

# The Combined Psychoactive Effects of Caffeine, Nicotine and MDMA on the Prefrontal Cortex of Juvenile Experimental Male Wistar Rats

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## Abstract

The developing brain at the adolescent stage is prone to the effects of various psychoactive agents and environmental influences. Caffeine, nicotine and MDMA influences on the developing brain and the possible consequences of such on fundamental mental attributes that determine brain health primarily including memory, cognition, behavior and learning among others. This research studied the structural and functional changes attributable to the combined use of caffeine, nicotine and MDMA on the prefrontal cortex including learning, cognition and anxiety tendencies. Male juvenile experimental Wistar rats, thirty-two [n=32] were grouped and administered Caffeine, nicotine and MDMA using oral gavages, whereas an untreated group was used as control (Group A). Group B received caffeine [100mg/kg body weight] and nicotine [50mg/kg body weight], C received Caffeine [100mg/kg body weight] and MDMA [10mg/kg body weight] and Group D received Nicotine [50mg/kg body weight] and MDMA [10mg/kg body weight]. Each group had eight rats. Treatments as described lasted thirty days after which animals were sacrificed. The Neurobehavioral tests carried out which were Barnes maze to access spatial learning and memory and elevated plus maze to access the anti-anxiety effects of pharmacological agents. The prefrontal cortex was washed in PBS and processed for histological and immunohistochemistry. Cryostat sections [~20µm] of the fixed tissue (prefrontal cortex) were mounted on glass slides followed by GFAP and histochemistry procedures as well as the routine H&E procedure. Photomicrographs were analyzed using ImageJ. Caffeine, nicotine and MDMA had effects on the brain cells. Neurons morphologies were distorted in certain instances as well as the expression of Nissl bodies and myelin sheath, most especially in group D which was administered Nicotine + MDMA. Results show that the combined effect of caffeine, nicotine and MDMA influences the activities of enzymes and vital neurotransmitters in the brain and alters the brain chemistry relative to the tested enzymes and neurotransmitters as well as its morphology.

**Keywords:** Nicotine • Caffeine • Prefrontal cortex • MDMA

## Introduction

Psychoactive drugs are chemicals that change the state of consciousness, perception and mood [1]. The brain is prone to the effects of various psychoactive agents and other agents and environmental influences at these stages of development [2]. Caffeine is a purine alkaloid present in high concentrations in beverages such as tea and coffee and also in Coca-Cola. In addition, it is also an important source of caffeine for children includes chocolate and soft drinks. This alkaloid is a behavioral stimulant. In the last decade, several reports have noted the beneficial effects of caffeine [3].

Nicotine is notably addictive. It is one of the maximum commonly abused drugs. Nicotine is found in the leaves of *Nicotiana rustica*; in the tobacco plant, which is a potent parasympathomimetic stimulant and an alkaloid found in the nightshade family of plants. Nicotine acts as a receptor agonist at most nicotinic acetylcholine receptors (nAChRs) [4].

3,4-Methylenedioxymethamphetamine (MDMA) is a powerful hallucinogenic drug and it has the ability to induce neurotoxicity. MDMA has stimulatory and hallucinogenic properties that make its psychoactive effects distinctive and different from other psychostimulant and hallucinogenic agents [7]. MDMA has shown to reduce the Serotonin levels in the terminals of axons of rats and mice. It produces degenerate neurons in discrete areas of the brain

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such as insular and parietal cortex, thalamus, tenia tecta and bed nucleus of the terminal stria. There are evidences that indicate the long-term MDMA abuse is associated with cognitive impairment and mood disturbances, which can last for months after cessation of drug intake [5].

Various research on the effects of caffeine, nicotine and MDMA effects on the brain especially in humans and experimental models have been done, but knowledge of the effects of these substances especially on the developing brain- both during the early stages of life are still inadequate. This effort would contribute to knowledge on the nature of caffeine, nicotine and MDMA influences on the developing brain and the possible consequences of such on fundamental mental attributes that determine brain health primarily including memory, cognition, behavior and learning among others. A number of previous investigations have reported structural and/or functional aberrations in the brain that are attributable to the effects of caffeine especially in the hippocampus, frontal cortex, cerebellum and choroid plexus.

