



Caffeine, Nicotine and MDMA Effects on Retina suggestive of Potential Functional Consequences

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors made substantial contributions to the conception and design of the work. Author JOO carried out the lab work including experimentation, acquisition, analysis, and interpretation of data for the work; Author POO supervised the work, conducted critical appraisal of the project, and contributed to data analysis and writing of the manuscript. Both authors contributed to writing the final reports and approved it for publication. Both authors read and approved the final manuscript.

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ABSTRACT

The implications of the use of psychoactive substances, that are usually abused on the retina are yet to be adequately explored. The retina is neural in nature. Most investigations on psychoactive agents have only studied their effects on the brain and behaviour. The mechanisms employed by these agents in producing their effects on the brain suggest that the retina, being neural, might also be significantly affected by the use of the substances. This research investigated the effects of the prolonged use of caffeine, nicotine and 4-methylenedioxymethamphetamine [MDMA] on the retina. Juvenile male experimental Wistar were grouped and administered the lower and higher dose of each agent while a reference group remained as the Group A. Groups B and C received the lower [30mg/kg body weight] and the higher [50mg/kg body weight] doses of caffeine respectively; Groups D and E received the lower [10mg/kg body weight] and higher [20mg/kg body weight] doses of nicotine respectively while Groups F and G received the lower [20 mg/kg body weight] and the higher [40 mg/kg body weight] doses of MDMA respectively. The substances had effects on the thickness of the retina with higher doses in each instance causing reductions in retina thickness; the

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patterns of GFAP expression were also aberrant with the MDMA treated groups being most aberrant. There was no sign of extensive loss of any type of retinal cells. Rhodopsin expression generally demonstrated active rods and provided insight into relatively healthy cones. There is evidence that these agents altered retina thickness and GFAP expression but without extensive disruptions to serve as pathological hallmarks of retina degeneration. The consequences of these might be further investigated.

Keywords: Retina; caffeine; nicotine; MDMA; vision.

1. INTRODUCTION

The aim of the investigation was to observe possible specific structural, histochemical, and biochemical changes associated with various doses of prolonged use of caffeine, nicotine and MDMA on the retina of experimental animals, thus modelling the potential effects of these substances on vision. The retina, especially in the mammal, is a highly developed structure. It forms the innermost eye coat that is primarily responsible for photoreception and the processing thereof. Retinal degeneration is a relatively frequent occurrence that has been linked to ageing [1], genetic factors [2,3] and number of risk factors including medications [4], drugs and nutrition [5]. Interestingly, the specific roles and mechanisms of the risk factors require conscientious investigation to establish more specifically their effects and the consequences of such. Caffeine and nicotine (in addition to alcohol) are world's most popular or rather notorious psychoactive agents that are legally consumed [6]. MDMA [4-methylenedioxymethamphetamine] is illegal, yet still largely consumed by individuals [7], and as such remains a major drug of research and health interest.

Retina degeneration is generally considered a neurodegenerative disease. Cases were also found to be characterized by ischemia-induced excitotoxicity and loss of retinal neurons. It has been postulated that psychoactive substances, due to their abilities to influence neurotransmitters and neuronal communications and functions, could have effects on retinal structural and functional integrity, especially as it concerns its toxicity-induced degeneration. Caffeine is the world's most popularly ingested legal psychoactive agent or stimulant, ingested as an additive to soda, food drinks and pharmaceutical drugs including analgesics. Its use during pregnancy has been reported to have effects on the neural tissues. Caffeine can influence macular circulation [8,9]; and the implications of this on retinal integrity should be

understood in habitual users. Also, it was suggested that there could be significant links between caffeine and central serous chorioretinopathy (CSCR) [10]. Caffeine LD 50 has been reported to be about 150-200mg/kg body weight in humans and Wistar rats [11,12]. The average coffee drinker in the United States for instance consumes about 4mg/kg body weight per day [13]. It is however logical to appreciate the fact that this value could vary greatly among individuals based on caffeine sources. There is a probability of higher values in certain instances especially when caffeine is available in purified forms that can be used at higher doses.

