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Original Research Article

Effects of the Combination of Caffeine, Nicotine, and 3,4 Methylenedioxymethamphetamine (MDMA) on the Hippocampus of Experimental Wistar Rats

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ABSTRACT

Caffeine, nicotine, and MDMA are generally referred to as psychoactive substances which contain chemical substances that change the function of the brain and result in alteration of mood, perception, consciousness, cognition, or behavior. These substances are both rewarding and positively reinforcing which indicates a potential for inducing addiction. The hippocampus is an important part of the brain whose primary function is learning and memory. This study aimed to investigate the impact of combining caffeine, nicotine, and MDMA on the hippocampus of Wistar rats. A total of 32 Wistar rats were randomly divided into four groups, each consisting of eight animals. Group A served as the control and received normal saline (2 ml/kg.bw). Group B received a combination of caffeine (100 mg/kg) and nicotine (50 mg/kg), Group C received a combination of caffeine (100 mg/kg) and MDMA (10 mg/kg), and Group D received a combination of nicotine (50 mg/kg) and MDMA (10 mg/kg). The administration of substances continued for a duration of 21 days. The result showed that there was a significant increase in the level of neurotransmitters, Neuroarchitecture using different staining techniques and neurostructural changes were observed on the hippocampus, especially in the dentate gyrus across the co-administered groups. The co-administration of caffeine, nicotine, and MDMA did not cause any extensive hippocampal degeneration or localized neurodegeneration, however, there was an increase in the number of cells in the dentate gyrus which suggested a possible neurogenesis. Neurobehavioural analysis showed impairment in learning and memory.

***Keywords*:** Learning, memory, hippocampus, MDMA, caffeine and nicotine.

Introduction

Psychoactive substances are compounds that influence the nervous system, resulting in shifts in consciousness, alterations in perception, and fluctuations in mood. These substances are often used to seek pleasure or alleviate discomfort, but they can also lead to physical dependence and tolerance, requiring larger doses over time to achieve the desired effects. [1] Regular consumption of these substances can result in an increased tolerance and dependence, and discontinuation of their use may trigger symptoms like sleep disturbances, shaking, nausea, increased blood pressure, heightened anxiety and sadness, faster heart rate, and seizures. [2]

Psychoactive drugs are categorized into three main groups: depressants, stimulants, and hallucinogens. Depressants decrease both physical and mental functions, represented by substances like alcohol, narcotics, barbiturates, and tranquilizers.

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Stimulants, on the other hand, boost central nervous system activity, encompassing drugs like amphetamines, cocaine, caffeine, ecstasy, and nicotine. Hallucinogens alter perceptions and induce unusual visual experiences; examples include marijuana, lysergic acid diethylamide (LSD), and similar substances. [3,4] Nicotine and caffeine stand out as the most widely used psychoactive substances globally. [5] Studies have revealed that nicotine adversely affects the body's genetic composition. [6]

Caffeine enhances energy levels and attentiveness and is found in various sources such as coffee, tea, cola, chocolate, and beverages. [7] Studies indicate that short-term caffeine intake enhances cognitive recognition. [8] However, prolonged consumption of caffeine at low doses (0.3 g/L) has been linked to a decline in hippocampus-related learning abilities and the impairment of long-term memory. [9] Research suggests that the activation of nicotine receptors by low doses of nicotine causes apoptotic cell death in primary hippocampal progenitor cells; [10] the impact of nicotine on the brain varies between different age groups, such as adolescents and adults, with evidence pointing to a heightened sensitivity to the rewarding effects of nicotine in certain age brackets. [4] Prolonged MDMA exposure in humans has been found to cause significant neurodegeneration in serotonergic axon terminals in areas such as the striatum, hippocampus, prefrontal cortex, and occipital cortex, [11] even low doses of MDMA have been associated with adverse changes in brain microvasculature and white matter, impacting neuroplasticity. [12] Long-term users exhibit worldwide decreases in gray matter volume, thinning of the parietal and orbitofrontal cortices, and reduced hippocampal activity. [13] Studies have demonstrated that caffeine can amplify the effects of nicotine, either through addictive mechanisms or synergistically, affecting memory function, motor skills,

and certain biochemical markers linked to brain function in male mice. [14] While both caffeine and nicotine independently increased heart rate, their combination seemed to have opposing effects. [15] A significant concern associated with MDMA misuse involves its concurrent use with other stimulants such as alcohol, amphetamines, cocaine, cannabis, or nicotine. [16] MDMA exhibits an affinity for neuronal nicotinic acetylcholine receptors (nAChRs) and functions as a partial agonist of alpha7 nAChR. [17] MDMA impacts locomotor activity in mice sensitized to nicotine. [18]

Materials and Methods

Ethical Considerations

The study adhered to the guidelines outlined in the National Institute of Health Guide for the care and use of laboratory animals, [19] and all procedures and experiments were conducted in compliance with these regulations. Approval for the study protocol was obtained from the Babcock University Health Research Ethics Committee (BUHREC) under approval number (BU-218444).

Animal Care and Housing

Thirty-two (32) healthy Juvenile Wistar rats, weighing approximately (110-130g), were obtained from and accommodated in the Babcock University Animal House, situated at Babcock University in Ilishan Remo, Ogun State, Nigeria. The rats were randomly assigned to four (4) groups, each containing eight (8) animals, and placed in well-ventilated large plastic cages. They were kept under natural light-dark cycles at room temperature and provided with unrestricted access to food and water. Prior to the commencement of the experiment, a seven-day acclimatization period was observed for the animals.