## Materials and Methods

### Animals Treatment

Thirty-two male juvenile experimental wistar rats weighing between 100-120g were housed at the Institutional Animal Holding Facility in standard seized cages throughout the duration of treatment under suitable environmental conditions and given time to acclimatize. They were divided into four groups and administered Caffeine, nicotine and MDMA using oral gavages, whereas an untreated group was used as control (Group A). Group B received caffeine [100mg/kg body weight] and nicotine [50mg/kg body weight], C received Caffeine [100mg/kg body weight] and MDMA [10mg/kg body weight] and Group D received Nicotine [50mg/kg body weight] and MDMA [10mg/kg body weight]. Each group had eight rats. Animals were administered substances daily between the hours of 07: 00 and 08: 00 using suitable oral gavages. Treatments lasted thirty days after which animals were euthanized. During experiment, animals were housed under conditions of controlled temperature and humidity, with 12h light/dark cycles. All activities involving the use, handling, treatment, and management of the experimental animals were carried out in compliance with ethics and standard institutional research practices.

### Neurotransmitter and Enzyme Assay

After sacrificing the animals, the tissues of interest were excised and kept in plain sample bottles containing phosphate buffer saline (PBS) till the end of the exercise. Tissues were thereafter homogenized in sucrose was then added to the tissue in the mortar with a pestle each tissue was homogenized and poured into fresh sample bottles, after homogenizing, the bottles containing the samples were kept in a centrifuge and left for 15 minutes at 4000G. After centrifuging, the bottles were brought and the supernatant was extracted and poured in fresh sample bottles. At the completion of this process, the supernatant were taken for neurotransmitter assay and the neurotransmitter tested for are: dopamine, serotonin and GABA. (The same homogenate was used for enzyme succinate dehydrogenase, Cytochrome C oxidase and lactate dehydrogenase). The Reagent used was Phosphate buffer. Solution A's concentration

was 2.27g of  $\text{KH}_2\text{PO}_4$ +250ml of distilled water and it was shaken well to help dissolve  $\text{KH}_2\text{PO}_4$ . While Solution B was 2.365g of  $\text{Na}_2\text{HPO}_4$  +250ml of distilled water shaken well to help the dissolving of  $\text{Na}_2\text{HPO}_4$ . Solution A and B were then mixed together.

### Histology

After harvesting the brain of the experimental animals, the prefrontal cortex was grossed and sectioned. The tissues were then processed for histological slides. Brain tissue was fixed in formal saline for 48 hours, It was then dehydrated in the following order of alcohol: 70% for one hour, 80% for one hour, 90% for two hours, 95% for two hours and two changes of 100% absolute alcohol for two hours each, It was then cleared in two changes of xylene for two hours each, It was then infiltrated with two changes of paraffin wax for two hours for the first change and four hours for the second change, The processed tissue was placed in paraffin wax for embedding using a plastic mould, The embedded tissue was sectioned using Cambridge rocker microtome at 5µm and picked on an albuminized slide in a warm bath at a regulated temperature lower than 58 °C which is the regular melting point of wax. Slides were stained with Hematoxylin and Eosin processing to check the histoarchitectural organization of the cells and tissue of the Prefrontal Cortex, Cresyl Fast Violet to check for Nissl substance, Luxol Fast Blue Technique to check for the integrity of the myelin sheath

### Immunohistochemistry

Samples were assayed for glial fibrillary acidic protein (GFAP) which was used as a principal marker for brain astrocytes. The tissues were fixed with 10% Formalin and the Paraffin sections cut at 4 microns. Sections were deparaffinized thoroughly in three changes of xylene, 3 minutes each and hydrated through two changes each of 100% and 95% ethyl alcohols, 10 dips each. Then washed well with distilled water. Sections were rinsed in distilled water, excess water was tapped off, Sections were circled with pap pen liquid blocker to reduce reagent usage and ensure tissue coverage, Endogenous peroxidase was blocked with freshly made 3% hydrogen peroxide and then incubated for 5 minutes. Slides were rinsed gently in distilled water, and then rinsed in two changes of Tris Buffered Saline.