Nicotine is contained in a number of natural herbs, fruits and products especially tobacco, the kola nut, and other plants. While smoking is a habit-and-lifestyle health challenge globally, kola consumption is, in addition, a major issue in certain other places like Nigeria. Nicotine has been reported to have potential to compromise retinal functional integrity [14]. It is however still important to examine its specific effects on the retina structural and functional integrity. In fact the ECV [14] called 'for further research to be done into the effects of nicotine on the human eye'. Nicotine LD 50 vary between humans and research mammal models- 50mg/kg body weight, 3mg/kg body weight and 0.5-1.0 mg/kg body weight for rat, mice and humans respectively [15]. MDMA is a highly addictive psychostimulant drug [16]. It is commonly abused. It has the potential to damage the retina. Yet, it remains poorly studied in terms of specificity and nature of damage [17]. It is therefore important to understand its specific effects on the developing retina. MDMA LD50 with intraperitoneal administration is 55 in rat and 57 mg/kg in mouse, respectively [18]

2. MATERIALS AND METHODS

2.1 Experimental Animals and Grouping

Juvenile Wistar rats, male, fifty-six(n=56) and weighing 120g on the average were distributed into seven (7) groups of eight animals. The

Groups were named A-G with the Group A as the control group animals that were only fed ad libitum and given placebo in order to serve as the standard reference for the results that were obtained from other animal groups. Animals in the Groups B-G were treated with the agents of study dissolved in water to achieve the standards regimen as indicated in the research design. Animals were administered the daily dosage once daily between the hours of 16:00 and 18:00 for the sixty (60) days that the treatment lasted. Administration was done by using oral gavage.

The grouping patterns, dosage administration and the rationale are provided as follows:

2.1.1 Group A-Control

The control animals were fed ad libitum and given a placebo. This group serves as the reference group for the other treated groups.

2.1.2 Group B- Animals were administered the lower dose of caffeine

The animals in this group were administered the caffeine lower dose of 30mg/kg body weight daily using suitable oral gavage for 60 days. This treatment was to model and provide information on the nature of caffeine effects, at the lower dose, on the retina of the animals.

2.1.3 Group C- Animals were administered the higher dose of caffeine

Animals were administered 50mg/kg body weight daily, for the 60 days of caffeine administration to observe the effects of the higher dose of caffeine on the retina of the animals.

2.1.4 Group D- Animals are administered the lower dose of nicotine

Animals were administered 10mg/kg body weight to assess the specific effects of the substance on the retina.

2.1.5 Group E- Animals are administered the higher dose of nicotine

Animals were administered 20 mg/kg body weight to assess the specific effects of the substance on the retina.

2.1.6 Group F- Animals are administered the lower dose of MDMA

The lower dose of MDMA – 20 mg/kg body weight were administered to the Group F animals daily for the duration of treatment.

2.1.7 Group G- Animals are administered the higher dose of MDMA

The higher dose of MDMA - 40mg/kg body weight was administered to the Group C animals, orally, daily throughout the period of animal treatment.

2.2 Animal Care, Treatment, Tissues Excision

Animals were euthanised after the period of treatment and remains were properly disposed using the institutional facility. Following sacrifice, the eyes were carefully exercised and fixed for a short time in formal saline. The dissection microscope was used to dissect the eye and separate the lens from the eye coats. The tissues were thereafter prepared for cryofixation and cryosectioning (~ 10µm thickness). Sections were mounted on histological slides and arranged in plastic slide boxes and preserved in the deep freezer until further treatments for histological and histochemical demonstrations. The mounted and fixed cryostats sections were processed using suitable histological and histological procedures as follows:

2.3 Specific Research Techniques

(1). Haematoxylin and Eosin staining technique (Sheehan and Hrapchak, 1980) for structural demonstration of the retinal overall histological histoarchitecture in order to measure the relative thickness and observe the structural organisation of the tissue.

