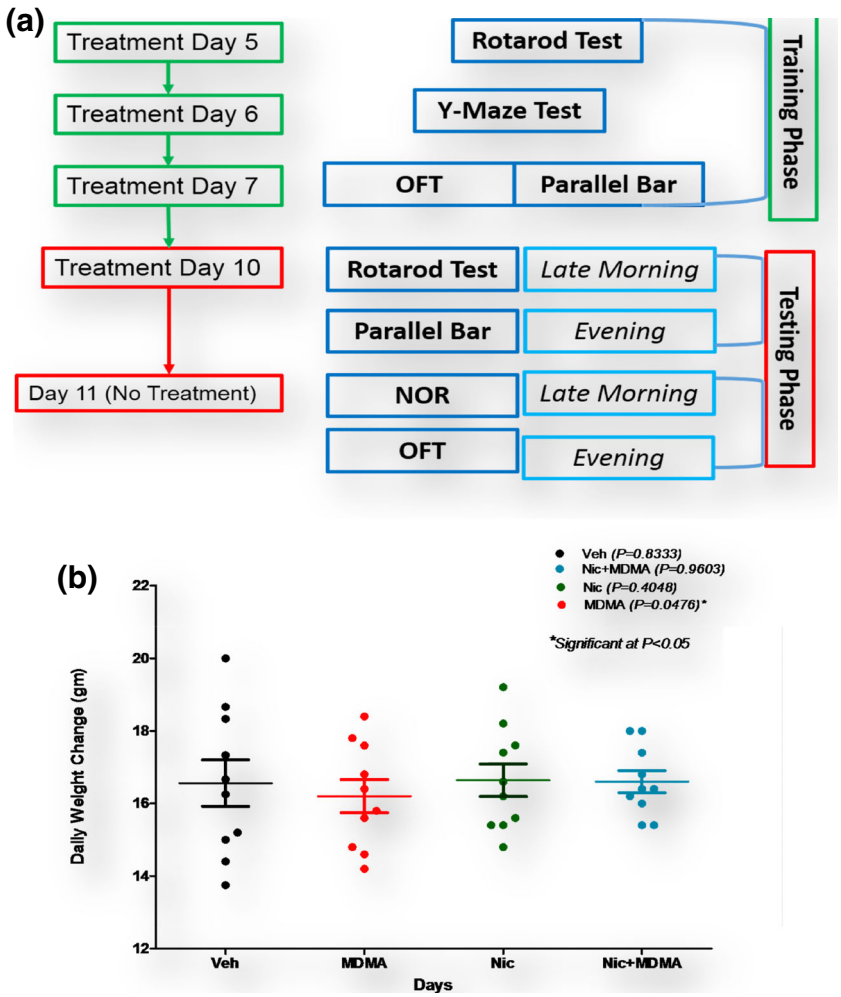


Fig. 1 **a** Schematic illustration of behavioural tests for motor and memory function **b** Scatter plot showing daily weight change for 10 days (acute treatment). The control group recorded a steady increase in body weight for the treatment duration; with a *P value* of 0.8333. MDMA treatment caused a significant decrease in body weight when compared to the control ($P = 0.0476$; *Significant at* $*P < 0.05$) at 95 % *confidence interval*. However, the Nicotine ($P = 0.4048$) and Nicotine-MDMA ($P = 0.9603$) treatment showed no significant change in body weight over this period when compared with the control. The Nicotine and Nicotine-MDMA treatment groups recorded an increase in body weight between Day 3 and 7 when compared with the control; a decline in body weight was observed thereafter (Day 8–10)

Parallel bar test Two metal bars, each 1 m long and 2 mm thick, were placed on an elevated wooden platform 60 cm high above a padded floor. The metal bars were equidistant to each other (3 cm apart) throughout the length of the apparatus. Subsequently, the animal was placed perpendicular to the axis of the metal bars at the midpoint of their length (0.5 m mark). With the use of a timer, the duration (seconds) for each animal to complete a 90° turn on the double bar was recorded (latency of turn; LOT). Similarly, we determined the time taken for an animal to cover 0.5 m to one end of the bars. In subsequent analysis, we compared the LOT and the time taken to cover 0.5 m on the metal bars (as described earlier) for the control and treatment groups.

Y-Maze test for motor function The Y-Maze set up was placed in an isolated sound-proof room with adequate illumination. The animals (mice) were gently placed into the central triangle of the Y-Maze and allowed to explore the three arms A, B and C of the maze 10 min following which the video was analysed to determine the frequency of arm entries for each arm of the maze. Subsequently, we determined the frequency of alternation between the three arms of a Y-Maze (*A*, *B and C*) as a measure of exploratory motor function associated with spatial working memory. The total number of arm entries/animal/group was determined and compared for the control and treatment groups.

Y-Maze test for memory function Using the procedure described above for Y-Maze, we estimated spatial working memory function for the control and treatment groups. The memory index was presented as a function of the percentage alternation between successive arms of the maze (number of Right Decisions/total arm entries−2 × 100).

Novel object recognition test (NOR) This was done to measure the non – spatial working memory in the control and treatment groups. Each animal was subjected to two tests T_1 and T_2 for 4 min each; an inter-trial time of 60 min was observed between T_1 -T_2. In T_1 the animals were allowed to explore two identical objects for 4 min following which the animals were returned to their cages. The animals spent the inter-trial interval in their cages following which they were

returned to the testing area for the second test T_2 (4 min). In T_2, one of the old objects (familiar objects) was replaced by a new object (novel object). The animals explored the objects for 4 min while the scientists recorded the time spent exploring the old and novel objects respectively. The memory index was calculated thus;

$$\frac{\text{Time Spent on New Object}}{\text{Time spent of New object} + \text{Time spent on Old Object}} \times 100$$

Oxidative stress and lipid peroxidation markers Whole brain tissue was preserved in cold 0.25 M Sucrose at 4 °C and homogenized using a mortar and pestle. Subsequently, the tissue homogenates were centrifuged at 3000 rpm and the supernatant was collected for colorimetric assay of GPx (glutathione peroxidase), SOD (superoxide dismutase), MDA (Malondialdehyde) and ALP (alkaline phosphatase) using the appropriate kits. All assay protocols were in accordance with the manufacturer's specifications and guidelines.

Oxidative protein damage The total protein content in the brain homogenate was measured using the Biuret method (Kingsley 1939) to determine the extent of oxidative protein damage in animals treated with Nicotine, MDMA and Nicotine + MDMA.