Excess buffer was tapped off; Glial Fibrillary Acidic Protein primary antibody was applied and incubated at room temperature for 30 minutes. Slides were rinsed in two changes of buffer, Excess buffer was tapped off, and amplifier was applied and then placed in incubator for 10 minutes then were rinsed in two changes of buffer. Excess buffer was tapped off; HRP polymer was applied and incubated for 10 minutes. The Slides were rinsed in two changes of buffer; The Required quantity of DAB chromogen was prepared in 4ml of deionized water then 2drops acetate buffer after which 1 drop AEC chromogen was added then 1 drop 3% hydrogen peroxide, Excess buffer was tapped off, DAB was applied and incubated for 5 minutes and Slides were rinsed in four changes of distilled water and were counterstained lightly with Mayer Haematoxylin stain for 2 minutes. The slides were rinsed in warm tap water to blue sections, dehydrated in 2 changes each of 95% and 100% ethyl alcohol and cleared in 3 changes of xylene, 10 dips each; Glycerol gelatin or other compatible aqueous mounting media was applied and carefully covered with a coverslip.

## Results

### Neurotransmitter: Gaba Neurotransmitter Assay Result

The mean level of the control group was  $0.2270 \pm 0.002113$ . The mean level of the Caffeine + Nicotine group was slightly higher than the control group at  $0.2355 \pm 0.003888$ . The mean level of the Caffeine + MDMA group was significantly higher than control and Caffeine + Nicotine at  $0.2357 \pm 0.004364$ , while the mean level of the Nicotine + MDMA group was higher than all the remaining three groups at  $0.2360 \pm 0.004074$ .

\*: Shows a statistical significance when compared to the control group at  $p < 0.05$

### Glutamate Neurotransmitter Assay Result

The mean of control group was  $0.2382 \pm 0.005009$ . The mean of Caffeine + Nicotine was slightly lower than the control group at  $0.2323 \pm 0.004462$ . The mean of Caffeine + MDMA was significantly higher than control and Caffeine + Nicotine at  $0.2433 \pm 0.006206$ . The mean of Nicotine + MDMA was higher than all the groups at  $0.2482 \pm 0.007040$ .

There was no statistical significance at  $p < 0.05$

### Dopamine Neurotransmitter Assay Result

The mean of control group was  $0.1308 \pm 0.002971$ . The mean of Caffeine + Nicotine was slightly higher than the control group at  $0.1390 \pm 0.005354$ . The mean of Caffeine + MDMA was significantly higher than control and Caffeine + Nicotine at  $0.1472 \pm 0.007565$ . The mean of Nicotine + MDMA was higher than all the groups at  $0.1483 \pm 0.008381$ .

There was no statistical significance at  $p < 0.05$

### Serotonin Neurotransmitter Assay Result

The mean of control group was  $0.1363 \pm 0.003159$ . The mean of Caffeine + Nicotine was higher than the control group at  $0.1413 \pm 0.004971$ . The mean of Caffeine + MDMA was significantly higher than control and Caffeine + Nicotine at  $0.1458 \pm 0.006327$ . The mean of Nicotine + MDMA was higher than all the groups at  $0.1473 \pm 0.006216$ .

\*: Shows a statistical significance when compared Caffeine + Nicotine vs. Nicotine +

### Enzyme Assay

#### Cytochrome Oxidase Enzyme Assay Result

The mean of control group was  $0.0100 \pm 0.001461$ . The mean of Caffeine + Nicotine was higher than the control group at  $0.01183 \pm 0.001956$ . The mean of Caffeine + MDMA was significantly higher than both control and Caffeine + Nicotine at  $0.01483 \pm 0.002868$ . The mean of Nicotine + MDMA was higher than all the groups at  $0.0165 \pm 0.003695$ . There was no statistical significance at  $p < 0.05$

