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

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ABSTRACT

The juvenile is vulnerable to psychoactive agents and environmental influences. Caffeine, nicotine, and 3,4-Methylenedioxymethamphetamine (MDMA) could influence the developing brain with potential consequences on mental attributes including memory, cognition, behaviour, and learning. This research studied the structural and functional changes attributable to the combined use of caffeine, nicotine and MDMA on the prefrontal cortex. Thirty-two juvenile male Wistar rats were grouped into four (n=8) and administered water-dissolved caffeine, nicotine and MDMA by oral gavages. An untreated group served as control. The second group received caffeine (100mg/kg bw) and nicotine (50mg/kg bw), the third group received caffeine (100mg/kg bw) and MDMA (10mg/kg bw) and the last group received nicotine (50mg/kg bw) and MDMA (10mg/kg bw). Treatments lasted thirty days, after which animals were sacrificed. The prefrontal cortices were fixed in formal saline, processed, and demonstrated using eosin and haematoxylin (H&E), Cresyl fast violet, and Luxol fast blue histological methods as well as glial-acidic fibrillary protein (GFAP) immunohistochemistry. Representative photomicrographs were obtained and analysed. Combined ingestion of caffeine, nicotine and MDMA affected the brain causing neuronal morphological aberrations, aberrations in Nissl expression, and astrocyte reactions especially with the combination of caffeine + nicotine as well as caffeine + MDMA with mild aberrations in myelin integrity. Effects also included significant elevations in GABA and serotonin activities with the combination of nicotine and MDMA ($p<0.05$). Results showed that the combined use of psychoactive agents could elicit peculiar effects.

Keywords: *Nicotine, Caffeine, MDMA, Prefrontal cortex, Brain*

INTRODUCTION

Psychoactive drugs are chemicals that change the state of consciousness, perception and mood (Hassan et al. 2017). The brain is prone to various psychoactive agents and other environmental influences both at the prenatal and the postnatal stages of development (Klomp et al. 2012). Caffeine is a purine alkaloid present in beverages such as tea, coffee and the Coca-Cola, as well as chocolate and other soft drinks. This alkaloid is a behavioural stimulant, which can be beneficial (Juárez-Méndez et al. 2006). However, there is also a need to critically evaluate any consequence of its consumption and effects.

Nicotine is notably addictive, and one of the most commonly abused drugs (Sajja et al. 2016). Nicotine is found in the leaves of Nicotiana rustica, a tobacco plant, which is a potent parasympathomimetic stimulant, and an alkaloid found in the nightshade family of plants (Benowitz 2009). Nicotine acts as a receptor agonist at most nicotinic acetylcholine receptors (nAChRs) (Malenka et al. 2009).

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3,4-methylenedioxymethamphetamine (MDMA) is a powerful hallucinogenic drug that can induce neurotoxicity. It has psychoactive effects distinctive and different from other psychostimulants and hallucinogenic agents (Hagino et al. 2011). MDMA has been 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 including the insular and parietal cortex, thalamus, tenia tecta, and bed nucleus of the terminal stria (Sarkar and Schmued 2010). Certain reports have indicated that long-term MDMA abuse is associated with cognitive impairment and mood disturbances, which can last for months after cessation of the drug intake (Morgan 2000; Parrott 2002).

Reports of the combined effects of these substances especially on the developing brain are still largely inadequate. The primary aim of this study was to observe the nature of the effects of psychoactive agents on the frontal cortex structural and functional integrity when the psychoactive agents, caffeine, nicotine and MDMA are combined and ingested, thus providing insight into their combined effects on specific brain health attributes. This research would contribute to existing knowledge on the possible consequences of brain exposure to the psychoactive agents on fundamental mental attributes that determine brain health, especially in terms of behaviour and learning.

MATERIALS AND METHODS

Ethical Approval

The experimental procedures were conducted following the National Institute of Health of the United States of America guidelines for the care and use of laboratory animals in line with guidelines of the Department of Anatomy, Babcock University, Ilishan-Remo, Ogun state, and ethical approval for this study was obtained from Babcock University Health Research Ethical Committee (BUHREC) Babcock University, Ilishan-Remo, Ogun State (BUHREC872/19).

Animal Treatment

Thirty-two male juvenile Wistar rats weighing 100-120 g were housed at the Babcock University animal holding facility in standard cages throughout treatment, under suitable environmental conditions. They were divided into four groups and administered caffeine (anhydrous; Powder City, USA; Batch Number 151219 and Lot Number CAFAH20151012), nicotine (Sigma-Aldrich, USA; Product no. CAS 22083-74-5) and MDMA [3,4-methylenedioxymetamphetamine; a controlled drug obtained by permission through the National Drug Law Enforcement Agency (NDLEA)] using oral gavages, whereas an untreated group was the control. The caffeine + nicotine group received caffeine (100 mg/kg body weight) and nicotine (50 mg/kg body weight); caffeine + MDMA group received caffeine (100 mg/kg body weight) and MDMA (10 mg/kg body weight) and nicotine + MDMA group received Nicotine (50 mg/kg body weight) and MDMA (10 mg/kg body weight). Each group had eight rats (Table 1). Animals were administered the psychoactive substances daily between 07:00 and 08:00 using suitable oral gavages. Treatments lasted thirty days, after which animals were euthanized using cervical dislocation. During the experiment, animals were housed under suitable temperature and humidity conditions, with 12 h light/dark cycle. 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.