Histology The animals were anaesthetized with Ketamine (100 mg/Kg) and Diazepam (5 mg/Kg) (intraperitoneal) following which a transcardial perfusion was done through the left ventricle using normal saline. Subsequently, a freshly prepared perfusion fixative [90 ml of 10 % Formalin and 90 ml of Picric acid] was injected gradually through the left ventricle with the animal inverted (gravity) to percolate the cranial cavities and cerebral blood vessels. The whole brain was then removed and fixed overnight in the perfusion fixative; following which it was transferred into a cryopreserving fixative [240 ml of 10 % Formalin and 30 % Sucrose] at 4 °C for 48 h. Coronal (whole brain) sections were obtained using stereotaxic markings to expose the various neural systems of interest. Tissue sections taken at 0.84 mm anterior to the bregma revealed the motor cortex (M1), the striatum (CPu) and the medial forebrain bundle (mfb). At 1.71 mm anterior to the bregma, the various part of the hippocampus were seen; CA1, CA2, CA3 and dentate gyrus (DG). The amygdaloid area and anterior amygdaloid nucleus (AAn) were shown at 0.72 mm anterior to the bregma. Tissue processing was done routinely to obtain paraffin wax embedded sections stained in Haematoxylin and Eosin.

Cell count Histological images were acquired using Cameroscope (5.1 MP) connected to an Olympus Binocular Research Microscope (Olympus, New Jersey, USA. Subsequently, the images were rendered in grayscale using Image J (NIH, USA). The cells were counted at different microscopic fields ($n = 7$) for $n = 5$ sections for all groups using the method of Going (1994). Cell count score was analysed in One-way ANOVA (with Tukey post-hoc test) and expressed in a bar chart (mean) with error bar (SEM).

Statistical analysis For the body weight, brain weight, behavioural studies, cell count and enzyme assays, data were analysed in one way ANOVA, with Bon Ferroni post-hoc test. The outcome for the control, Nicotine, MDMA and Nicotine + MDMA treatments were compared and presented in a bar chart (mean) with error bars (standard error of mean). The values were considered significant at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Results

Body weight

The effect of Nicotine, MDMA and Nicotine + MDMA treatment on the body weight was measured daily for a duration of 10 days. No significant change in body weight was recorded for the Nicotine ($P = 0.4048$) and Nicotine-MDMA ($P = 0.9603$) treatment groups. However, the MDMA ($P = 0.0476^{*}$) treated animals recorded a significant decrease in body weight when compared with the control ($P = 0.8333$) at 95 % confidence interval ($^{*}P < 0.05$). Generally, a fluctuation in body weight was seen in the treatment groups while the control recorded a steady increase in body weight for this duration (Fig. 1b). Between Days 3 and 8, the control group showed a steady rise in body weight; similarly, Nicotine and Nicotine-MDMA treatment recorded an increase in weight higher than that of the control. During this phase, the MDMA treatment group recorded a sharp decline in body weight when compared with the control, Nicotine and Nicotine-MDMA treatments (Fig. 1b).

Motor function and coordination

Rotarod test for motor function The latency of fall (LOF) was determined to measure motor function in the treatment and control groups after prolonged treatment with Nicotine, MDMA and Nicotine + MDMA. The Nicotine treatment group recorded the most significant increase in motor function when compared with the control ($P < 0.05^{*}$). Although no significant change in LOF was recorded when MDMA was compared with Nicotine treatment, however, the Nicotine treatment groups recorded a significant LOF versus the Nicotine-MDMA treatment ($P < 0.01^{**}$). Thus, nicotine treatment improved motor function (increased LOF) when compared with the control and other treatment groups (Fig. 2).

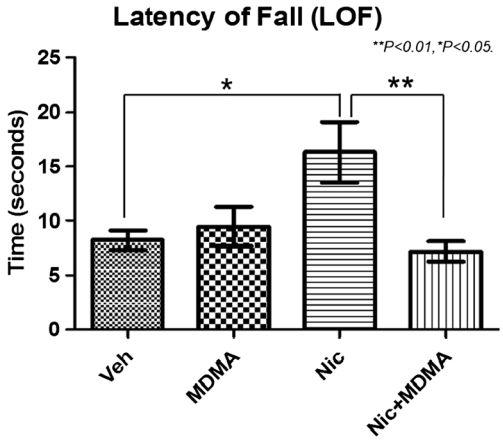


Fig. 2 Rotarod test for motor function; latency of fall (LOF). The highest LOF was recorded in the Nicotine treated animals ($P < 0.05$) when compared with the control. MDMA and Nicotine-MDMA treatments caused no significant change in LOF when compared with the control. Although no significant change was seen when Nicotine was compared with MDMA treatment, however, Nicotine treatment caused a significant increase in motor function (LOF) when compared with the Nicotine-MDMA treatment ($P < 0.01$**)

Parallel bar test for motor coordination The time taken for an animal to turn on two raised parallel bars was analysed to determine the latency of turn (LOT; motor coordination). Similar to our observations in the Rotarod test, the Nicotine treatment group had a LOT value higher than the control ($P < 0.05$*) while MDMA recorded a significant decrease in LOT when compared with the Nicotine treatment ($P < 0.001$*) and Nicotine-MDMA treatment ($P < 0.01$**) (Fig. 3a). In the same test, the frequency of lateral movement on the bar was determined to measure motor function in the animals. Similar to our observations in the LOT, the Nicotine treatment group showed no significant change in motor coordination while MDMA ($P < 0.01$**) and Nicotine-MDMA ($P < 0.05$*) recorded a significant decrease in motor coordination versus the control (increased speed of movement on bar; Fig. 3b). It is noteworthy, to mention that the most significant decline in motor function and coordination was seen in the MDMA treatment group.

Y-Maze test for motor function Motor activity, associated with spatial working memory, was measured as a function of total number of arm entries in Y-Maze (frequency of arm alternation). No significant change was observed when the treatment groups were compared with the control. However, the Nicotine treatment group recorded the highest number of entries and was significant when compared with the MDMA treatment ($P < 0.05$*) (Fig. 4a).

Memory and cognitive function

Y-Maze test for memory function The percentage alternation between the arms of the Y-Maze was analysed to determine the spatial working memory for the control and treatment groups. No significant change was seen when a treatment group was compared with other treatment groups and control (Fig. 4b).

Novel object recognition This was done to measure non – spatial working memory in treated animals (10 days treatment duration). MDMA, Nicotine and Nicotine-MDMA treatments caused a decline in memory function when compared with the control. Treatment with MDMA caused a significant decrease in memory function when compared with the control ($P < 0.001$). Interestingly, the Nicotine treatment group showed a less significant decline in memory function versus the control ($P < 0.05$*) when compared with MDMA versus the control ($P < 0.001$***). Similar to the outcome of MDMA treatment, when Nicotine was co-administered with MDMA, a decline in memory function was also observed versus the control ($P < 0.01$**) (Fig. 5a). Thus, we inferred that MDMA administered singly or combined with Nicotine facilitated a decline in memory function.