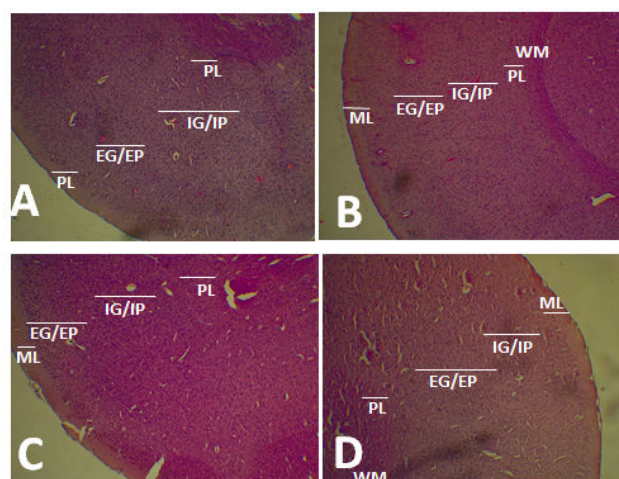
#### Succinate Dehydrogenase Enzyme Assay Result

The mean of control group was  $0.0135 \pm 0.001204$ . The mean of Caffeine + Nicotine was higher than the control group at  $0.01517 \pm 0.001108$ . The mean of Caffeine + MDMA was significantly higher than both control and Caffeine + Nicotine at  $0.0195 \pm 0.002579$ . The mean of Nicotine + MDMA was higher than all the groups at  $0.02383 \pm 0.003928$ . \*: Shows a statistical significance when compared Control vs. Nicotine + MDMA at  $p < 0.05$

#### Lactate Dehydrogenase Enzyme Assay Result

Figure 4.7. Bar graph showing the mean Level of Lactate Dehydrogenase across all the experimental groups.

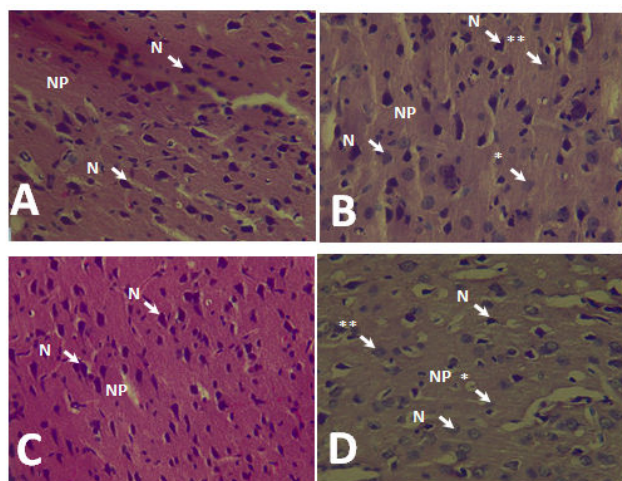
The mean of control group was  $0.007667 \pm 0.001116$ . The mean of Caffeine + Nicotine was higher than the control group at  $0.01033 \pm 0.002028$ . The mean of Caffeine + MDMA was significantly higher than both control and Caffeine + Nicotine at  $0.0135 \pm 0.003074$ . The mean of Nicotine + MDMA was higher than all the groups at  $0.01767 \pm 0.004112$ . MDMA at  $p < 0.05$ . \*: Shows a statistical significance when compared Caffeine + MDMA vs. Nicotine + MDMA at  $p < 0.05$ .



**Figure1:** Photomicrographs of the prefrontal cortex of the experimental animals in Groups A-D. The Hematoxylin and eosin histological technique [H&E] is being used to demonstrate the brains' prefrontal cortices in cross sections to observe the cortical structural integrity across the layers [H&E, X40].