Psychoactive substance procurement and preparation

The psychoactive substances (nicotine, caffeine, and MDMA) were purchased in crystalline from Sigma-Aldrich, U.S.A. Caffeine was administered at 100 mg/kg, nicotine at 50mg/kg, and MDMA at 10mg/kg.

Experimental Design and Drug Administration

The experiment utilized 32 test subjects, which were divided into four (4) groups, denoted as A, B, C, and D, with each group containing 8 animals (n=8). Group A served as the control and received distilled water at a dosage of 2 ml per kg of body weight. Group B was administered caffeine at a dose of 100 mg/kg and nicotine at a dose of 50 mg/kg. Group C received caffeine at 100 mg/kg and MDMA at 10 mg/kg, while Group D was given nicotine at 50 mg/kg and MDMA at 10 mg/kg. The administration of the substances lasted for 21 days and was carried out orally using a cannula.

Neurobehavioral Assessments

On the 19th day of the experiment, anxiety and stress levels of the animals were evaluated using the elevated plus maze. This maze is widely utilized in rodent neurobehavioral studies and has been validated for assessing the anxiolytic effects of pharmacological substances and steroid hormones, as well as for elucidating the brain regions and mechanisms involved in anxiety-related behaviors. [20]

The EPM consisted of two open arms (50 × 10cm), opposite each other, crossed by two enclosed arms (50 × 10× 40cm) with an open roof. [21] In other words, it has four arms supported by a stand that raises the arms above the ground level and sizes that could vary for various murine types and sizes especially the rat and the mouse. The testing area, where the maze is located is prepared by cleaning, drying, and proper positioning of the video tracking device. Each rat that underwent the test was positioned at the junction of all four arms facing an open arm. The entry of each animal into each arm was observed and recorded for 5 minutes; immediately after every rat, the maze was cleaned with 70% alcohol to prevent cues for the new animal. Other etiological parameters are also considered and these may include head dips, rears, and stretched-attend postures. Animals with lower levels of anxiety spend more time in the open arm.

The Barnes maze, employed to assess alterations in spatial learning and memory, comprises a circular platform with 20 openings arranged around its perimeter, illuminated by overhead lighting. Distinct visual cues like colored patterns and shapes are strategically positioned within the maze's vicinity. The primary objective of this maze is to gauge the rat's capacity to learn and retain the positions of these distant visual cues within the testing environment. [22] The Barnes maze was conducted in two phases: adaptation and spatial acquisition.

The Adaptation phase was characterized by a training and adaptation period from day 7 to day 9; The rat was placed in a start chamber in the middle of the brightly lit maze for 10 seconds, then the chamber was gently lifted and the rat was carefully guided towards the escape box to not stress the rat, after 2 minutes the rat was successfully in the escape box. The bright light was then turned off and the rat was allowed to stay in the box for 3 minutes. The training and adaptation period was from day 7 to day 9.

For the spatial acquisition phase (day 11), the maze was then cleaned with 70% ethanol before the commencement of the acquisition period and the maze was turned on its central axis to control the possibly remaining odorless. The rat was then placed in the middle of the maze within a chamber that was removed after 10 seconds and then the rat was allowed to explore the maze for 3 minutes. During the 3 minutes, the number of primary errors total errors, total errors, and primary latency was measured again and this was done to all the rats in each group. The experiment was recorded using a webcam for further analysis.

Animal sacrifice and tissue processing

The experimental animals were sacrificed 12 hours after the last administration on day 21 by cervical dislocation. The skulls of the rats were opened and the whole brain tissue extracted weighed. For each group, five (5) whole brain tissues were fixed in 10% formol saline for histology and immunohistochemistry while the other three (3) were homogenized for enzymes and neurotransmitter assays.

Histological and Immunohistochemical Analysis

Following four days of brain tissue fixation, a thin coronal section of the hippocampus was prepared at the level of the optic chiasm and subjected to rapid routine tissue processing. This section was then stained with Hematoxylin and Eosin to illustrate the general histological appearance, Luxol fast blue to visualize myelin, and Cresyl fast violet to highlight the Nissl substance within neuronal cytoplasm. Immunohistochemistry was performed on the hippocampus using GFAP (glial fibrillary acidic protein) to detect astrocytic reaction. The staining protocol for Hematoxylin and Eosin followed the established procedure outlined in Adetunji *et al.*, 2020.

The mounted tissues were deparaffinized, cleared in three different solutions of xylene, dehydrated by using descending graded concentrations of alcohol (100% and 95%) and water, and then stained with hematoxylin for ten (10) minutes. Afterward, the slides were rinsed under running water and then differentiated in 1% hydrochloric acid alcohol solution; the slides were dipped in the alkaline bluing agent and rinsed again under running water for five (5) minutes. The tissues were counterstained with 1% eosin stain and then dehydrated in increasing concentrations of alcohol. The tissues were then mounted and coverslips were placed.

Cresyl Fast violet

The reagents used were 70% and 95% ethanol concentration, differentiation solution which consists of 2 drops glacial acetic acid in 95% ethanol and cresyl violet acetate 0.2% in filtered acetate buffer. The method used was for paraffin-embedded wax; the tissue section after deparaffinization and rehydration was stained in 0.1% Cresyl violet for fifteen (15) minutes and then rinsed under running water. The sections were washed in 70% ethanol and immersed in differentiation solution for two (2) minutes. Dehydration was done about three (3) times in absolute ethanol; clearing was done in xylene and mounted with a coverslip. Drying was allowed to occur in the fume hood.