Histology

Following animal sacrifice, their skulls were dissected to expose the brain. Their prefrontal cortices were excised, washed in phosphate buffered saline (PBS) and fixed in formal saline for 48 h. Tissues meant for biochemical assays were not fixed but homogenised fresh and processed as described under neurotransmitters and enzymes assay methods section. Sections for histology were then dehydrated, cleared, paraffin wax embedded, and sectioned using Cambridge rocker microtome at 5 µm. Sections on slides were processed and stained with Haematoxylin and Eosin for histoarchitectural organization of the prefrontal cortex, cresyl fast violet stain for Nissl substance, and Luxol fast blue technique for the integrity of the myelin sheath

Immunohistochemistry

Tissue sections on slides were blocked off endogenous peroxidase with freshly prepared 3% hydrogen peroxide for 5 min. Excess was washed off in distilled

Table 1: Grouping of the Experimental Animals as well as the Administration and Doses of the Agents used for the Experiments

Group	Number of Animals	Agent/Dose	Rationale
A- Control	8	No agent	Served as control
B- Caffeine + Nicotine group	8	caffeine (100mg/kg body weight) and nicotine (50mg/kg body weight)	To observe the combined effects of caffeine and nicotine
C- Caffeine + MDMA group	8	Caffeine (100mg/kg body weight) and MDMA (10mg/kg body weight)	To observe the combined effects of caffeine and MDMA
D- Nicotine + MDMA group	8	Nicotine (50mg/kg body weight) and MDMA (10mg/kg body weight).	To observe the combined effects of nicotine and MDMA

water and then rinsed in two changes of Tris buffered saline. Sections were incubated with anti-glial fibrillary acidic protein primary antibody at room temperature (25℃) overnight, and subsequently biotinylated immunoglobulin secondary antibody for 30 min. Sections were further incubated for 10 min in horseradish peroxidase polymer, and developed in 3,3′-diaminobenzidine for 5 min. The sections were counterstained in Mayer's haematoxylin for 2 min, and carefully cover-slipped with glycerol gelatine mounting media.

Neurotransmitters and Enzymes Assay

Fresh prefrontal cortex tissues were kept in plain sample bottles containing phosphate buffered saline (PBS, pH ~7.4); and thereafter homogenized in sucrose. For each sample, the homogenate was centrifuged and the supernatant collected. The supernatant was assayed for neurotransmitters: dopamine, serotonin, and γ-amino butyric acid (GABA). Also assayed were selected enzymes- succinate dehydrogenase, cytochrome C oxidase and lactate dehydrogenase. Elisa bioassay kits were used for the tests and the procedure for each specific assay type was followed.

Statistical analyses

Results were analysed and presented as mean values using the Graphpad prism (version 8) with one-way analysis of variance (Tukey's post-hoc test was further carried out) to consider the levels of statistical significance of values in the treated groups relative to the control ($p<0.05$).

RESULTS

Neurotransmitters

Gamma-Butyric Acid

The mean GABA level of the control (0.2270± 0.002) was significantly lower ($p<0.05$) compared with the caffeine + nicotine (0.2355±0.003), and the nicotine + MDMA groups (0.2360±0.004). The caffeine + MDMA group GABA level (0.2357±0.004), was not significantly different ($p>005$) compared to the control group (Fig. 1A),

Glutamate

The mean glutamate levels of the control (0.2382±0.005), caffeine + Nicotine (0.2323±0.004), caffeine + MDMA (0.2433±0.006) and the nicotine + MDMA (0.2482±0.007) were not statistically different ($p>0.05$) (Fig. 1B)

Dopamine

The mean dopamine levels of the control (0.1308±0.003), caffeine + nicotine (0.1390±0.005), caffeine + MDMA (0.1472±0.008), and the nicotine + MDMA (0.1483±0.008) were not statistically different ($p>0.05$) (Fig. 1C).