(2). Glial acidic fibrillary protein [GFAP] special histochemical technique for structural identification of cellular morphologies and integrity of processes. Cryostat sections (~20µm) of the frozen and fixed tissue (retina and supporting tissues) were mounted on glass slides; followed by GFAP protocol as described in the work of Benarroch *et al.*, [18]. The blocking reagent was applied to the tissue specimen followed by incubation in an enclosed chamber for 5 minutes. Specimen on each slide was gently rinsed with 1X Rinse Buffer for a 15-30 seconds. Dilution of primary antibody was applied over the specimen followed by incubation in an enclosed chamber at room temperature overnight. Specimen was rinsed. The secondary antibody was applied to the specimen and incubated in an enclosed chamber for 30 minutes. Specimen was rinsed. The DAB (chromogen reagent) was applied to the

specimen and incubated in an enclosed chamber for 30 minutes. Specimen was rinsed. The Haematoxylin counterstain solution was applied to the tissue and incubated in an enclosed chamber for 1 minute. Tissue specimen was rinsed. Tissue slides were drained of excess water and a coverslip was mounted using albumin.

(3). Immunohistochemical demonstration and analysis of rhodopsin using special histochemical technique in order to use the same to evaluate its expression as marker of rod health or degeneration tendencies and consequently the vulnerability of the cones. Rhodopsin was demonstrated using the immunohistochemistry method as described by Bobu *et al.*, [19]. Frozen eye sections, containing demonstrated the retina and the supporting tissues were cryo-sectioned (20m) and mounted on glass slide. These sections were taken through the IHC protocol for rhodopsin demonstration. Sectioned were first washed and blocked; then incubated overnight in the primary antibody. The sections were thereafter washed and incubated in the secondary antibody and counter-stained in DAPI. Following washing and draining if excess water, the slides were cover-slipped and examined under the microscope.

3. RESULTS

Histological and histochemical results were presented as photomicrographs across the animal groups with adequate labelling to illustrate the structures of interest and any changes that are observed to illustrate differences. Data were recorded and analysed as mean values per group. Values across the groups were compared with the control and other groups using the two-way ANOVA. The Version 6 of the GraphPad Prism statistical software was used to analyse results. Animals weights and the weights of the eyes were taken at the point of sacrifice; the mean relative eye (organ) weight (REW) was compared for possible statistical significance in

deference between values across the groups. Photomicrographs of the retina in its cross sections were taken. Thickness of the retina and its layer were taken in micrometres, using the Image J Software. The values are presented in bar charts with signs to indicate statistical significance wherever applicable ($P \leq 0.05$).

The current research shows that the thickness of the retina (Fig. 1) was affected in manners that showed that it could be attributable to the effects of the administered substances (Figs. 1A, 1B, 1C, 1D). The thickness of the retina was reduced whenever high doses of caffeine, nicotine and MDMA were administered (Fig. 1A). These are indications that these agents had effects on the neural retina and the effects included the relative thinning of the retina. It could therefore be stated that high doses of the agents caused relative thinning of the retina. Not only was the thickness of the retina reduced relative to the standard control that was not administered these agents; they were also reduced relative to each instance where the lower doses of the agents were administered to the animals. This would imply that the use of high doses of caffeine, nicotine and MDMA over a relatively prolonged period of time could result in relative thinning of the retina both relative to non-treated experimental animals as well as other who took less quantities.

4. Legend

- Group A-** Control Group.
- Group B-** Animals were administered the lower dose of caffeine.
- Group C-** Animals were administered the higher dose of caffeine.
- Group D-** Animals are administered the lower dose of nicotine.
- Group E-** Animals are administered the higher dose of nicotine
- Group F-** Animals are administered the lower dose of MDMA
- Group G-** Animals are administered the higher dose of MDMA

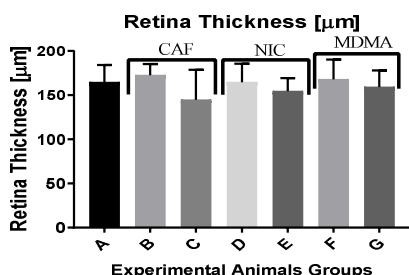


Figure 1A: Bar chart showing retina thickness across the experimental animal groups A-G (µm). Retina thickness varied in the treated groups relative to the control, Group A. Retina thickness was generally reduced when higher doses of caffeine, nicotine and MDMA were administered relative to the control and the group administered the lower doses in each instance. (CAF- Caffeine-treated groups; NIC- Nicotine-treated groups; MDMA- MDMA-treated groups)