Luxol Fast Blue

The reagents used are absolute alcohol, xylene, distilled water, luxol fast blue, 0.2% cresyl fast violet, and 0.05% lithium carbonate (aq). After the sections were deparaffinized and rehydrated, the slides were

immersed in LFB solution and then incubated at 60°C for four (4) hours; differentiation was done in lithium carbonate and rinsed under running water. The slides were counterstained with cresyl fast violet for eight (8) minutes, dehydrated through 95% and absolute ethanol; cleared in xylene. The tissues were then mounted and coverslips were placed.

Glial Acid Fibrillary Protein (GFAP)
Hippocampal tissue sections underwent deparaffinization in xylene and subsequent hydration using absolute alcohol followed by 95% ethyl alcohol. Next, endogenous peroxidase activity was inhibited with two drops of freshly prepared 3% hydrogen peroxide for a duration of five (5) minutes. The slides were then rinsed with distilled water and tris buffer saline. Subsequently, the primary antibody against glial fibrillary acidic protein was applied and allowed to incubate for thirty (30) minutes. After incubation, the slides were rinsed in buffer and then washed under running water for ten (10) minutes, repeated twice. Following this, HRP polymer was applied and incubated for ten (10) minutes. DAB substrate chromogen was added, and the slides were subsequently rinsed in deionized water. Mayer's hematoxylin was used for counterstaining. After rinsing the slides in water, dehydration was carried out using 95% and 100% ethyl alcohol. Finally, glycerol gelatin mounting media was applied, and coverslips were used to complete the process.

Statistical analysis
The findings of the study were expressed as mean ± SEM and assessed using a one-way ANOVA test for comparisons across multiple groups. Statistical analysis was performed using GraphPad Prism software (version 8), with a significance level set at $p < 0.05$.

Results and Discussion

Caffeine, Nicotine, and MDMA are well-known psychoactive stimulants. These substances are consumed by so many people and several individuals even combine these substances without any medical prescription. It is consumed to enhance performance, alertness, wakefulness, etc. by the world's population. Research has shown that the prolonged intake of these substances can inhibit hippocampal neurogenesis, and memory impairment. This study aimed to evaluate the impact of combining caffeine, nicotine, and MDMA on the hippocampus of juvenile Wistar rats, with behavioral, histological, and immunohistochemical assays conducted. The results from the experiments are as follows:
The open arm entry (OAE) for Group A (control) was 3.75±0.83. Group B (caffeine + nicotine) showed a higher entry rate compared to the control at 6.00±1.00. Group C (caffeine + MDMA) also exhibited a higher entry rate than the control at 8.33±1.99. Similarly, Group D (nicotine + MDMA) had a higher entry rate than the control at 6.75±1.93. However, no significant difference was observed across all groups compared to the control (Figure 1A).
The open arm duration (OAD) for Group A (control) was 26.00±9.91. Group B (caffeine + nicotine) demonstrated a longer duration compared to the control at 60.33±15.33. Similarly, Group C (caffeine + MDMA) and Group D (nicotine + MDMA) showed longer durations than the control, at 37.50±11.86 and 54.60±13.93, respectively. Nevertheless, no significant difference was noted across all groups compared to the control (Figure 1B).
The closed arm entry (CAE) for Group A (control) was 3.62±10.67. Group B (caffeine + nicotine) exhibited a higher entry rate than the control at 7.50±1.74. Similarly, Group C (caffeine + MDMA) also showed a higher entry rate than the control at 5.50±1.84. Group D (nicotine + MDMA) displayed a significantly higher entry rate than the control (*=P<0.05) at 9.80±2.22 (Figure 1C).
The closed arm duration (CAD) for Group A (control) was 261.4±16.36. Group B (caffeine + nicotine) demonstrated a shorter duration compared to the control at 234.8±14.93. Similarly, Group C (caffeine + MDMA) showed a shorter duration compared to the control at 246.8±3.816. Group D (nicotine + MDMA) displayed a longer duration than the control at 275.2±17.59. However, no significant difference was observed across all groups compared to the control (Figure 1D).