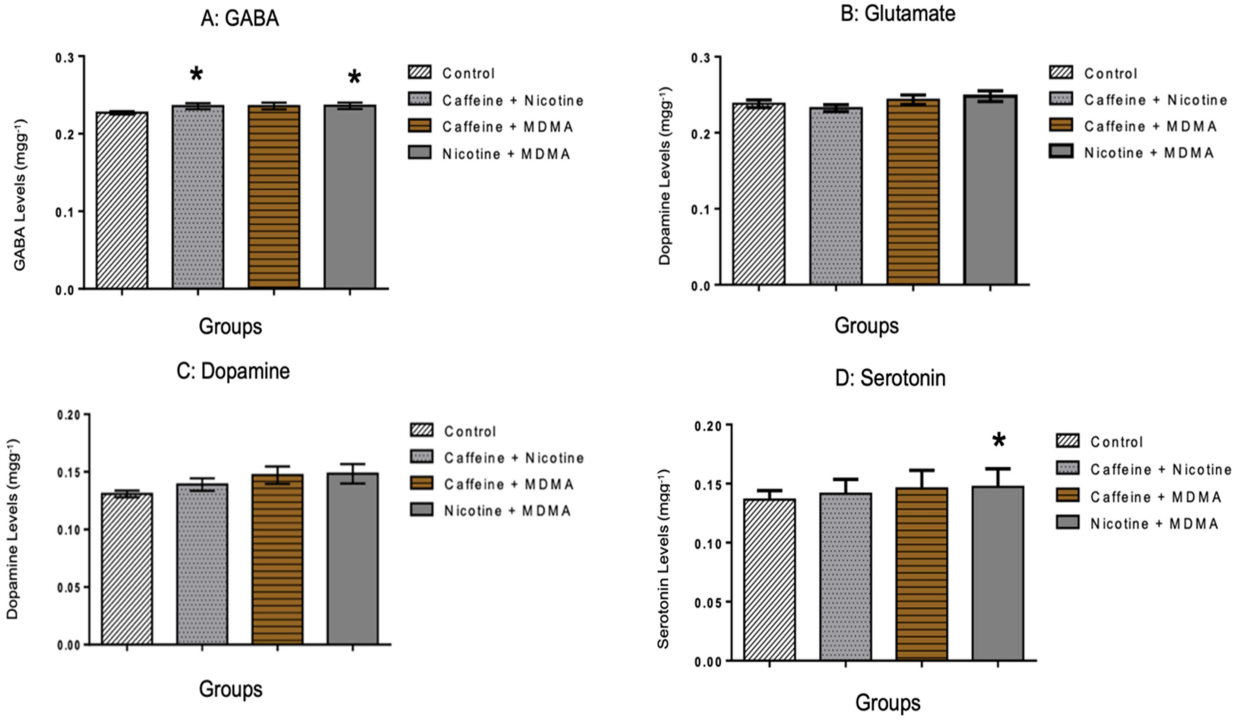

Fig. 1: The mean levels of specific neurotransmitters: GABA, glutamate, dopamine and serotonin in the prefrontal cortex of the control and treated groups. * Statistically different ($p<005$) compared to the control group using the GraphPad prism software

Serotonin
The mean serotonin level of the control (0.1363±0.003) was significantly lower ($p<0.05$) compared with the nicotine + MDMA (0.1473±0.006). The mean serotonin levels of the caffeine + Nicotine (0.1413±0.004) and the caffeine + MDMA (0.1458±0.006) were no statistically different (p>005) (Fig. 1D).

Enzymes
Cytochrome Oxidase
The mean cytochrome oxidase activities of the control (0.0100±0.001), caffeine + nicotine (0.0118±0.002), caffeine + MDMA (0.0148±0.003), and the nicotine + MDMA (0.0165±0.004) were not significantly different (p>005).

Succinate Dehydrogenase
The mean succinate dehydrogenase activity of the control group (0.0135±0.001) was significantly less than the nicotine + MDMA (0.0238±0.004) The mean succinate dehydrogenase activities of the caffeine + nicotine (0.0152±0.001) and caffeine + MDMA (0.0195±0.003) were not statistically different (p>0.05) when compared with the control group (Fig. 2B)

Lactate Dehydrogenase
The mean lactate dehydrogenase activity of the control group (0.0077±0.001) was significantly less than the Nicotine + MDMA group (0.0177±0.004). The mean lactate dehydrogenase activities of the Caffeine + Nicotine (0.0103±0.002) and Caffeine + MDMA (0.0135±0.003) groups were not significantly different (p>0.05) when compared with the control group (Fig. 2C).

Histology and Histochemistry
The cortical histoarchitecture was demonstrated using the H&E technique. Evidence of cellular morphological heterogeneity and trauma suggestive of karyorrhexis was observable in the caffeine + nicotine and nicotine + MDMA groups (Fig. 3). On the other hand, the caffeine + MDMA group did not present any evidence of morphological heterogeneity. There was evidence of reduced and aberrant patterns of Nissl expression in the caffeine + MDMA and nicotine + MDMA groups. The caffeine + nicotine also showed mild aberrations (Fig. 4).
There were areas of localised demyelination, suggestive of deleterious effects in the caffeine + MDMA and nicotine + MDMA groups when Luxol fast blue was used to demonstrate cortical myelin (Fig 5). The glial fibrillary acidic protein (GFAP) immunolabelling showed observable astrocyte reactions, indicative of neuroinflammation in the caffeine + nicotine and caffeine + MDMA groups (Fig. 6)

DISCUSSION

Analysis of cortical GABA was considered important. GABA, an amino acid, serves as the main inhibitory neurotransmitter in the brain; modulating functions that help ensure smooth movements in striatal neurons in the direct and indirect basal ganglia