Oxidative stress markers

Total protein content MDMA or Nicotine treatment caused an increase in protein content in the brain tissue homogenate when compared with the control ($P < 0.001$). No significant change in total protein was seen when Nicotine was compared with MDMA; similarly, a combined Nicotine-MDMA treatment caused no significant change versus the control. In addition, Nicotine-MDMA total protein content was lower than the values recorded for either Nicotine or MDMA treatment ($P < 0.001$) (Fig. 6a). We deduced from these outcomes that Nicotine or MDMA treatment increased total protein content in brain tissue while a combined Nicotine-MDMA treatment had no effect on protein content after a prolonged treatment duration.

Oxidative stress Nicotine treatment caused a significant increase in percentage superoxide dismutase (SOD) when compared with the control ($P < 0.05$) while MDMA and Nicotine-MDMA caused no change in SOD expression versus the control. These findings suggests that Nicotine treatment induced mitochondrial stress and radical formation (increased SOD) while MDMA did not increase radical level for this period of treatment. Going further, co-treatment of Nicotine-MDMA caused no significant increase in SOD levels (radical); suggesting that MDMA blocks the radical formation pathway utilized by nicotine (Fig. 6b).

Lipid peroxidation Although, Nicotine-MDMA treatment caused no significant change in SOD level and total protein when compared with the control, this treatment group was characterized by an increase in neuronal lipid peroxidation

versus the control ($P < 0.05$) (Fig. 6c). Subsequent analysis involving GPx assays show that Nicotine-MDMA treatment also increased the level of GPx; further confirming an increase in the rate of lipid peroxidation (Fig. 6d). Similar to our observation in MDA analysis, the MDMA or Nicotine treatment group recorded a lower GPx value (reduced rate of lipid peroxidation) when compared with the control ($P < 0.05$) (Fig. 6d).

ALP After Nicotine treatment, no significant change in ALP level was observed while MDMA increased ALP when compared with the control ($P < 0.001$). Although, nicotine did not significantly alter ALP levels, a combined Nicotine-MDMA treatment increased ALP significantly when compared with MDMA ($P < 0.001$), Nicotine ($P < 0.001$) and the control group ($P < 0.001$) (Fig. 6e).

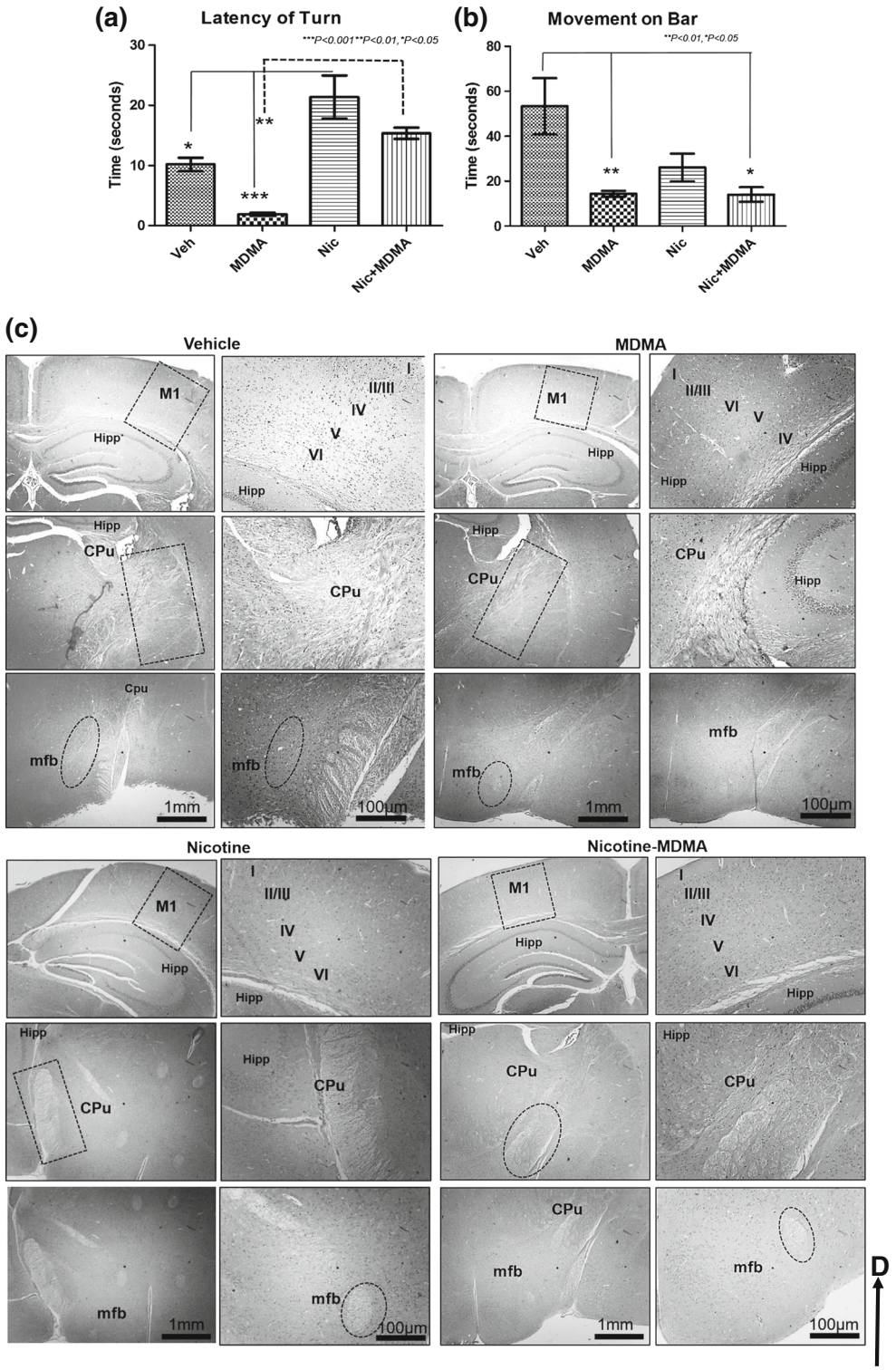
In summary, Nicotine-MDMA treatment caused no notable change in total protein/SOD but caused an increase in lipid peroxidation (MDA/GPx/ALP). On the other hand Nicotine increased total protein and SOD while MDMA increased brain protein but had no significant effect on SOD. Interestingly, both Nicotine and MDMA caused a reduction in MDA and had no significant effect on GPx expression level. Thus Nicotine or MDMA treatment elevated total brain protein but had no effect on lipid peroxidation. Furthermore, Nicotine-MDMA increased ALP significantly versus the control and other treatment groups; this was also associated with significant effects on lipid peroxidation (increased MDA/GPx) (Table 1).