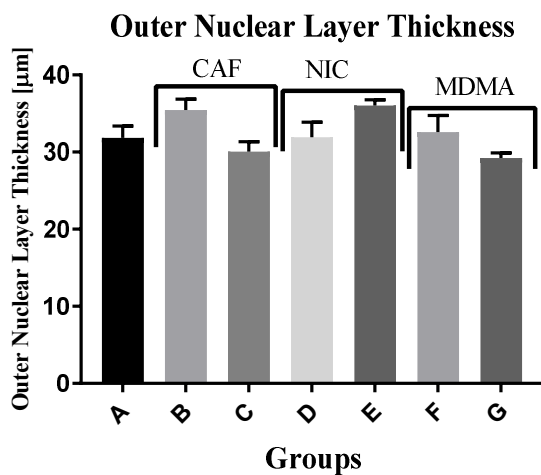


Figure 1B: Bar chart showing the thickness of outer nuclear layer (ONL) of the retina across the experimental animal groups A-G (µm). ONL thickness varied in the treatment groups relative to the control, Group A. In each instance, dose dependent changes are observable for the agents. ONL was relatively thicker when lower dose of caffeine and MDMA were administered. (CAF- Caffeine-treated groups; NIC- Nicotine-treated groups; MDMA- MDMA-treated groups)

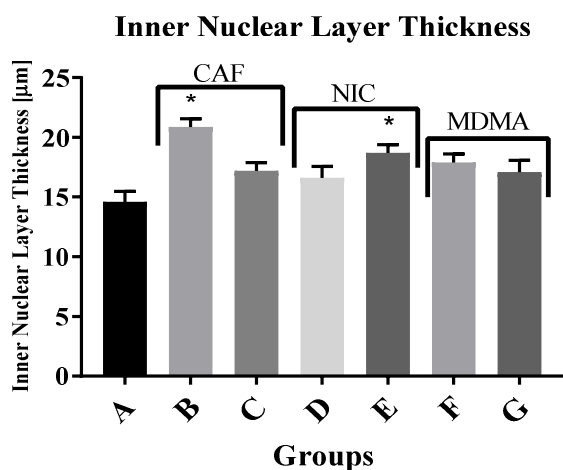


Figure 1C: Bar chart showing the thickness of inner nuclear layer (INL) of the retina across the experimental animal groups A-G (µm). There was general increase in the INL thickness in the treated groups relative to the control, thus attributable to the treatment(* indicates statistical difference in value relative to the control, group A). Also, pattern of aberration varies for each agent administered. (CAF- Caffeine-treated groups; NIC- Nicotine-treated groups; MDMA- MDMA-treated groups)

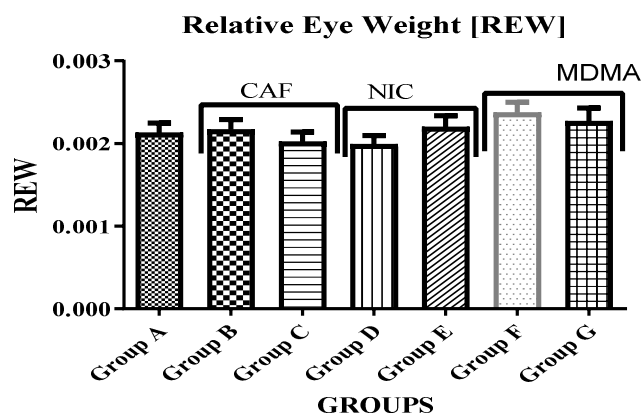


Figure 1D: Bar Chart illustrating the Relative Eyes Weight of the Groups A-G experimental groups of animals; values vary across the groups based on regimen, but variations were not statistically significant ($p \leq 0.05$). (CAF- Caffeine-treated groups; NIC- Nicotine-treated groups; MDMA- MDMA-treated groups)

Fig. 1. Morphological and histological results including Whole Retina Thickness; Outer Nuclear Layer Thickness and Inner Nuclear Layer Thickness