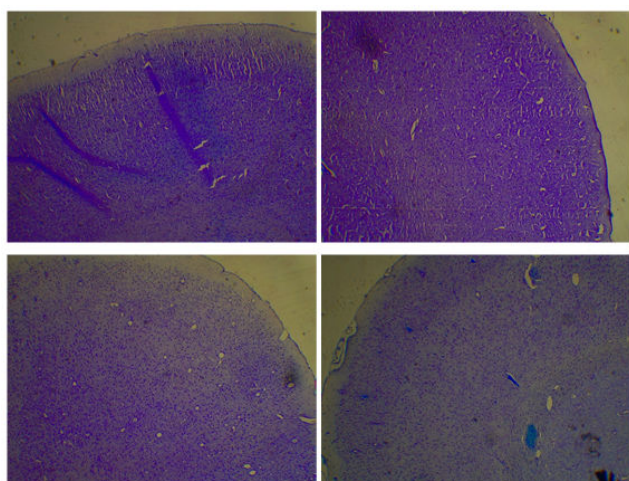
LEGEND: ML = Molecular Layer, EGL = External Granular Layer, EPL = External Pyramidal Layer, IGL = Internal Granular Layer, IPL = Internal Pyramidal Layer, PL = Plexiform Layer, WM = White Matter



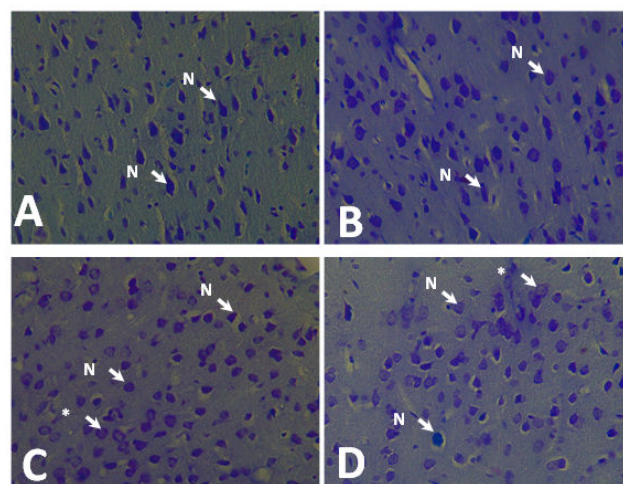


**Figure2:** Photomicrographs of the prefrontal cortex of the experimental animals in Groups A-D. The Hematoxylin and eosin histological technique [H&E] is being used to demonstrate the brains' prefrontal cortices cells and neuropil to observe the structural integrity of the cells and the tissues. Certain cortical cells showed signs of cellular trauma including karyolysis [\*] and karyorrhexis [\*\*]. [H&E, X400].

LEGEND: N = Neuron, NP = Neuropil

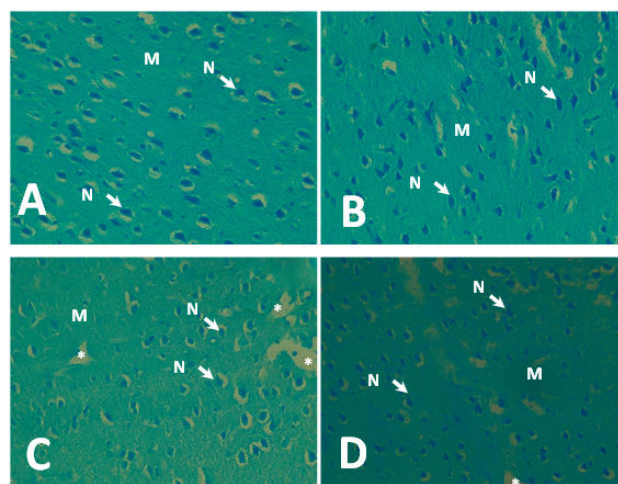


**Figure3:** Photomicrographs of the prefrontal cortex of the experimental animals in Groups A-D. The Cresyl Fast Violet histological technique [CFV] is being used to demonstrate the brains' prefrontal Nissl bodies to observe its distribution across the cortical layers as a marker for the functional integrity of the cortex [CFV, X400].