Brain morphology

Motor neural axis Behavioural analysis (*Rotarod*, *Y-Maze and Parallel bar tests*) show that MDMA treatment reduced motor function while Nicotine increased motor function for the same treatment duration. Going further, a combined Nicotine-MDMA treatment had a less significant effect on motor function when compared with the control and MDMA treatment (Figs. 2 and 3). Sequel to these findings, we examined the general morphology of the motor neural axis (*M1*, *CPu* and *mfb*) to show associated structural alterations (histology) after treatment with Nicotine, MDMA and Nicotine + MDMA. MDMA treatment induced degenerative changes and cell loss in the M1 when compared to the control ($P < 0.01$). In addition, prominent loss of fibres was observed between the hippocampus and the Layer 6 of the M1 (Fig. 3c-MDMA). Similarly, MDMA treatment caused striatal degeneration which was seen as a reduction in CPu cell count per unit area (Fig. 3c, d MDMA). By contrast, Nicotine treatment caused an increase in cell count in the CPu ($P < 0.01$); although it decreased M1 cell count ($P < 0.001$) when compared with the control (Fig. 3d). Similar to the observations in Nicotine treatment, a combined Nicotine-MDMA treatment caused a reduction in M1 cell count ($P < 0.001$) but increased CPu count ($P < 0.001$) versus the control (Fig. 3c, d). Subsequent analysis of behavioural data (Nicotine and Nicotine + MDMA) revealed that an increase in CPu cell count was associated with an improved motor function despite cell loss in the M1. This was further supported by the observations in MDMA treatment. A decline in cell count for M1 and CPu was recorded in this group (Fig. 3c, d); the animals also showed a decline in motor function versus the Nicotine and Nicotine-MDMA treatment groups (Fig. 3a, b).

Hippocampus In behavioural analysis, MDMA, Nicotine and Nicotine-MDMA treatment groups showed no significant change in spatial working memory (*Y-Maze*; Fig. 4b) but recorded a significant decline in non-spatial working memory function (NOR; Fig. 5a) when compared with the control. Histological analysis of the hippocampus showed that MDMA treatment induced a reduction in CA1 ($P < 0.01$) and CA2 ($P < 0.001$) cell counts (degeneration) when compared with the control. Interestingly, the CA3 and DG cell counts were relatively unchanged versus the control (Fig. 5b, c MDMA). By contrast, degenerative changes (cell loss) were recorded in the CA3 ($P < 0.001$) and DG ($P < 0.001$) of Nicotine treated animals, while no significant change were recorded in CA1 and CA2 cell counts versus the control (Fig. 5b, c Nicotine). Combined Nicotine-MDMA

Fig. 3 **a-b** Parallel bar test for motor function and coordination. **a** Latency of turn (LOT) depicts the average duration in seconds taken by animals in each group to complete a turn on two raised parallel bars (motor function). The highest LOT was recorded in the Nicotine treatment group when compared with the control ($P < 0.05$*). Similarly, the Nicotine treatment recorded a significant increase in LOT when compared with the MDMA treatment- which recorded the lowest LOT ($P < 0.001$***). No significant change in LOT was seen between the Nicotine treatment and Nicotine-MDMA treatment. Interestingly, the Nicotine-MDMA treatment gave higher LOT scores when compared with the MDMA treatment group. **b** The average time taken by animals in a particular group to complete movement to either ends of the elevated parallel bars. Decreased motor coordination was observed in the MDMA ($P < 0.01$) and Nicotine-MDMA treated groups when compared with the control ($P < 0.05$). In this test no significant difference in motor coordination was observed between the MDMA, Nicotine and Nicotine-MDMA treated animals. **c-d** Morphology of the motor cortex (M1), striatum/basal ganglia (CPu) and the medial forebrain bundle (mfb). **c** Cell loss was observed in the M1 of the treatment groups; MDMA: $P < 0.01$, Nicotine: $P < 0.001$, Nicotine-MDMA: $P < 0.001$ when compared with the control. This was also associated with degenerative changes between layer 6 (VI) and the hippocampus. The most prominent change in CPu was observed in the Nicotine-MDMA treatment group; this was seen as an increase in cell count per unit area when compared with the control and other treatment groups ($P < 0.001$). Degenerative changes were also observed in the CPu of the MDMA treatment when compared with the control and the Nicotine treatment group ($P < 0.01$). *Hipp* hippocampus, *CPu* striatum, *M1* motor cortex, *mfb* medial forebrain bundle. *Scale bars 1 mm and 100* μm. **d** Cell count analysis (mean) and reconstruction of the M1 and CPu using Image J



treatment caused a reduction in CA1 ($P < 0.001$), CA2 ($P < 0.001$) and DG ($P < 0.05$) cell counts while the CA3 remained unchanged when compared with the control (Fig. 5b, c Nicotine-MDMA). These findings suggest selective susceptibility of the hippocampal cells and regional variation in pattern of degeneration following the various treatments. Interestingly, degenerative changes were seen in one or more regions for all treatments and translated into a decline in memory function for all treatment groups versus the control (Fig. 5a).

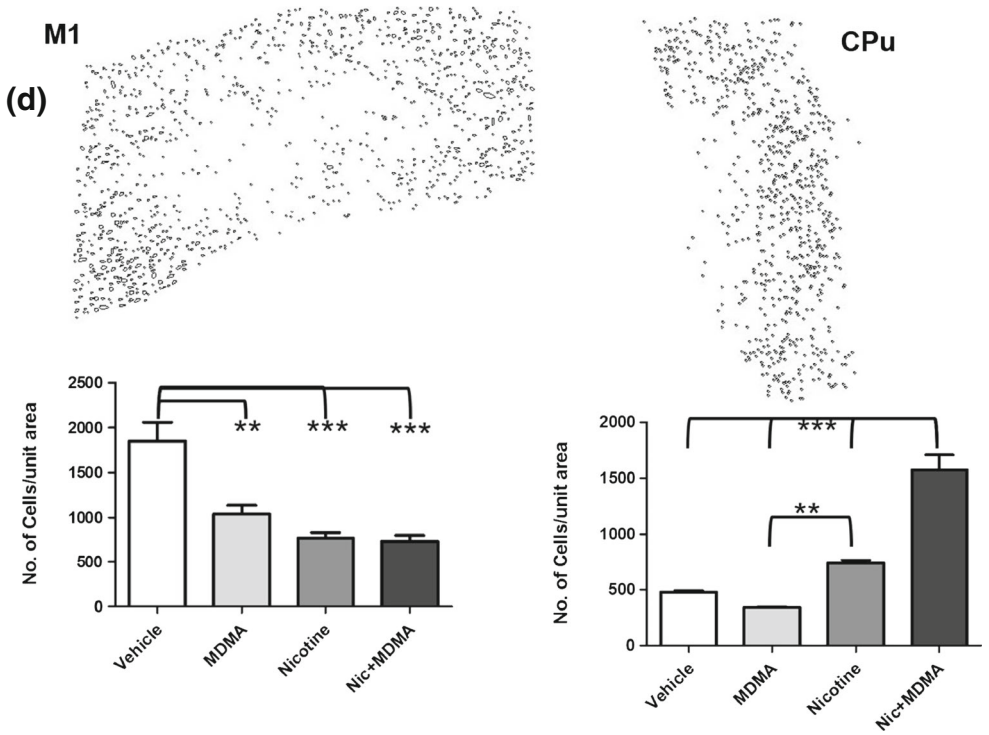


Fig. 3 (continued)