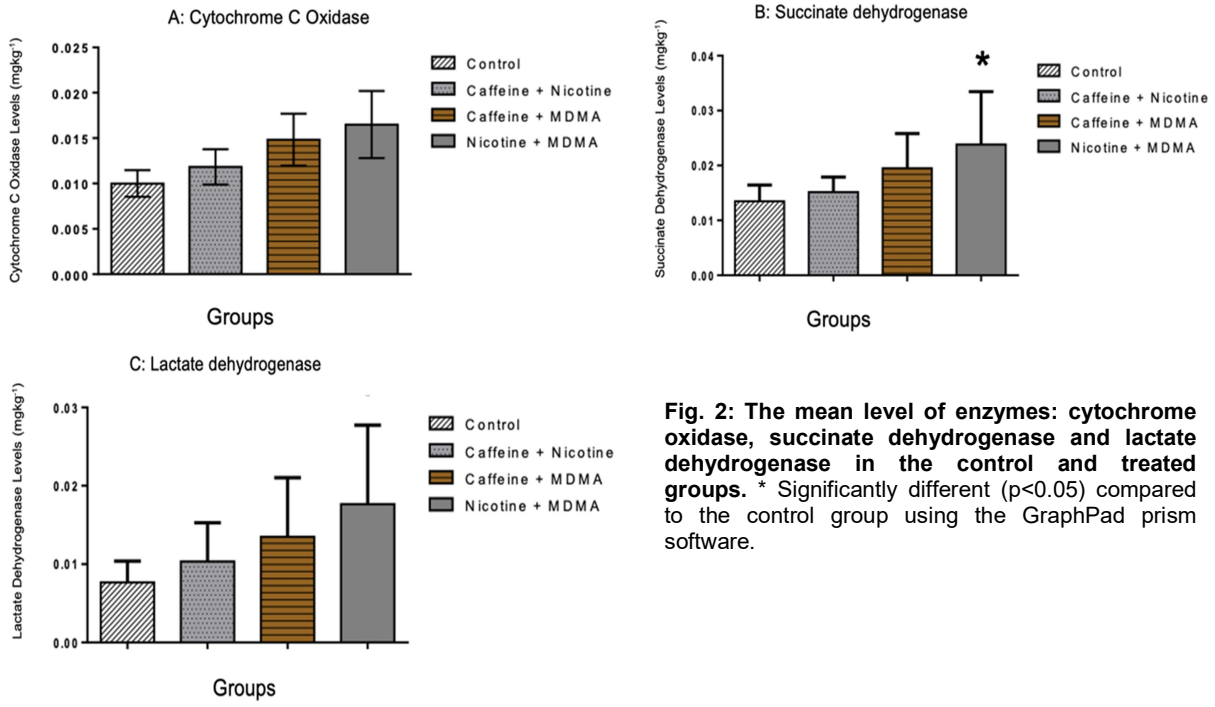

Fig. 2: The mean level of enzymes: cytochrome oxidase, succinate dehydrogenase and lactate dehydrogenase in the control and treated groups. * Significantly different ($p<0.05$) compared to the control group using the GraphPad prism software.

pathways, and at the level of the spinal cord while participating in and maintaining respiratory rate (Jewett and Sharma 2021). The level of GABA in the prefrontal cortex was statistically significa-nt higher in the caffeine + nicotine and nicotine + MDMA groups compared with the control. These results aligned with the report that administration of two psychoactive substances caused an increase in the levels of GABA (Owolabi et al. 2017); also, nicotine over-stimulates the glutamate receptors in GABAergic neurons, which in turn increases their output (Kim et al. 2019).

because of its broad modulatory roles in the neural circuitry of behaviour and emotion. Its analysis is therefore quite relevant to this study and its inferences (Berger et al. 2009). Serotonin level was significantly higher in the nicotine + MDMA group when compared with the control group. However, there were non-significant increase among the other test groups compared to the control group, which could be attributed to MDMA indicted in the greater release of serotonin (Rothman et al. 2000), and that MDMA enhances the release of neurotransmitters-