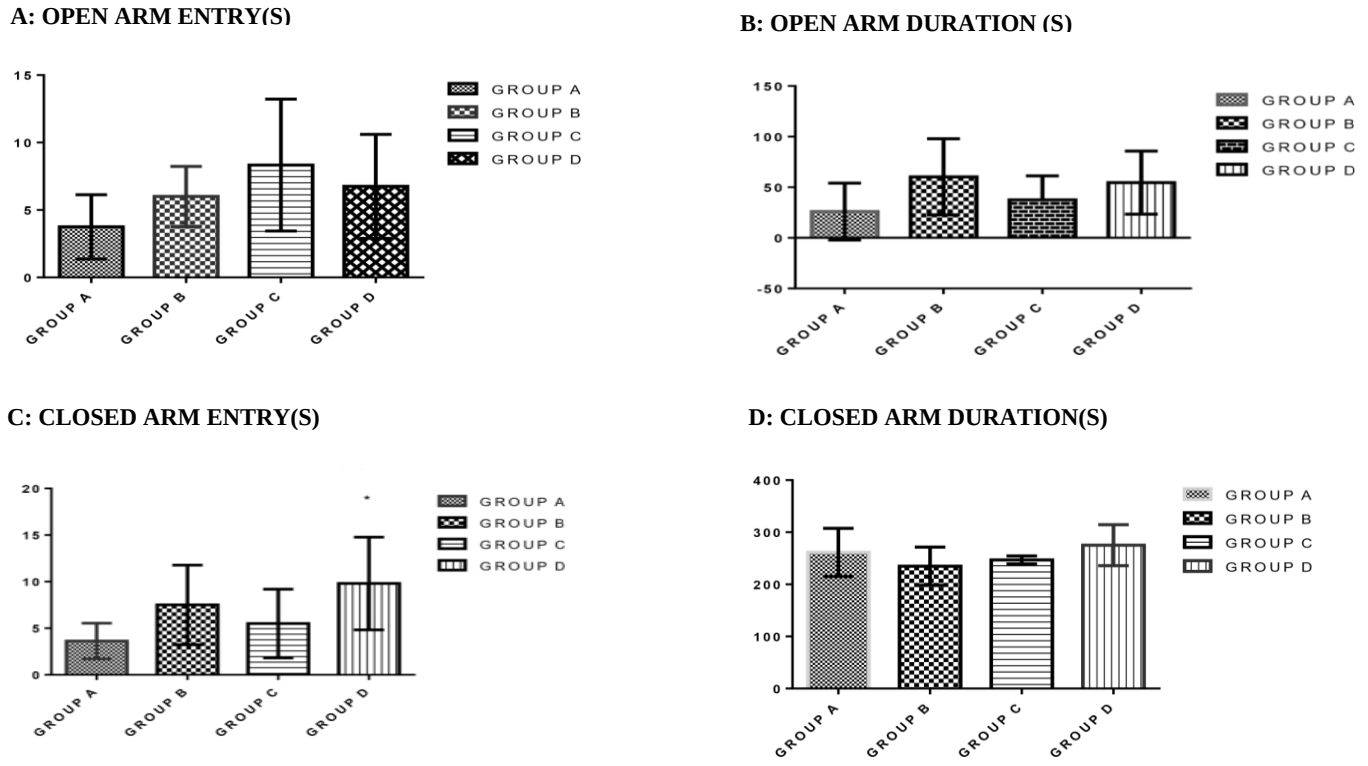

Figure 1: (A, B, C & D): Bar graph illustrating the open arm entry, open arm duration, closed arm entry, and closed arm duration for both the control and treated groups. No statistically significant differences were observed compared to the control group at P<0.05.

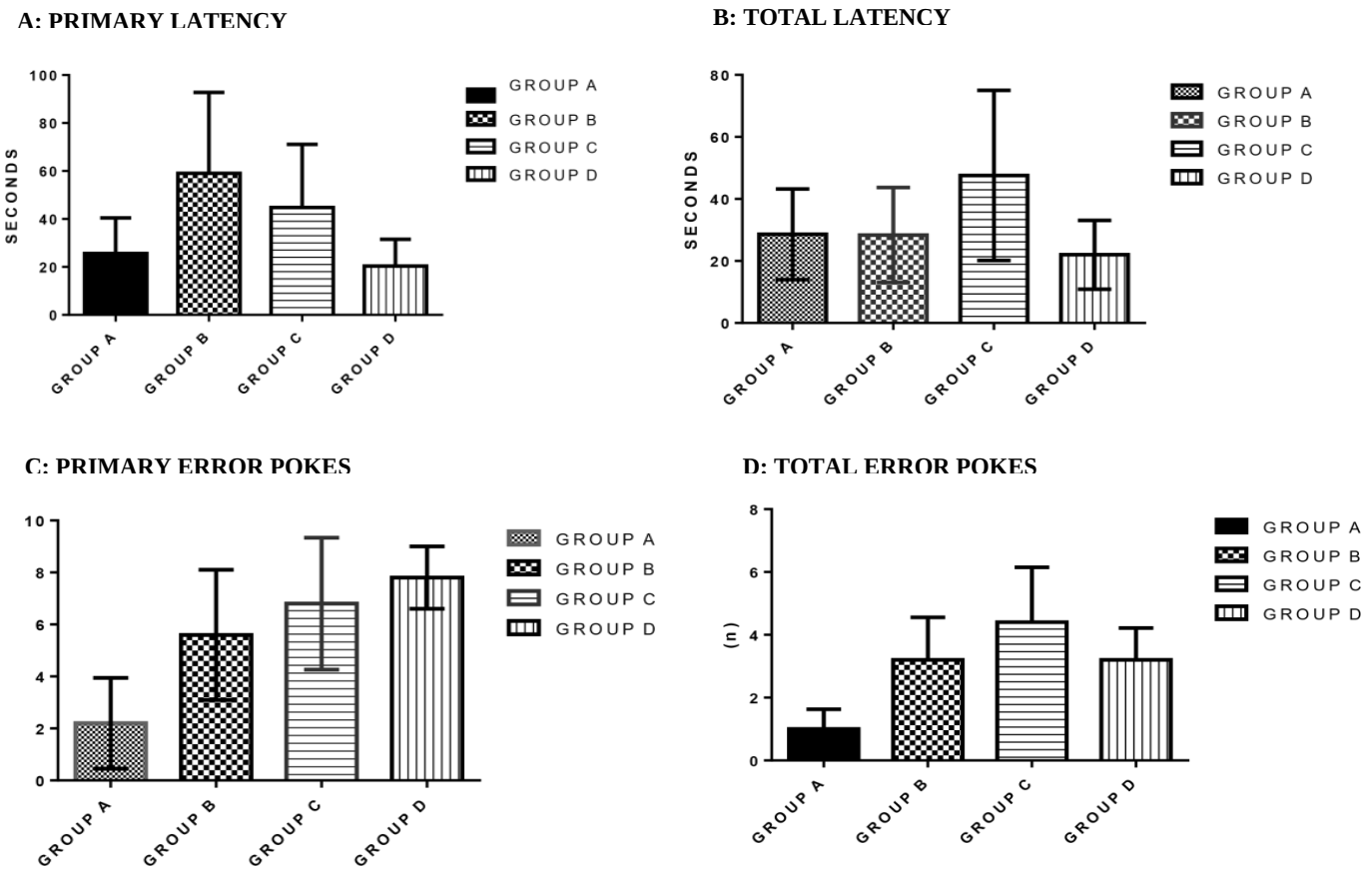

Figure 2: (A, B, C & D): A bar graph illustrating the primary latency, total latency, primary error pokes, and total error pokes. No statistically significant differences were observed compared to the control group at P<0.05.