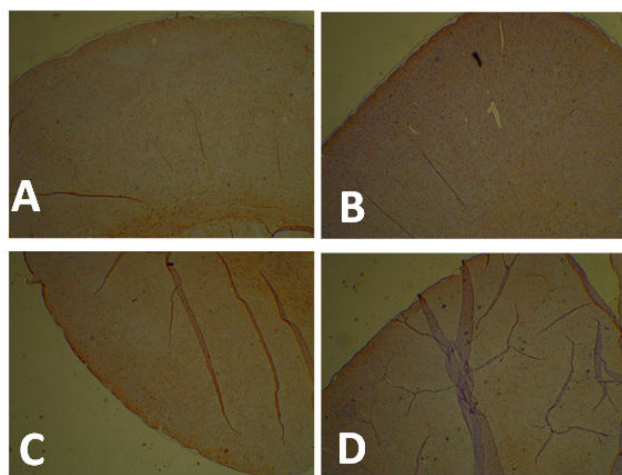
**Figure4:** Photomicrographs of the prefrontal cortex of the experimental animals in Groups A-D. The Cresyl Fast Violet histological technique [CFV] is being used to demonstrate the brains' prefrontal Nissl bodies to observe the functional integrity of the cortical cells [CFV, X400]. Nissl expression in certain cells was compromised [\*].

LEGEND: N = Neuron

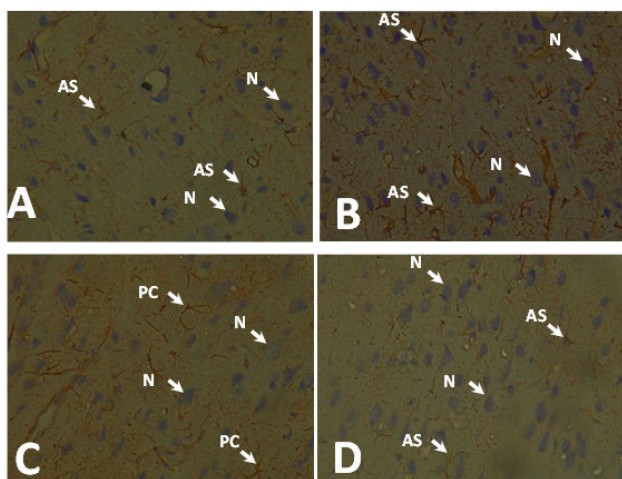


**Figure5:** Photomicrographs of the prefrontal cortex of the experimental animals in Groups A-D; demonstrated using the Luxol Fast Blue histological technique to observe the myelin material of the neuronal axons. There are areas of localized demyelination within the tissue in Groups C and D[\*] [LFB, X400].

LEGEND: N = Neuron, M = Myelin

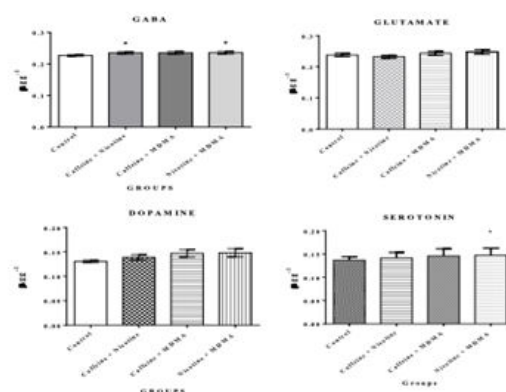


**Figure6:** Photomicrographs of the prefrontal cortex of the experimental animals in Groups A-D; demonstrated using the glial fibrillary acidic protein immunohistochemistry technique which is expressed by astrocytes. The photomicrographs demonstrate the entire cross section of the prefrontal cortex to observe the expression of GFAP across the cortical layers and possible localized lesions or trauma. [GFAP, X40].

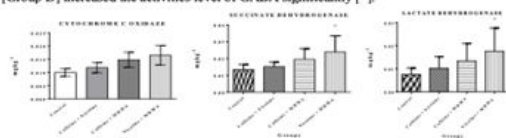


**Figure7:** Photomicrographs of the prefrontal cortex of the experimental animals in Groups A-D; demonstrated using the glial fibrillary acidic protein immunohistochemistry technique which is expressed by astrocytes. It provides information on astrocytes reaction to tissue trauma and assault. Astrocytes were prominently demonstrated in Groups B and C compared to the control [GFAP, X400].

LEGEND: N = Neuron, AS = Astrocytes



**Figure 8.1:** Bar charts showing the mean activity levels of neurotransmitters GABA, Glutamate, Dopamine and Serotonin in the brain of the experimental animals. Relative to the control, caffeine and nicotine combination [Group B] and nicotine and MDMA combination [Group D] increased the activities level of GABA significantly [\*].