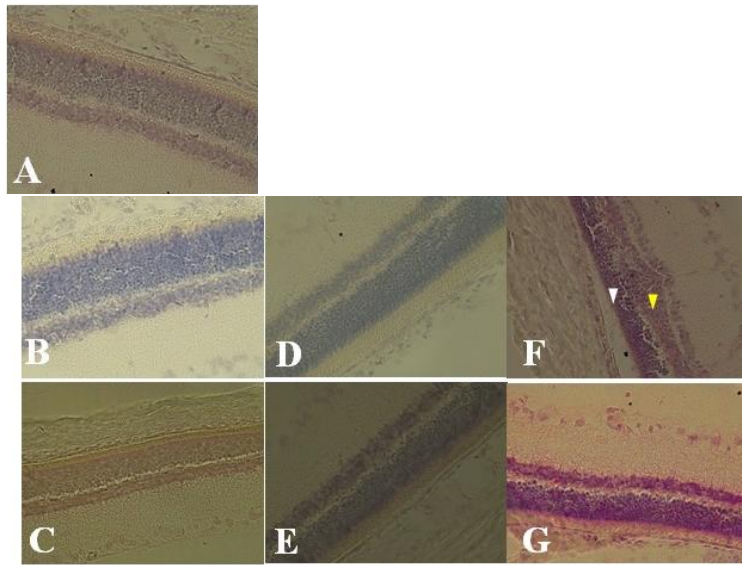


Fig. 2. Photomicrographs of the retina of the experimental animals showing the layers of the retina and its characteristic features at various magnifications. Results does not show specific observable extensive changes across the groups especially in terms of the retinal cells, their layers and spatial organizations as well as the morphologies of the cells. There is relative thinning in groups that were administered the high dose of psychoactive agents compared to those administered the lower dose. Partial retinal detachment with accompanying histoarchitectural disruption is also observable in the group treated with the lower dose of MDMA (Group F-yellow and white arrow heads) (Magnification = x400)

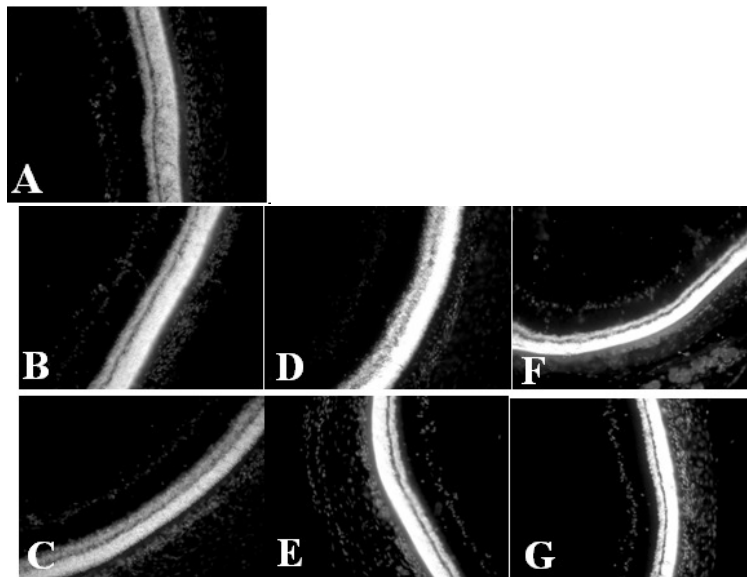


Fig. 3. Photomicrographs of the retina of the experimental animals suing DAPI (counterstain for use in multicolor fluorescent techniques) to demonstrate the cells in the layers of the retina. Results do not show any observable significant changes across the groups especially in terms of the retinal layers cells and their densities, their layers and spatial organizations. (Magnification = x400)