Discussion

Taken together the outcomes of this study suggests that Nicotine, MDMA and Nicotine + MDMA treatment alters the metabolism of the brain and the structure of neural systems involved in motor function (M1, CPu and mfb) and memory consolidation (hippocampus).Our findings suggests that the mechanism of tissue damage and behavioural changes in Nicotine-MDMA treatment are intrinsically linked with oxidative stress patterns peculiar to each treatment category (MDMA, Nicotine and Nicotine-MDMA). Furthermore, the observed variations in oxidative stress involved changes in brain total protein, ROS formation, phosphatase activity and brain tissue lipid peroxidation. In this study, we have shown the effects of these changes in neural system structure, motor function and memory function after a prolonged treatment period (10 days).

Behavioural changes The outcomes of this study show that Nicotine, MDMA and Nicotine + MDMA treatment

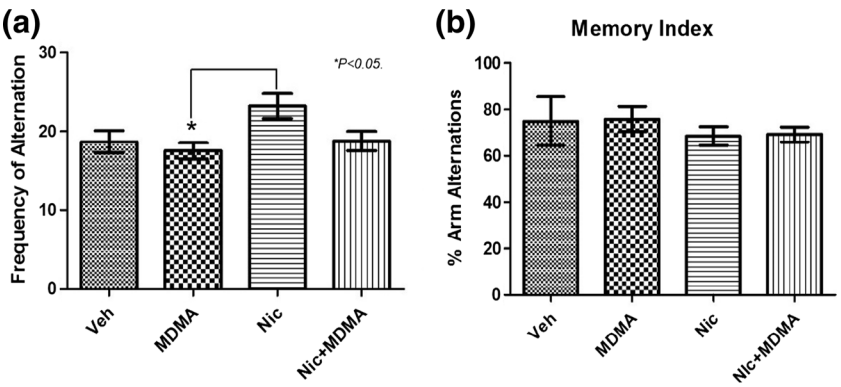


Fig. 4 **a**-**b** Motor-Cognitive function determined in Y-Maze test. **a** The total number of arm entries in the *Y-Maze* was determined to measure exploratory motor function associated with spatial memory after an acute treatment duration. No significant change was observed in exploratory motor function when the treatment groups were compared with the control. Similar to the outcomes of Rotarod and parallel bar tests for motor function, the Nicotine treated animals showed a significant exploratory motor function (number of arm entries) when compared with the MDMA treated animals ($P < 0.05$*). **b** The percentage change in Y-Maze arm alternations were plotted to determine the spatial working memory index. No significant change in spatial working memory was observed when the treatment groups were compared with the control for this treatment duration (10 days)