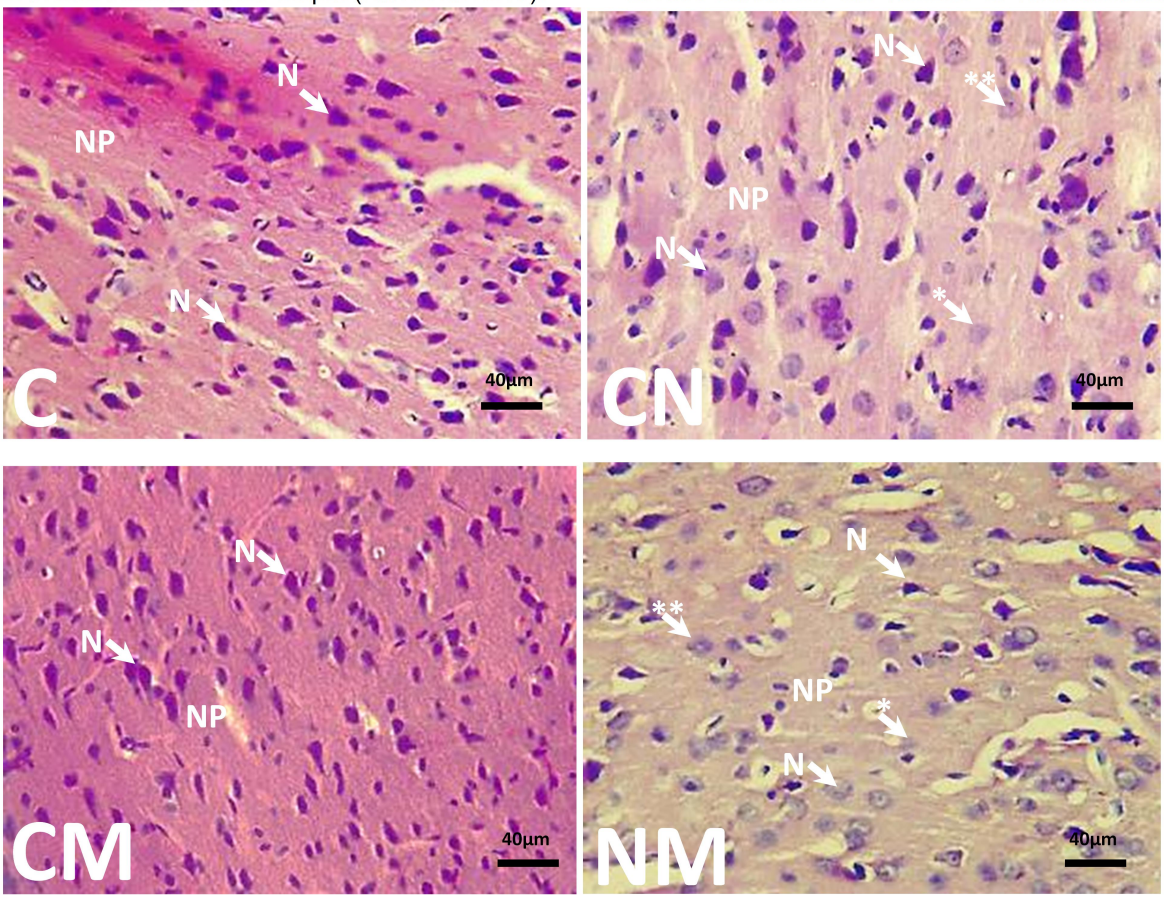

Fig. 3: Photomicrographs of the prefrontal cortex of the experimental animals across all groups. Some cortical cells showed signs of cellular trauma including karyolysis (*) and karyorrhexis (**). C = Control, CN= Caffeine + Nicotine, CM= Caffeine + MDMA, NM= Nicotine + MDMA, N= Neuron, NP= Neuropil. H&E, ×400

Dopamine is an organic chemical that functions as a hormone and a neurotransmitter (Drozak and Bryła 2005). It plays several crucial roles in the brain and body. Dopamine's activity can be influenced by the effects of caffeine and MDMA. Analysis of dopamine levels showed non-statistically significant increase in all groups compared to the control group. Despite the fact that the observed difference in this study was not statistically significant, it has been reported that the co-administration of both substances had greater effect on the extracellular dopamine level than when administered separately (Górska et al. 2018).

In the central nervous system, serotonin plays critical roles in regulating almost every human behaviour

dopamine and serotonin (5-HT) (Gough et al. 1991) and blocks their reuptake (Berger et al. 1992), resulting in increased dopamine and serotonin levels within a synaptic cleft. Nevertheless, nicotine alone reduces brain serotonin levels (Bhalsinge et al. 2017).

Glutamate is involved in synaptic plasticity and in cognitive functions such as learning (Kennedy 2013). In the present study, there was no significant difference in glutamate level among the groups. The caffeine + MDMA and nicotine + MDMA groups showed a non-significant increase in the glutamate level when compared to the control. This is similar to the findings that MDMA 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 (Anneken et al. 2016).

Succinate dehydrogenase is an enzyme complex that is responsible for cellular respiration (Rutter et al. 2010). It also plays a role in oxygen level sensing and tumour suppression, and is known as markers for oxidative stress (Tretter et al. 2016). Results showed that succinate dehydrogenase activity was statistically significant in the nicotine + MDMA group showed a statistically significant increase in the nicotine + MDMA group. This result indicates the neurotoxic effects of MDMA (Walker et al. 1999), which might induce tissue damage. On the contrary, nicotine can reduce the levels of lactate dehydrogenase (Ding et al. 2012). The lactate dehydrogenase activity of the caffeine + nicotine, and caffeine + MDMA groups were non-significantly elevated compared to the control group, which could be as a result of caffeine being able to increase the level of lactate dehydrogenase (Machado et al. 2010).