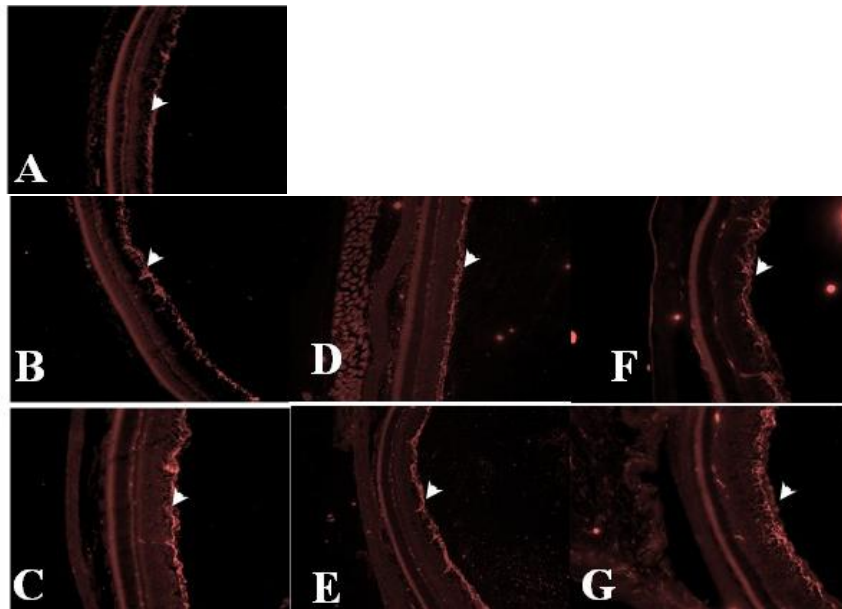


Fig. 4. Photomicrographs of the retina of the experimental animals using GFAP to demonstrate the ganglion cells in the layers of the retina using the digital confocal microscope. Results do not show any observable significant aberrations across the groups especially in terms of the retinal GFAP expression on the ganglion cells (arrowhead). Patterns of expression however very relatively based on agent and dose. GFAP expression was relatively more prominent with higher doses of caffeine, nicotine and MDM (C, E and G) relative to the lower doses (B, D and F respectively). The Groups E and G administered MDMA had increased prominence of GFAP expression in a relatively less compact pattern when compared with B, C, D and E in their patterns of GFAP. (Magnification = x400)

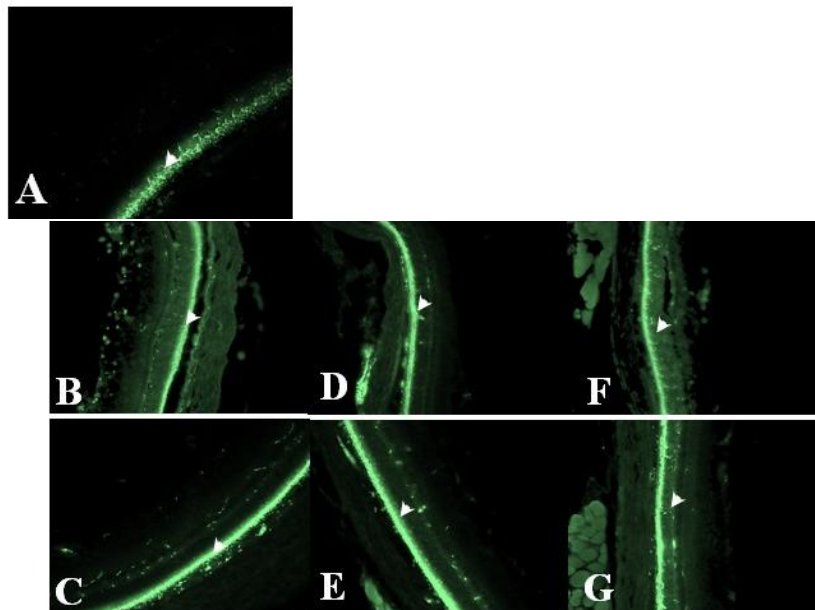


Fig. 5: Photomicrographs of the retina of the experimental animals demonstrating rhodopsin (arrowhead) in the retina using the digital confocal microscope. Results showed expression of rhodopsin in all the groups without observable significant aberrations. (Magnification = x400)

DISCUSSION

4.1 Caffeine, Nicotine and MDMA Effects on Retina Thickness and REW

Not many investigations have considered the effects of psychoactive agents on the retina and this current research might be one of the very few ones about this time. One of the few reports available, on human, reported that retinal nerve fibre layer (RNFL) thinning was associated with chronic alcohol and tobacco use [20]. MDMA was also reported to have influence on retina physiology in rats [21]. Another research during which experimental mice were administered nicotine reported that nicotine altered retinal pigment epithelial cells morphology and function, caused thinning of the outer nuclear layer and a damaged photoreceptor–RPE interface in manners that pointed to possible pathological effects of nicotine on retina [22]. Furthermore, there are reports that associated relative thinning of the retina with the chronic use of certain drugs including the hydroxychloroquine therapy in human subjects [23]. It was stated in the same report that this might be an early sign of retinal toxicity. In another investigation, which considered the retina thickness in eyes with autoimmune retinopathy the retina and certain of its layers exhibited thinning that was found to be statistically significant [24]. The current experiment, using experimental animals, which were administered selected psychoactive agents also exhibited thinning of the retina but that was not statistically significant. Relative thinning therefore might have to do with severity of the case in terms of higher dose ingestion and possibly accompanying longevity of exposure.