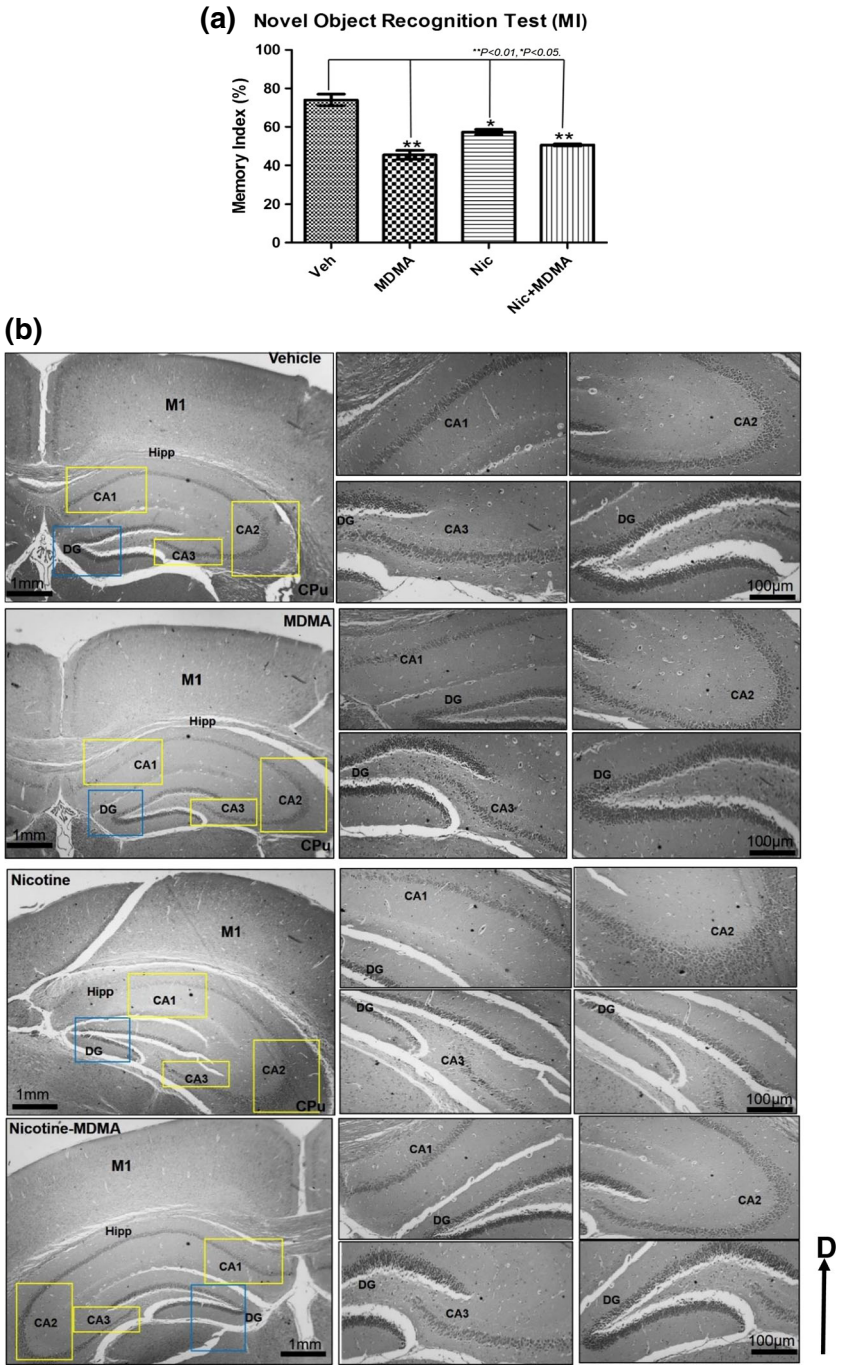


Fig. 5 **a** Novel object recognition test (recognition memory). Nicotine ($P < 0.05$*), MDMA ($P < 0.01$**) and Nicotine-Cadmium ($P < 0.01$**) treatment caused a decline in object recognition memory when compared with the control. A more significant decline in memory function was recorded in the MDMA treatment ($P < 0.01$**) when compared with the control. Similarly, when MDMA was co-administered with Nicotine, a further decline in memory function was also observed versus the control ($P < 0.01$**). Nicotine treatment caused a less significant decline in memory function versus the control ($P < 0.05$*). No significance was found when a treatment group was compared with the other treatment groups (Nicotine, MDMA or Nicotine-MDMA). **b-c** Structure of the Hippocampus; CA1, CA2, CA3 and dentate gyrus (DG). **b** MDMA treatment caused a reduction in cell count in the CA1 ($P < 0.01$) and CA2 ($P < 0.001$) regions of the hippocampus when compared with the control. Interestingly, the CA3 and DG cell count remained relatively unchanged. Nicotine treatment caused no significant change CA1 and CA2 cell count but was characterized by a reduction in CA3 ($P < 0.001$) and DG ($P < 0.001$) counts versus the control. Combined Nicotine-MDMA treatment reduced the cell count in the CA1 ($P < 0.001$), CA2 ($P < 0.001$) and DG ($P < 0.05$) while the CA3 count did not significantly change versus the control. In behavioural analysis, the MDMA, Nicotine and Nicotine-MDMA treatment groups were characterized by a reduction in object recognition memory function and is linked with reduction in cell count for one or more regions of the hippocampus. *Hipp* hippocampus, *CPu* striatum, M1 motor cortex, *DG* dentate gyrus. *Scale bars 1 mm and 100* µm. **c** Cell count analysis and reconstruction of the CA1, CA2, CA3 and DG using Image J

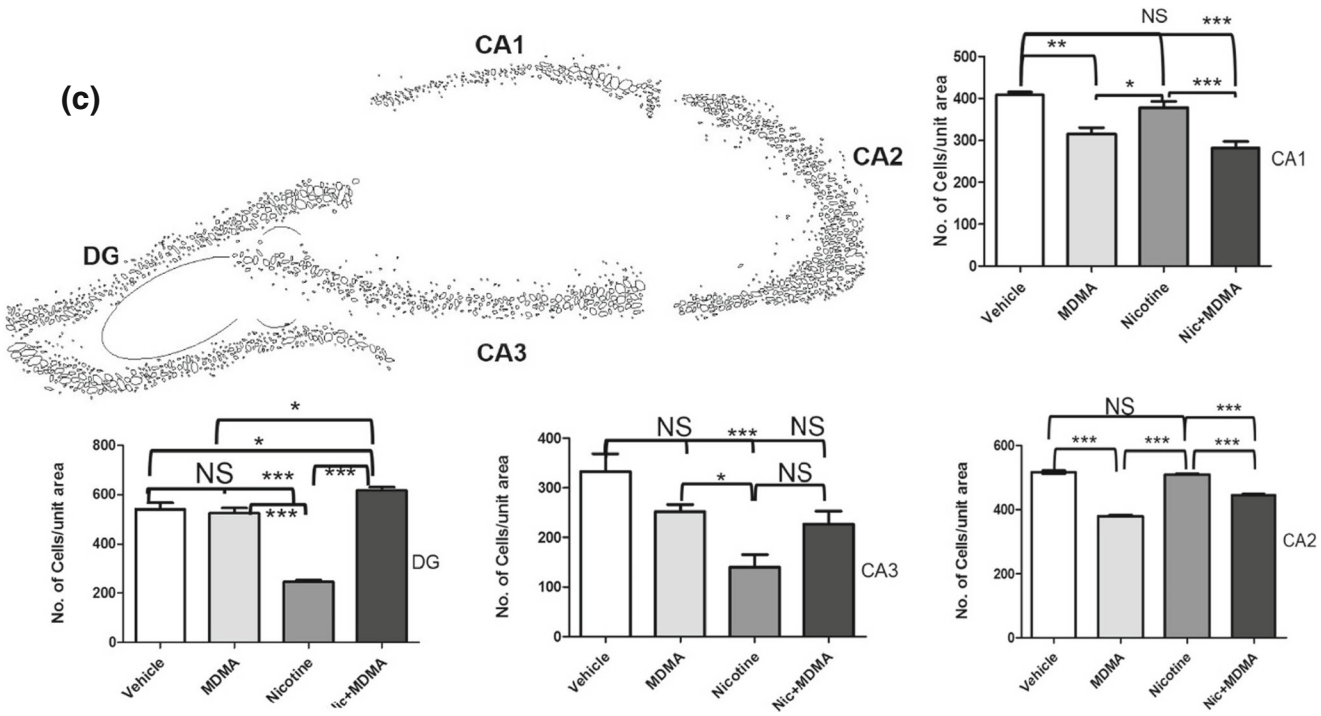


Fig. 5 (continued)

significantly induced changes in motor and cognitive functions in mice after a period of prolonged exposure. Going further, the observed behavioural changes were seen to corroborate structural changes in the various brain regions concerned with integration of motor function (CPu, M1 and mfb) and memory function (Hippocampus). Through their effects on various neurotransmitters in the central and peripheral nervous systems, MDMA and Nicotine influences neuronal activities and synaptic plasticity leading to impairments or enhancement of motor and memory related functions. The effect of Nicotine and MDMA on 5-HT, Glutamergic, Cholinergic and Dopaminergic neurotransmission is important in defining the role of these drugs of addiction in the development of neural functions in the mammalian brain during the periadolescent period and how it influences cognition and motor function in the early adolescent period of life. This period is important in neural development as it represents the final phase of synaptic consolidation of various brain networks governing these behaviours in the adolescent (Galiñanes et al. 2009).

Other studies have shown that MDMA affects synaptic plasticity at glutamergic and 5HT nerve endings in the hippocampus thereby impairing memory processing and consolidation (Capela et al. 2009; Callaghan et al. 2006; Li et al. 2006). In this study, periadolescent (P.21) mice treated with MDMA showed a significant decline in non-spatial working memory when examined in NOR in the early adolescence period (P.28-P.31; Fig. 5a). Surprisingly, MDMA caused no significant change in spatial working memory of these animals during the early adolescence (Y-Maze; Fig. 4b). Similarly, MDMA treatment significantly affected motor function in these animals. This was associated with a decline motor coordination when compared with the control and the nicotine treated animals (Figs. 2, 3a, b). On the other hand, prolonged nicotinic stimulation increased dopaminergic and 5HT activity through the brain reward systems, thus, indirectly creating an excitatory effect in the motor neural pathway (Collo et al. 2013; Scherma et al. 2012). Increased motor coordination observed in Nicotine treatment is often mediated through the central effect of Nicotine on cholinergic receptors in the brain, and its peripheral effects at neuromuscular junction and peripheral cholinergic nerve endings (Wang et al. 2014). Similar to our findings, other studies have reported an increase in motor function due to a prolonged use of nicotine (Bruijnzeel et al. 2014; Lenoir et al. 2013). However, our results show that prolonged treatment with Nicotine had no significant effect on spatial working memory (Fig. 4b; Y-Maze) but significantly reduced non-spatial working memory function ($P < 0.05$) when compared with the control (Fig. 5a; NOR). In subsequent analysis, we examined the effect of Nicotine treatment on the histology of the hippocampus and observed no change in CA1 and CA2 cell counts, but a reduction in CA3 and DG cell counts ($P < 0.001$) (Fig. 5b-Nicotine).