**Figure 8.2:** Bar charts showing the mean activity levels of key enzymes cytochrome C oxidase, succinate dehydrogenase, and lactate dehydrogenase in the brain of the experimental animals. There were general aberrations in the levels of these enzymes and exposure to psychoactive agents tends to increase their activity levels. The combination of nicotine and MDMA increased succinate dehydrogenase and lactate dehydrogenase activities levels significantly [\*].

## Discussion

### Neurotransmitters

Results gotten from GABA neurotransmitter level analysis showed statistically significant difference among all groups when compared with the control group. Group D (Nicotine plus MDMA) showed the highest level of GABA when compared to control group, this confirms the statement reported that the administration of two psychoactive substances caused an increases in the levels of GABA across the treated groups relative to the control group also nicotine over-stimulates the glutamate receptors in GABAergic neurons which in turn increases GABAergic output.

Dopamine is an organic chemical that both functions as a hormone and a neurotransmitter. It plays several important roles in the brain and body. Dopamine is a primary neurotransmitter whose activity can be influenced by the effects of Caffeine and MDMA. Analysis on dopamine level showed statistical significant increase in all groups. When compared to the control group with group D (Nicotine plus MDMA) and group C (Caffeine plus MDMA) being the highest when comparing to the control group. This is also reported that the co-administration of both substances had a significantly stronger effect on the extracellular dopamine level than when administered separately.

Assay results gotten from serotonin neurotransmitter level analysis showed statistically significant difference among all groups when compared with the control group although the increase among all groups were slight, group D (Nicotine plus MDMA) had the highest when comparing to the control group. Which could be attributed to the fact ha MDMA causes greater release of serotonin and that MDMA enhances the release of neurotransmitter's and/or blocks their reuptake, resulting to increased neurotransmitters level within a synaptic cleft. But also contrary that nicotine reduces brain serotonin



levels which may be as a result of MDMA's ability to enhance the release of neurotransmitters.

Glutamate a role in synaptic plasticity and is involved in cognitive functions such as learning and memory. From the analysis on the glutamate level. There was no significant difference among all the groups. Group C (caffeine + MDMA) and group D (nicotine + MDMA) showed an increase in the dopamine level when compared to group A. Group B (caffeine + nicotine) was lower than the control group. This is similar to the findings that MDMA has been shown to promote excessive glutamate release specifically in the hippocampus, and the glutamate receptors expressed by GABA neurons in this brain region are especially vulnerable to overstimulation.

## Enzyme

Succinate dehydrogenase is an enzyme complex that is responsible for cellular respiration. They also play roles in oxygen levels sensing and tumor suppression and are known as markers for oxidative stress. Results gotten from the succinate dehydrogenase enzyme level showed statistically significant increase among all the groups when compared to the control group, which is similar to previous findings that nicotine and MDMA induces oxidative stress, which increases the production of reactive oxygen species (ROS) is associated with carcinogenic transformation, cell toxicity, and DNA damage.

Lactate dehydrogenase is found in all living things, it catalyzes the conversion of lactate to pyruvic acid and back. It serves as a marker for tissue damage and as such is released when there are injuries. Results gotten from the lactate dehydrogenase enzyme level statistically significant increase among the treated groups, with group D (Nicotine plus MDMA) being the highest when comparing to the control group. This result indicates the neurotoxic effects of MDMA [22], which might induce tissue damage. In contrary, nicotine can reduced the levels of lactate dehydrogenase. With groups B (caffeine plus Nicotine) and C (Caffeine plus MDMA), the levels of lactate dehydrogenase is elevated compared to the control group, which could be as a result of caffeine being able to increase the level of Lactate dehydrogenase.

Cytochrome c oxidase or complex IV, is a large transmembrane protein complex that can be found in the mitochondria of eukaryotes. This enzyme has an intermediate role in apoptosis, a controlled form of cell death used to kill cells in the process of development or in response to infection or DNA damage. The results obtained from the analysis shows that there was an increase in the levels of the enzyme in all the groups most especially in group C (Caffeine and MDMA) and group D (Nicotine and MDMA) observed which might be attributed to the findings that MDMA induces neuronal death.