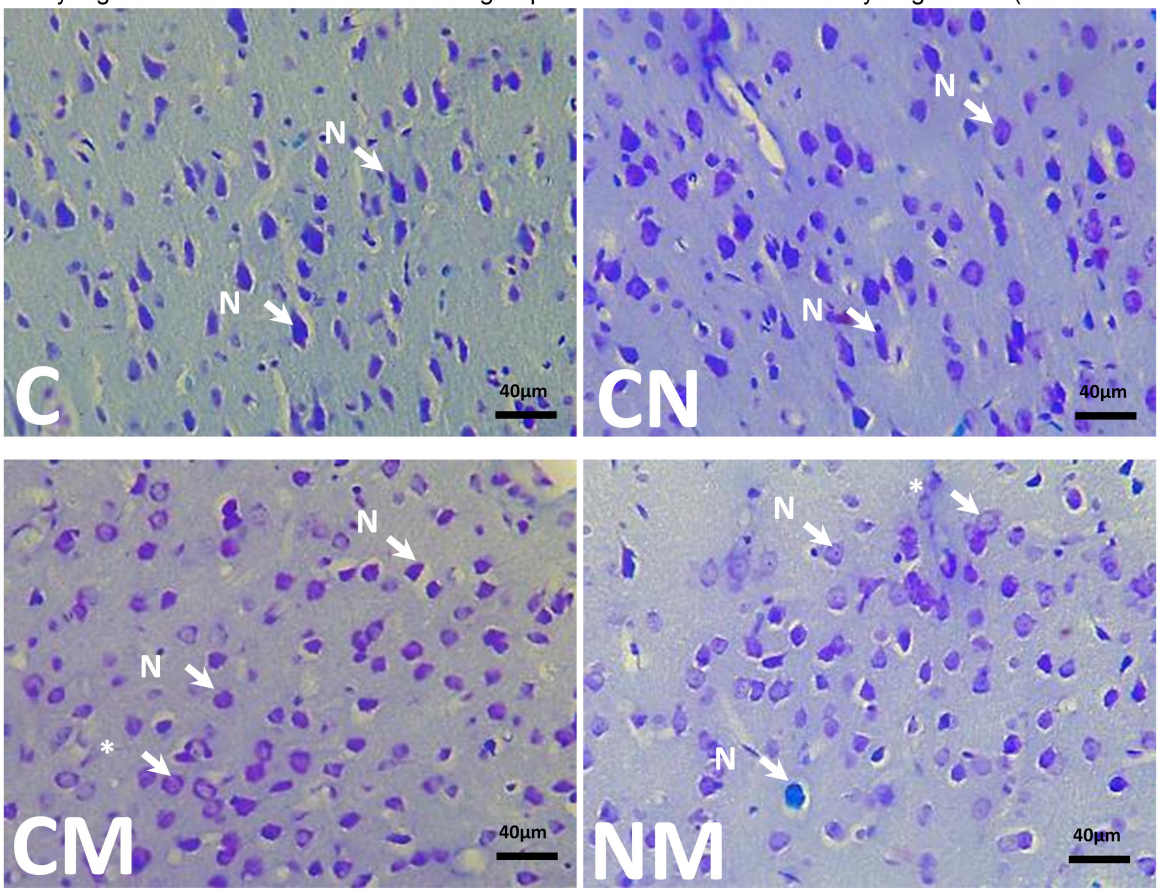

Fig. 4: Photomicrographs of the prefrontal cortex of the experimental animals across all groups. The cresyl fast violet (CFV) histological technique is being used to demonstrate the prefrontal cortices Nissl bodies to observe the functional integrity of the cortical cells (N). Nissl expression in certain cells was compromised (*). C = Control, CN= Caffeine + Nicotine, CM= Caffeine + MDMA, NM= Nicotine + MDMA, N= Neuron. CFV, ×400

relative to the control group. This might be an indicator of a deleterious effect of these agents. A previous finding had indicated that nicotine and MDMA induced oxidative stress, because they increased the production of reactive oxygen species, which in turn is associated with carcinogenic transformation, cell toxicity and deoxyribonucleic acid (DNA) damage (Han and Chen 2013).

Lactate dehydrogenase found in all living things, catalyses the conversion of lactate to pyruvic acid (Farhana et al. 2020). It serves as a marker for tissue damage and as such is released when there are injuries. Results of the lactate dehydrogenase activity

Cytochrome C oxidase or complex IV is a large transmembrane protein complex that can be found in the mitochondria of eukaryotes (Castresana et al. 1994). 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 (Liu et al. 1996). The results obtained from the analysis showed non-significant cytochrome C oxidase activity in the treated groups relative to the control, and which aligns with report that MDMA could induce neuronal death (Barbosa et al. 2016).

The prefrontal cortical histology of the caffeine + nicotine, and nicotine + MDMA groups showed effects on the neurons suggesting deleterious actions of the administered agents. There were also synthesis might be just functionally compromised in certain neurons. However, if these aberrations are taken in line with observations on the cellular morphologies, certain other cells might be showing