The effect of combined use of Nicotine and MDMA was examined on motor function and memory function. Similar to Nicotine or MDMA treatment, as discussed above, degenerative changes were seen in M1 and CPu after a prolonged

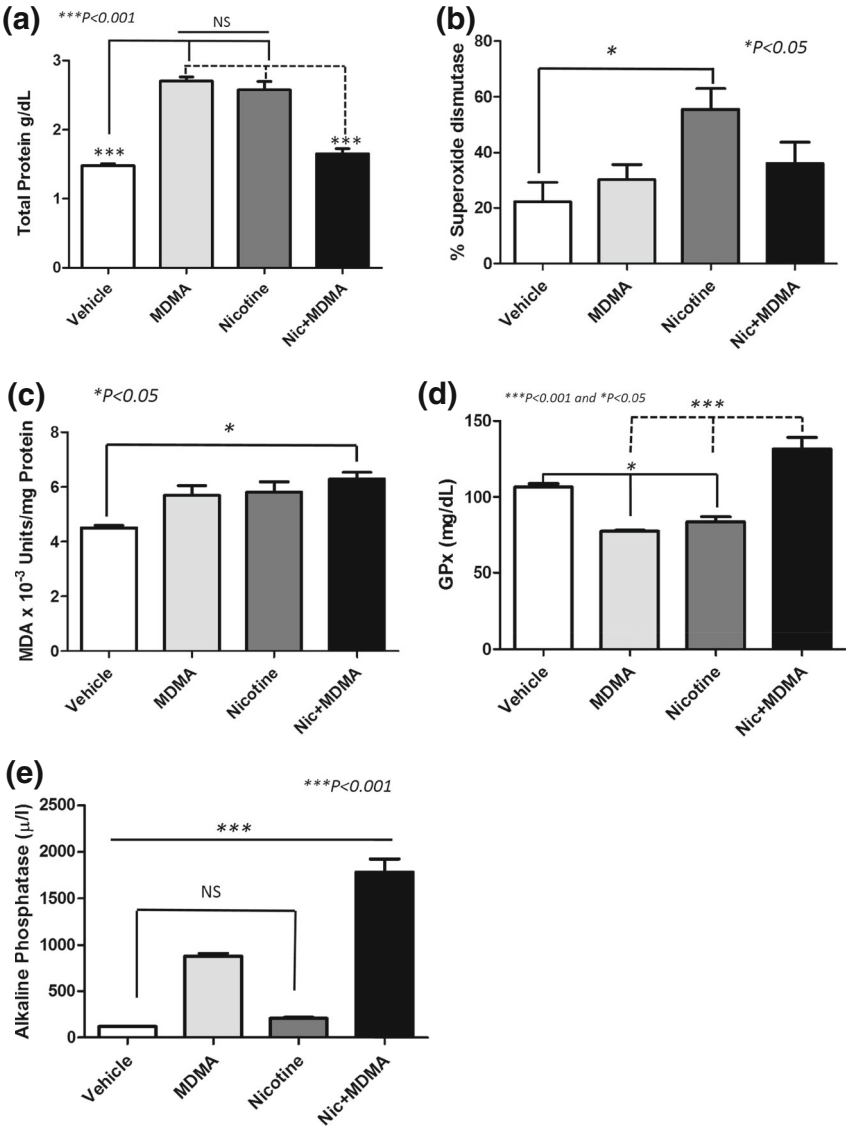


Fig. 6 **a**–**d** Colorimetric assay for oxidative stress markers and total protein content in brain tissue homogenate. **a** Total protein analysis show an increase in protein content after acute Nicotine or MDMA treatment when compared with the control ($P < 0.001$). No significant change in protein content was seen when the Nicotine-MDMA treatment group was compared with the control. In addition, the increase in total protein seen either in Nicotine or MDMA treatment was significantly higher than value recorded for the Nicotine-MDMA treatment group ($P < 0.001$). **b** An increase in superoxide dismutase was observed only in the Nicotine treatment group versus the control for the 10 days treatment duration ($P < 0.05$). MDMA and Nicotine-MDMA treatment caused no significant change in SOD when compared with the control. **c** Lipid peroxidation analysis show that neither Nicotine nor MDMA treatment caused a significant change in MDA when compared with the control. However, combined treatment with Nicotine-MDMA caused a significant increase in lipid peroxidation (MDA) when compared with the control ($P < 0.05$). **d** GPx analysis show that either MDMA or Nicotine treatment caused a significant reduction in GPx when compared with the control ($P < 0.05$). Nicotine-MDMA treatment increased GPx level when compared to the MDMA or Nicotine treatment groups ($P < 0.001$). No significant change in GPx was recorded when the Nicotine-MDMA was compared with the control. **e** Colorimetric assay for Alkaline Phosphatase (ALP). MDMA treatment significantly increased brain tissue ALP when compared with the control ($P < 0.001$). However, Nicotine treatment caused no significant change versus the control. Nicotine-MDMA combined treatment caused a significant increase in ALP when compared with the control ($P < 0.001$), Nicotine ($P < 0.001$) and MDMA ($P < 0.001$) treatment groups

treatment duration. These changes, however, correlates with a decline in motor function and coordination-similar to the pattern seen in MDMA treatment but significantly lower than that recorded by the nicotine treatment group. However, no significant change in motor function was observed when the Nicotine-MDMA treatment group was compared with the control (Figs. 2, 3a, b). Thus, Nicotine combined with MDMA treatment enhanced motor function when compared with MDMA treatment only (antagonizing effects) (Figs. 2, 3 and 4a). Gonzalez et al. (2005) and other groups have reported

Table 1 Schematic summary of ANOVA (with Bon Ferroni *post-hoc* test) comparing nicotine and (or) MDMA treatment versus the control and (or) other treatment groups

Treatment parameters	Nicotine	MDMA	Nicotine-MDMA
Total protein	***(Increase)	***(Increase)	NS
SOD	*(Increase)	NS	NS
MDA	NS	NS	*(Increase)
GPX	*(Decrease)	*(Decrease)	***(Increase)
ALP	NS	*(Increase)	***(Increase)

Statistical significance at 95 % confidence interval; *$P < 0.05$ and ***$P < 0.001$

similar effects in experimental models of Nicotine and MDMA treatment (Marston et al. 1999; van Wel et al. 2012).