The analysis from the elevated plus maze (Figure 1) indicated no significant difference in all arms compared to Group A, except for Group D (nicotine + MDMA), which showed a significant increase compared to Group A. Additionally, Group B (caffeine + nicotine) spent the least time in the open arm entry compared to the control group, possibly indicating anxiety and fearlessness. Conversely, Group C (caffeine + MDMA) spent the longest time in the open arm entry, which could be attributed to the known exacerbating effects of caffeine on anxiety and panic disorders. [23] The groups that were administered nicotine (caffeine + nicotine) and group D (nicotine +MDMA) spent less time in the open arm but the group that was not administered nicotine spent the highest time in the open arm. Group C (caffeine + MDMA). This was in contrast to research that shows that nicotine has anxiolytic properties which have been used to reduce anxiety in humans and experimental rats. [24]

The primary latency in Group A (control) was 25.60±14.87. Group B (caffeine + nicotine) exhibited a higher latency compared to the control at 59.00±33.82. Similarly, Group C (caffeine + MDMA) displayed a higher latency than the control at 44.80±26.34. In contrast, Group D (nicotine + MDMA) demonstrated a lower latency than the control at 20.40±11.17 (Figure 2A). The total latency in Group A (control) was 28.60±14.66. Group B (caffeine + nicotine) showed a slightly lower latency than the control at 28.40±15.31. Conversely, Group C (caffeine + MDMA) exhibited a higher latency than the control at 47.60±27.42. Group D (nicotine + MDMA) displayed a lower latency than the control at 22.00±11.10 (Figure 2B).

In terms of primary error pokes, Group A (control) had 2.200±1.744. Group B (caffeine + nicotine) showed a higher value than the control at 5.600±2.502. Similarly, Group C (caffeine + MDMA) and Group D (nicotine + MDMA) displayed higher values than the control at 6.800±2.538 and 7.800±1.200, respectively (Figure 2C). The total error pokes in Group A (control) were 1.000±0.632. Group B (caffeine + nicotine) exhibited a higher value than the control at 3.200 ±1.356. Similarly, Group C (caffeine + MDMA) and Group D (nicotine + MDMA) showed higher values than the control at 4.400±1.749 and 3.200±1020, respectively (Figure 2D).

Analysis of the results obtained from the Barnes maze test (Figure 2) revealed no statistically significant difference among all groups compared to Group A (control). Primary latency indicates the animal's ability to recognize the hole for the first time, while total latency reflects the animal's ability to enter the hole. Group B (caffeine + nicotine) and Group C (caffeine + MDMA) displayed increased primary latency compared to the control group, whereas Group D (nicotine + MDMA) exhibited decreased primary latency compared to Group A (control). In contrast to previous findings suggesting that nicotine enhances learning and memory by activating neurotransmitter receptors, Group B (caffeine + nicotine) and Group D (nicotine + MDMA) showed decreased total latency, while Group C (caffeine + MDMA) displayed increased total latency. This suggests that groups administered caffeine and MDMA experienced the highest latencies. This aligns with previous research indicating that prolonged consumption of MDMA can lead to deficits in various cognitive functions, such as attention, learning, and memory. [25] Caffeine has been shown to hinder hippocampus-dependent learning and impair long-term memory. [9]

There was no statistically significant difference observed in both the primary error pokes and the total error pokes across all groups when compared to Group A. All the groups were higher than group A which showed that they made more errors in recognizing the hole when compared to the control group.

The cytochrome c oxidase level for Group A (control) was 0.0100±0.0014. Group B (caffeine + nicotine) exhibited a slightly higher level than Group A at 0.0118±0.0011. Similarly, Group C (caffeine + MDMA) showed a slightly higher level than Group A at 0.0148±0.0028. Group D (nicotine + MDMA) demonstrated a slightly higher level than the control group at 0.0165±0.0036 (Figure 3A).