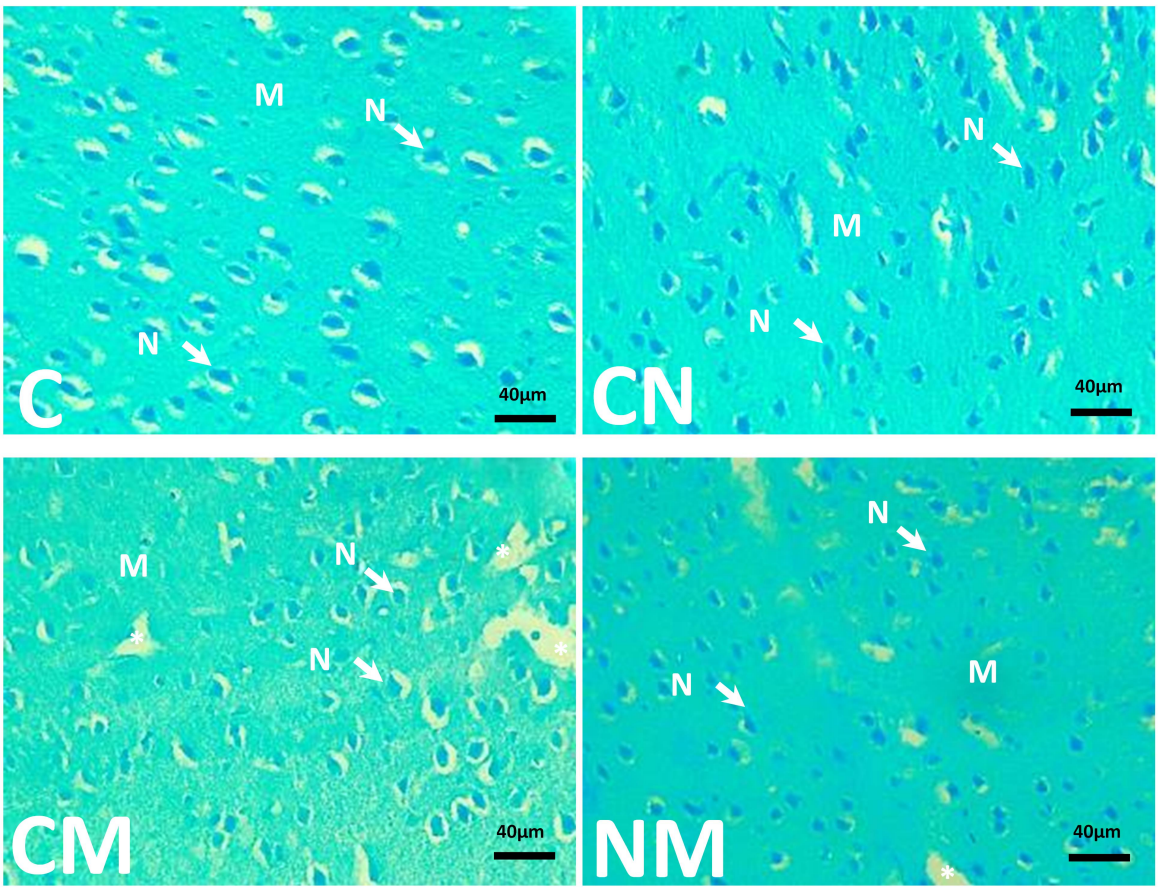

Fig. 5: Photomicrographs of the prefrontal cortex of the experimental animals across all groups. There are areas of localised demyelination within the tissue in the caffeine + MDMA and the nicotine + MDMA groups (*). C = Control, CN= Caffeine + Nicotine, CM= Caffeine + MDMA, NM= Nicotine + MDMA, N= Neuron, MY= Myelin. LFB, ×400

karyolysis and karyorrhexis of the cells. 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. While there is no previous study on the combined form of these psychoactive agents; a study on nicotine had previously suggested that its chronic use might cause neuronal death in the striatum and the hippocampus (Ijomone et al. 2015).

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. The cortex in the caffeine + MDMA, and nicotine + MDMA groups expressed Nissl less abundantly relative to the control. It is therefore suggestive that these cells were functionally compromised in these groups. The reduced protein 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. No study obtainable had previously considered the Nissl bodies under these conditions and similar context; however, useful is the work of Budzynska et al. (2018) that reported oxidative stress attributable to the combined ingestion of MDMA and nicotine.

Myelin is generally demonstrated in all the treated groups and there was only evidence that suggest mild disruptions in myelin distribution. In the caffeine+ MDMA group, there were localized areas of compromised myelin integrity which might indicate localized areas of fibre damage and consequently loss of myelin materials that could be associated with the processes. This is in line with the reported induced oxidative stress effects of the co-

administration of psychoactive agents, especially MDMA and nicotine (Budzynska et al. 2018).

The pattern of astrocyte demonstration using GFAP expression was random in the groups. There were no localized areas of abnormal astrocyte aggregations that could be characteristic of a major localized lesion. However, despite the random distribution of the astrocytes in all the groups, astrocytes were prominently demonstrated in caffeine + nicotine, and caffeine + MDMA groups compared to the control by virtue of their expressions of the GFAP. 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, this brain parts therefore responded to the drugs effect in manners that indicative of an assault. While the enhanced expression might be mild compared to situations of major intoxications such as heavy metal poisoning (Sofroniew 2020); it is still important to emphasize 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.