Morphology In this study, histological examination of the motor neural system after MDMA treatment revealed a reduction in cell count in the M1 ($P < 0.01$) and CPu ($P < 0.01$) (Fig. 3c, d-MDMA). Similarly, loss of fibres was seen between the layer 6 of the M1 and the hippocampus (Fig. 3c, d) after MDMA treatment. Subsequent analysis of behavioural tests for motor function suggests that these degenerative changes led to a reduction in motor coordination when the MDMA treatment was compared with the control or Nicotine treatment (Figs. 2, 3a, b). Similarly, MDMA also caused structural changes in various parts of the hippocampus which resulted in a decline in memory function (Fig. 5a–c; MDMA). A reduction in cell count was observed in the CA1 ($P < 0.01$) and CA2 ($P < 0.001$) while the CA3 and DG showed no change in cell count after a prolonged treatment period (Fig. 5c, d).

Some of the observed histological changes in the CPu elucidates the effect of nicotinic stimulation of motor function and coordination. Structural changes post nicotine treatment involved an increase in CPu cell count ($P < 0.01$) when the nicotine treated group was compared with the MDMA treatment group (Fig. 3c, d Nicotine). In support of these observations, we also recorded an increase in motor function and coordination when nicotine treated mice were examined in Rotarod, Y-Maze and Parallel bar tests for motor function (Figs. 2, 3a, 4b). Although Nicotine treatment improved motor function when combined with MDMA, this combination, however, caused a significant decline in memory function (Fig. 5a). This was in agreement with the outcome of memory function tests for Nicotine or MDMA treatment as both treatments caused a decline in memory function after a prolonged treatment (Fig. 5a; Nicotine, MDMA). Similar to the preposition by Bakhtiar et al. (2014) and Gulley and Juraska (2013), structural changes in the hippocampus were also associated with the decline in memory function seen in the Nicotine, MDMA and Nicotine + MDMA treatment group. Nicotine-MDMA treatment was characterized by a reduction in CA1 ($P < 0.001$), CA2 ($P < 0.001$), DG ($P < 0.05$) cell counts while the CA3 was relatively unchanged when compared with the control (Fig. 5c). Our finding here in suggests that MDMA treatment caused detrimental changes in neural structure and behaviour when compared with the Nicotine-MDMA treatment group.

Biochemical assay Aside the structural changes seen in the various brain regions, we recorded variation in tissue metabolism and oxidative stress pattern induced by Nicotine, MDMA and Nicotine + MDMA treatment. Nicotine treatment caused an increase in total brain protein when compared with the control group (Fig. 6b). To our surprise, the level of ROS formation (%SOD; Fig. 6b) did not increase significantly when compared with the control. As expected, from these findings, an insignificant ROS formation was associated with no significant change in lipid peroxidation markers (GPx and MDA) versus the control (Fig. 6c, d). Interestingly, MDMA treatment caused a significant increase in ALP when compared with the control, thus signifying certain level of nuclear damage and abnormal membrane transport in the cells after MDMA treatment. These findings suggest that MDMA treatment induced degenerative changes through excitotoxicity and increase in brain total protein rather than lipid peroxidation. Although an increase in brain protein (measured in CSF) has been attributed to inflammatory response (Orio et al. 2010), however, several other markers are required to confirm inflammation in oxidative stress (in addition to an increase in total brain protein). As a result of oxidative stress induced by MDMA, degenerative changes were seen in the various parts of the brain (Figs. 3c, 5b and 6c). This also translated into a decline in motor and memory function in the animals (Figs. 5a and 3a).

Similar to our observations in MDMA treatment, intraperitoneally administered Nicotine increased the brain total protein (Fig. 6a). However, contrary to MDMA treatment outcomes, radical formation, depicted by an increase in %SOD in brain tissue homogenate, was highly significant in Nicotine treatment when compared with the control. Despite the observed increase in ROS formation (%SOD), no change in lipid peroxidation (MDA/GPx) was observed when compared with the control (similar to the MDMA treatment) (Fig. 6a–d). In contrast to MDMA treatment, no significant change in ALP was seen in the Nicotine treatment group when compared with the control. Thus, we deduced that Nicotine treatment increased protein content and ROS while ALP and lipid peroxidation markers remained relatively unchanged. Similar to our observations in MDMA treatment, Nicotine also induced structural

alterations in the motor area and hippocampus; causing an increase in motor function and a decline in memory function.

Ultimately, a combined Nicotine-MDMA treatment reduced the total protein content and SOD level. In addition, Nicotine-MDMA treatment significantly increased the extent of lipid peroxidation depicted by an increase in GPx and MDA after a prolonged treatment. This was also linked with an increase in ALP; depicting a wide range membrane damage in combined treatment (Table 1). Contrary to what was observed in Nicotine or MDMA treatment, a combined Nicotine-MDMA treatment created opposing effects on lipid peroxidation and brain total protein. A decline in brain total protein was observed when the Nicotine-MDMA treatment was compared with Nicotine or MDMA treatment ($P < 0.001$) (Fig. 6a). Interestingly, this Nicotine-MDMA treatment significantly increased MDA ($P < 0.05$) and GPx ($P < 0.001$) (lipid peroxidation markers) when compared with the control (Fig. 6c, d). From these outcomes, we deduced that co-treatment with Nicotine-MDMA induced degeneration through lipid peroxidation (MDA/ALP/GPx) while Nicotine or MDMA treatment caused an increase in brain total protein. As a result of the induced oxidative stress, the most prominent structural change was observed in the CPu. Surprisingly, this treatment group showed no significant change in motor function versus the control but recorded a decline in motor function when compared with the Nicotine and MDMA treatment (Figs. 2 and 3).

Conclusion

In summary, these treatments induced oxidative stress and cellular damage through various mechanisms. Nicotine or MDMA treatment increased total brain protein but had no significant effect on lipid peroxidation while Nicotine-MDMA did not alter brain protein but increased the level of lipid peroxidation (GPx and MDA). Nicotine treatment increased motor function; MDMA reduced motor function while Nicotine-MDMA did not significantly change motor activity and coordination. Going further, the most prominent change in the motor neural axis was observed in the CPu and mfb. Nicotine, MDMA and Nicotine-MDMA treatments significantly reduced memory function and caused structural changes in one or more parts of the hippocampus.

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Conflict of interest The authors hereby declare there is no conflict of interest associated with this study or any of the procedures and materials used for the purpose of the study.

Author contributions PAA, PDS, AOI and BPO initiated the research and conducted the behavioural test for motor and memory function. OMO, BJL and OOB processed the tissues for histology and biochemical assays. In addition, OMO and AOI analysed the results while OMO, BJL and MOB did the cell counting and analysis and wrote the manuscript. OMO, PDS and PAA did the final proof and arrangement as appropriate.

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