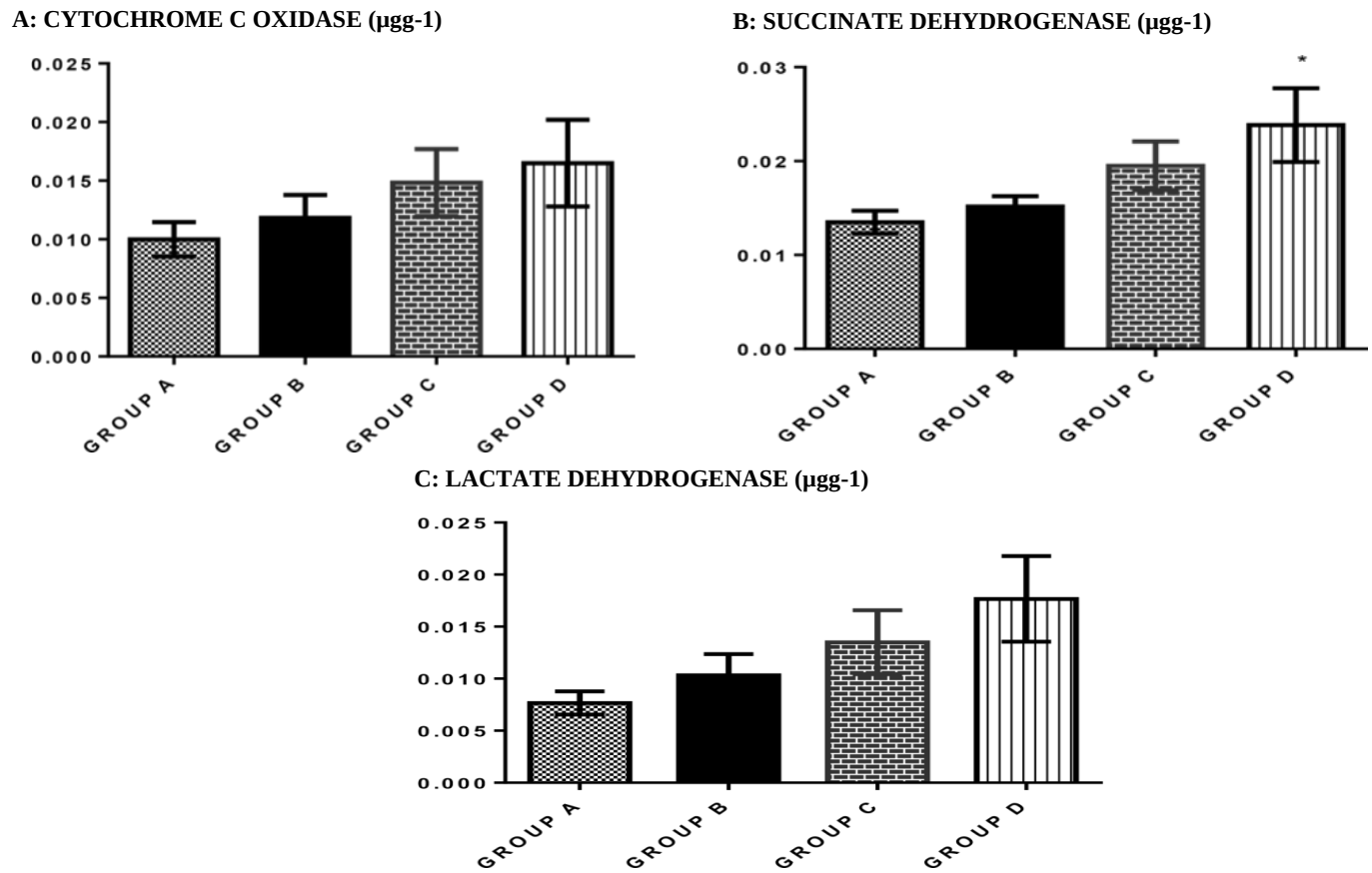

Figure 3: (A, B & C): Bar graph displaying the levels of cytochrome c oxidase, succinate dehydrogenase, and lactate dehydrogenase, highlighting statistical significance when compared with the control group (Group A) at P<0.05

For succinate dehydrogenase levels, Group A (control) displayed a level of 0.0135±0.0012. Group B (caffeine + nicotine) showed a slightly higher level than Group A at 0.0151±0.0011. Likewise, Group C (caffeine + MDMA) exhibited a slightly higher level than the control group at 0.0195±0.0025. Group D (nicotine + MDMA) had a significantly higher level than the control group (*=P<0.05) at 0.0238±0.0039 (Figure 3B).

Regarding lactate dehydrogenase levels, Group A (control) had a level of 0.0076±0.0011. Group B (caffeine + nicotine) demonstrated a slightly higher level than the control group at 0.0103±0.0020. Similarly, Group C (caffeine + MDMA) showed a slightly higher level than the control group at 0.0135±0.0030. Group D (nicotine + MDMA) exhibited a higher level than the control group at 0.0176±0.0041 (Figure 3C).

Across all enzyme tests, no significant difference was observed when compared with the control group.

The hippocampus was relatively preserved; there were no signs of significant cell loss to suggest hippocampal degeneration. The defining features in terms of the dentate gyrus and the cornu ammonis areas were preserved across the experimental groups. This showed that the agents administered and combined did not produce extensive hippocampal damage and did not induce acute hippocampal degeneration. Specific histological observation of the hippocampus dentate gyrus, however, showed signs of aberrations in the population and spatial distribution of the cells. In the treated groups, with Group C (administered Caffeine and MDMA) being affected the most, the aberrations were marked with increased cell density and alteration in the distribution of cells in this region which suggests that newer cells might have been added at a more rapid rate in response to the stimulations from the administered agents. Albeit, the cells were not yet naturally properly arranged in alignment with the typical dentate gyrus outline and the tip suggesting an ongoing process of dentate gyrus cellular reorganization. Although this effect was most prominent in Group C (caffeine +MDMA) the relative increase in cell density was also observed in Group B (caffeine + nicotine) and Group D (nicotine +MDMA). The agents administered produced effects that stimulated and altered the normal patterns of hippocampal neurogenesis to increase the cell population and alter their arrangement, particularly under the combined influences of caffeine and MDMA. This is in contrast with findings that MDMA diminishes neurogenesis. [26] However, the CA regions 1-4 did not show signs of hippocampal degeneration or aberrations in cell morphologies [Plate 1]. There were also no major aberrations in cell distributions and densities. This indicated that the observed effects in histoarchitecture majorly affected the dentate gyrus. This is in contrast to findings that nicotine decreases the number of neurons. [26] Myelin was generally demonstrated within the hippocampal formation and there was a lack of sufficient evidence of major disruptions in myelin to suggest extensive or major axonal degeneration within the hippocampus [Plate 2]. This could be attributed to the effects of the administered agents across the groups of research animals. Specific observations of the cornu ammonis areas 1-4 across the group did not present evidence of any major observable aberrations in myelin distribution; indicating that there were no major axonal degeneration and myelin loss in these regions. This is in contrast with findings which state that MDMA induces myelin disruption in the brain. [27, 28] Within the dentate gyrus, however, myelin distributions showed signs of alterations. In Group C [administered caffeine and MDMA] myelin distributions were not even and normal correlating with the observed changes in cellular distribution. Cells population showed that cells were not lost, but rather more populated; this observation suggested the presence of younger cells undergoing developmental processes as a consequence of the treatments or exposure to the agents. This would also show that caffeine and MDMA co-administration affected myelination in this region in addition to cell distribution and population density. Aberrations in Group D [nicotine + MDMA] show that myelin distribution was also less even and less intact; although not the same as the pattern of aberrations in Group C;