## Histology

**Observable Effects on Prefrontal Cortex Cells and Cortical Histoarchitecture**

The prefrontal cortex of the experimental animals is demonstrated in each group with its cortical layers relatively defined and observable in its typical outer molecular layer, external pyramidal and granular, internal pyramidal and granular as well as the deepest plexiform layer. There is no evidence of localized lesion that is characteristic of an established pathology [Figure 1]. The photomicrographs of the

Groups B and D showed that the combination of caffeine plus nicotine and nicotine plus MDMA had effects on neurons of the prefrontal cortex exhibited as heterogeneity suggesting deleterious effects of the administered agents on the cortical tissues. There are also observations that indicate significant cellular assault and impending karyolysis [\*] and karyorrhexis [\*\*]. These observations therefore suggest that the combination of these agents had significant deleterious effects on the cortical neurons in manners that might lead to apoptosis possibly upon continual exposure to the agents. This might further imply reduction in cortical number of neurons owing to death of certain neurons.

### Observable Effects on Prefrontal Cortex Cells Nissl Bodies

The expression of Nissl by cortical cells is an indication of functional integrity as Nissl bodies are active rough endoplasmic reticula in conjugation with ribosome, thus actively involved in protein synthesis. Furthermore, Nissl bodies are characteristic of nervous tissues. A general overview of the cortex in cross section shows that the cortex in Groups C and D expressed Nissl less abundantly relative to the control [Figure 3]. Further observation of the cortical neurons at a higher magnification shows that certain cells express Nissl less prominently and this might indicate their levels of functional activities. When compared with the control group, such cells are not present [Figure 4]. It is therefore evident that these cells were functionally compromised in the treated groups. The reduced protein synthesis might be just functional compromise in certain neurons. However, if these aberrations are taken in line with observations on the cellular morphologies, certain other cells might be showing signs of impending death, hence, compromised protein synthesis. The heterogeneity of these cells also indicates that certain cells are structurally and functionally compromised due to the effects of the administered agents.

### Observable Effects on Prefrontal Cortex Myelin Distribution

Myelin is generally demonstrated in all the treated groups and there is no evidence to suggest extensive demyelination. However, in Group C, when caffeine and nicotine were co-administered, the effects included localized areas of demyelination which might indicate localized areas of neuronal loss and consequently loss of myelin materials that could be associated with the processes [\*].

### Observable Effects on Prefrontal Cortex Astrocyte Reaction

Generally, the pattern of astrocyte demonstration using the glial fibrillary acidic protein [GFAP] expression as a marker for the astrocyte- was random in the groups. There are no localized areas of abnormal astrocyte aggregations that could be characteristic of a major localized lesion [Figure 6]. However, despite the random distribution of the astrocytes in all the groups, astrocytes were prominently demonstrated in Groups B and C compared to the control by virtue of their expressions of the glial fibrillary acidic protein. This implies that the administered agents interacted with the brain tissue in these parts and elicited astrocyte reaction. Since, enhanced astrocyte reaction is a marker of neural tissue assault or trauma, the brain tissue in this brain parts therefore responded to the agents' effects in manners that indicative of an assault. While the enhanced expression might be mild compared to situations of heavy metal poisoning and major intoxications; it is still important to emphasise that the co-administered agents elicited astrocyte reaction because of its negative effects on the brain tissue which could have deleterious

effects on the neurons and this is what the astrocytes reacted to.

## Conclusion

Various research on the effects of caffeine, nicotine and MDMA effects on the brain especially in humans and experimental models have been done, but knowledge of the effects of these substances especially on the developing brain- both during the early stages of life are still inadequate. This effort would contribute to knowledge on the nature of caffeine, nicotine and MDMA influences on the developing brain and the possible consequences of such on fundamental mental attributes that determine brain health primarily including memory, cognition, behavior and learning among others. A number of previous investigations have reported structural and/or functional aberrations in the brain that are attributable to the effects of caffeine especially in the hippocampus, frontal cortex, cerebellum and choroid plexus.

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