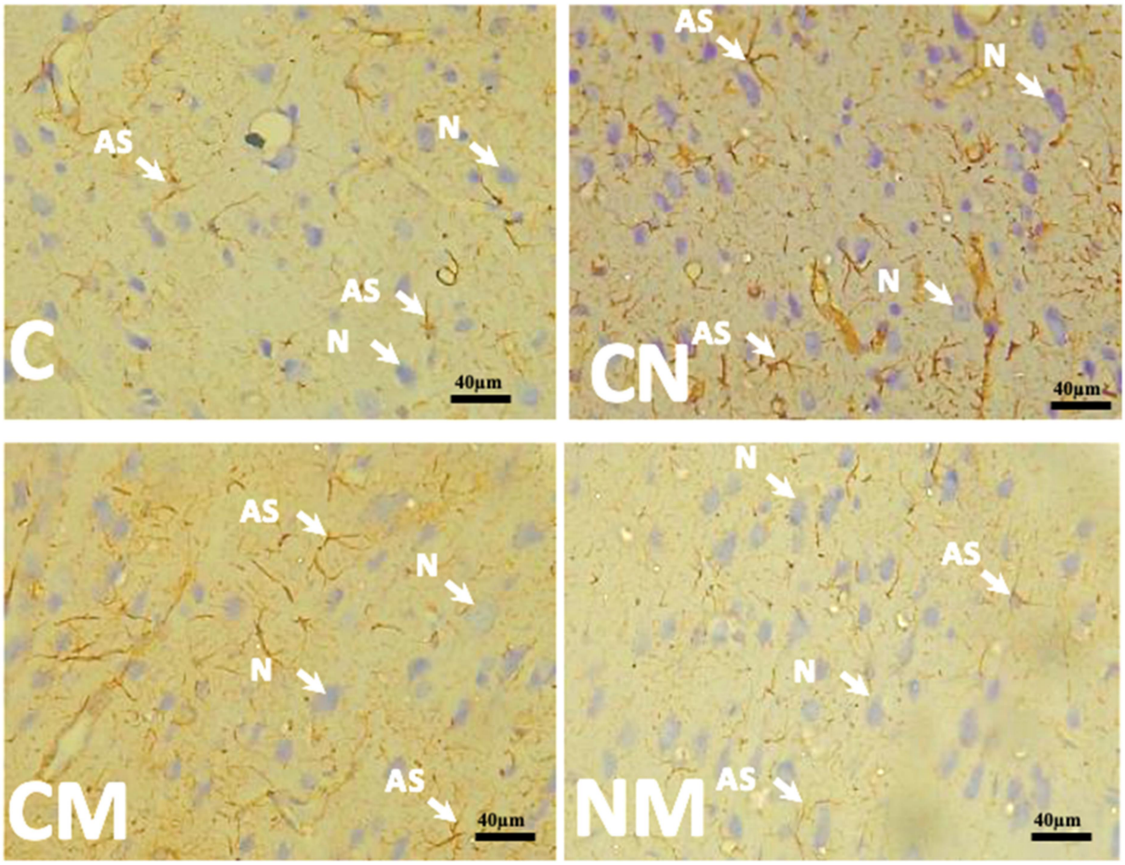

Fig. 6: Photomicrographs of the prefrontal cortex of the experimental animals across all groups. Astrocytes were prominently demonstrated in the caffeine + nicotine and the caffeine + MDMA groups compared to the control. These observations are indicative of astrocyte reactions. C = Control, CN= Caffeine + Nicotine, CM= Caffeine + MDMA, NM= Nicotine + MDMA, N= Neuron, AS = Astrocytes. GFAP, ×400

Administering caffeine, nicotine and MDMA had effects on brain cells. The neurotoxicity effects may be based on the disruption of neurotransmitters reuptake. This did not altogether lead to cell death in all instances. In this study neurotransmitters' imbalances were associated with aberrations in the morphology of neurons and aberrations in the expression of Nissl bodies and myelin substances. Neurotoxicity as observed was also linked to astrocyte reactions. When these astrocyte reactions are taken into consideration along with the observed effects on morphologies of the cells and Nissl expression; it is apparent that the administered agents had observable deleterious effects.

Conclusion

The combination of caffeine and nicotine as well as caffeine and MDMA caused observable neuroinflammation as marked by astrocyte reactions. Combination of psychoactive agents generally elevated neurotransmitters activity. Nicotine and MDMA combination also significantly elevated the activities of succinate dehydrogenase and lactate. Caffeine and nicotine or nicotine and MDMA significantly elevated GABA activities in the frontal

cortex. Nicotine and MDMA significantly elevated serotonin levels in the frontal cortex. These are indicative of synaptic dysfunctions. Morphological and functional changes included neuronal morphological heterogeneity, aberrations in Nissl expressions as well as mild aberrations in myelin integrity. Evidence collectively showed that combined ingestion of these psychoactive agents had deleterious effects on neurons, glia and neurotransmitters.

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Nil.

Conflict of Interest
None declared.

Authors Contribution
All authors, VCO, SOF, OEO, JOO, contributed equally to the study by participating in every stage of the work including conception, design, implementation, results analysis and writing of the article.

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