and might not be due to cell loss since cell population as observed was not reduced, it might be attributable to the changes caused by newer cells being rapidly added without fully developed myelinated axonal processes [Plate 2]. An overview of the hippocampal formation showed that neurons within the hippocampus express Nissl bodies [Plate 3]. This showed that the cells were living and active and there was little evidence suggestive of major hippocampal degeneration that could be interpreted to be histological hallmarks of hippocampal degenerations. This is in contrast with findings that exposure to MDMA can cause neuronal degeneration. [29] Within the dentate gyrus, cells prominently expressed Nissl bodies within the hippocampal regions. In the treated groups, the pattern of expression showed that cells were very actively involved in protein synthesis and over relatively wider areas within the hippocampal dentate gyrus. These observations suggest that the newly added cells might account for the relative abundance of Nissl bodies. Evidence, taken altogether points to the fact the treatment affected the protein synthesis activities in the dentate gyrus. Hence, the administered agents could affect the structure of the hippocampal dentate gyrus and its cellular activities. There are no major observable aberrations in the CA1-4 regions across the groups in terms of Nissl bodies' distribution. However, the treated groups appear to express Nissl bodies more prominently relative to the control, especially in Group B; suggesting that protein synthesis activities were stimulated and increased relative to the control [Plate 3]. Astrocytes within the hippocampal formation were generally demonstrated and the hippocampal formation was generally defined across the groups [Plate 4]. Relative to the control, astrocytes were more prominently demonstrated through their expressions of the glial fibrillary acidic proteins in Groups B and D dentate gyrus, but to a reduced extent in Group C. Astrocytes therefore responded more to the combined effects of caffeine and nicotine and nicotine and MDMA suggesting that the combination of nicotine with either caffeine or MDMA induced astrocyte reactions. Using this as a marker for tissue response to assault or stimulation, it could be inferred that the combined effects of these substances stimulated the dentate neural tissues in a manner that warranted astrocyte reaction, and hence might be hyper-stimulatory. These observations, when considered in line with the observed effect on cell morphologies, myelination, and Nissl expression do not mean cell death or pathological assault but more probably, hyperstimulation or hyper-excitation. This is similar to findings that caffeine affects myelination on set. [30] The pattern of astrocyte reaction or expression of the glial fibrillary acidic protein is also similar to the above in the CA regions with Groups B and D, administered caffeine and nicotine and nicotine combined with MDMA respectively [Plate 4]. The inference, in a similar fashion, would also be that the combined ingestion of the agents produced similar effects in the CA regions and the dentate gyrus.

Conclusion

The administered agents did not cause extensive hippocampal degeneration or localized neurodegeneration. However, effects are observed in the dentate gyrus including increased cell population in dentate gyrus and in cell densities and distributions in manners and patterns that suggest stimulated neurogenesis. This was accompanied by aberrations in myelination distribution, indicative of ongoing myelination of the newer cells in the dentate gyrus in particular. Astrocyte reactions suggest hyper-stimulation in the dentate gyrus and the cornu ammonis regions especially when there were caffeine and nicotine co-administration and nicotine and MDMA co-administration. The co-administration of agents altered dentate gyrus cellular organization, altered neurogenesis, altered myelination patterns, and included astrocyte reactions in certain instances. The results obtained from the neurobehavioral tests showed all treated groups had increased tendencies and impaired memory and cognition.

Further research should be done on dose-dependent combinations of nicotine, MDMA, and caffeine on learning and memory. Also, research should be conducted on the combined effects of these drugs on neurogenesis in the dentate gyrus.

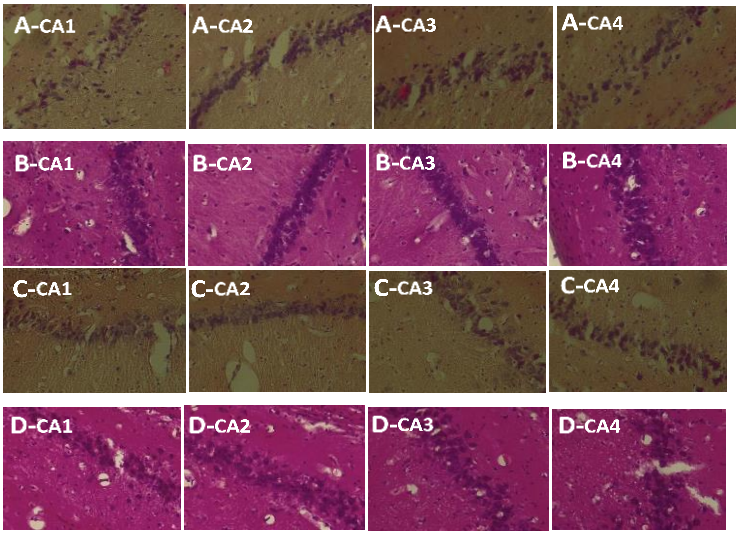

Plate 1: Photomicrographs depicting the hippocampus of experimental animal Groups A-D, showcasing the structural organization and cellular components of the cornu ammonis regions, including CA1-CA4, along with the neuropil. [H&E X400].

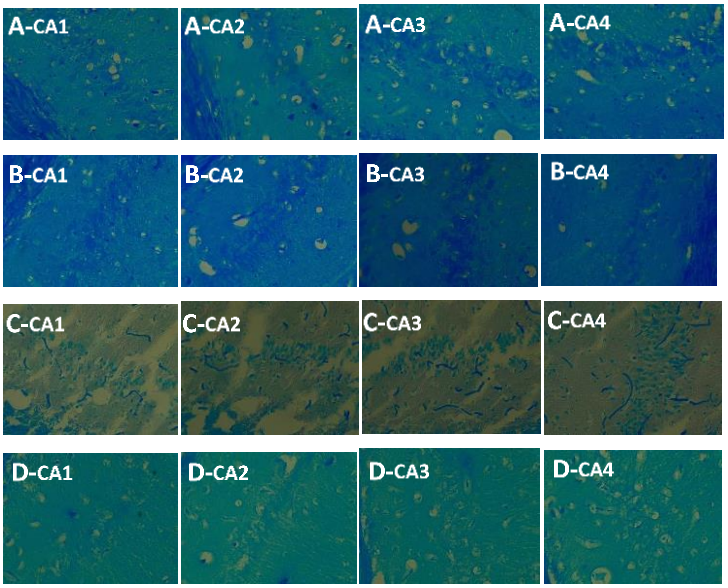

Plate 2: Photomicrographs illustrating the hippocampus of experimental animals in Groups A-D, displayed using the Luxol Fast Blue histological technique to examine the myelin of axons and bundles of nerve fibers associated with the hippocampus. The images capture the cornu ammonis regions, including CA1-CA4. [LFB X400].

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

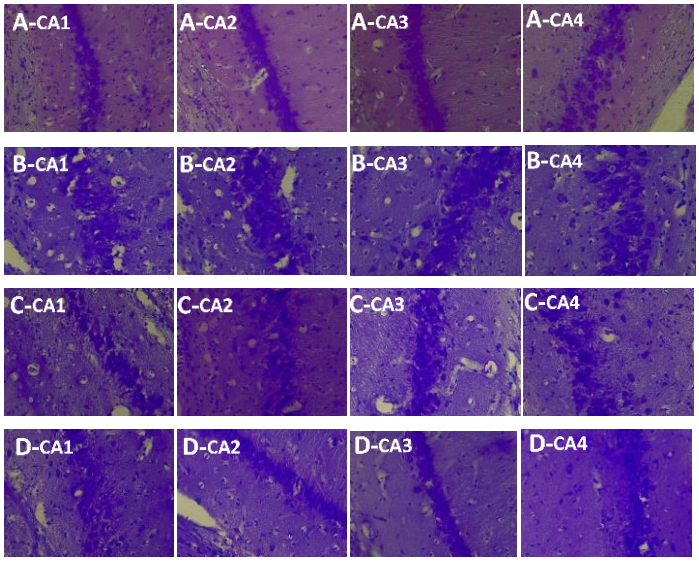

Plate 3: Photomicrographs displaying the hippocampus of experimental animals A-D, showcased utilizing the Cresyl Fast Violet histological technique to observe the expression of Nissl bodies in the cornu ammonis regions, including CA1-CA4. [CFV X400].

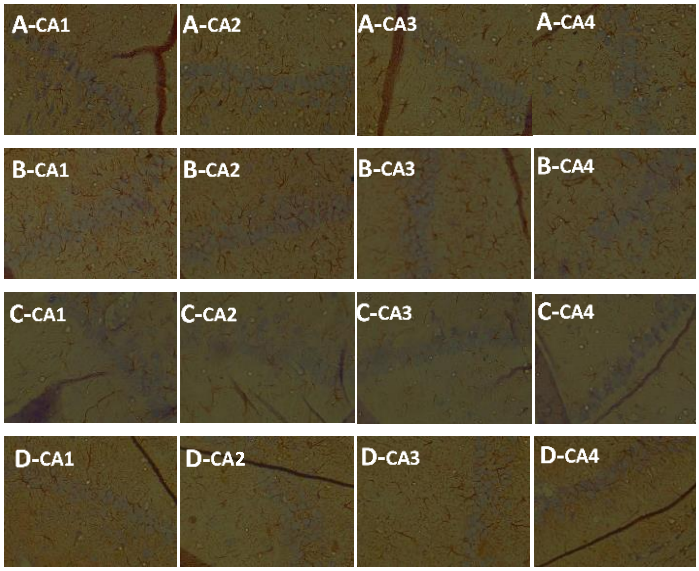

Plate 4: Photomicrographs depicting the hippocampus of experimental animals A-D, illustrated using the immunohistochemical technique for glial acidic fibrillary protein (GFAP) to observe GFAP expression in the cornu ammonis (CA1-CA4) regions. [GFAP X400].

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