There were also variations in the specific patterns of effects of these agents on the inner nuclear layers and the outer nuclear layers of the retina in this experiment (Figs. 1B and 1C). Caffeine and nicotine significantly altered the thickness of the inner nuclear layer by increasing it relative to the control and other groups administered MDMA (Fig. 1C). These variations are expectedly attributable to the variations in the natures of these agents and their mechanism of actions. Thus, the specific nature of the effects of psychoactive agents on the retina would vary based on the nature of the actions of the agents. The REW [relative eye weights] values also showed variations with the group administered the MDMA being generally higher. This could be attributable to lower body weights relative to the eye or increase eye weights relative to the body;

albeit variations were not found to be statistically significant.

4.2 Caffeine, Nicotine and MDMA Effects on Retina Histology

It is important to note the relative prominence of the ganglion cells in the groups treated with MDMA- both at low and the high doses (Fig. 2). Also, in each instance of the administration of the psychoactive agents, there is relative reduction in the thickness in the outer nuclear layer of the retina, which contains the photoreceptor cells in particular. Another observation that is peculiar to this group is the partial detachment of the retina in this group with accompanying disruption of the retinal pigment epithelium and outer nuclear layer cells (Fig. 2E). When this observation is considered in connection with rhodopsin expression; it is rather insightful to note that the cells relatively normal expression of rhodopsin would indicate that the rods were relatively normal; hence the relatively thinning could be possibly be due to compaction or cell shrinkage. It therefore further implies that the effects of psychoactive agents in the neural retina is observable. In other words, these agents have effects on the retina and possibly by extension, on vision. It is therefore important to appreciate the need to consider vision when the effects of these substances are being elucidated and considered for the purpose of education, research and awareness among others. There are facts that there are influences on the neural retina- whether with identifiable pathological implications or not- should be considered in the use of these substances. Furthermore, these results might be investigated further to understand the underlying mechanisms and to explore potential implications of these seemingly mild changes over longer period of time. This might provide insight into the effects of the psychoactive agents on the visions of perennial users and the might be considered alongside other effects attributable to their uses on human health.

DAPI demonstration showed the arrangement of retinal cells in their layers across the experimental groups of animals (Fig. 3). There is the preservation of the basic layering patterns of the retina in all the treated groups especially relative to the control. There is therefore evidence to state that the psychoactive agents did not cause extensive disruptions or cell death in any layer of the retinal; since there was not observable extensive cell loss. While this implied

that the ingestion and the effects thereof of the psychoactive agents namely caffeine, nicotine or MDMA would not have caused extensive photoreceptors or other cells degeneration, as they were observable; it does not provide exhaustive insight into their functional attributes and conditions. Another fact, however, that is inferential in this instance is that the retinal cells were largely preserved within their specific layers. This is to state the administration of these agents did not produce an extensive and radical structural disruption of the retina within the duration of treatment.

4.3 Caffeine, Nicotine and MDMA Effects on GFAP Expression in the Retina

The ganglion cells in the retinas across were demonstrated in all the experimental animal groups- using the GFAP technique (Fig. 4). This showed that the ganglion cells in these experimental animals were preserved, even in the treated groups. It is however important to note certain slight deviations in the pattern of ganglion cells GFAP expressions based on the regimens. There is relatively increased prominence of the GFAP expression when the higher doses of the substances- caffeine, nicotine and MDMA were administered (Fig. 4 C, E and G). This suggested that the relatively higher doses of the psychoactive agents had effects on the ganglion cells layer and was responsible for the relatively increased GFAP expression. Another consistent observation across the groups is the variation in the pattern of the GFAP expression based on the administered substances with the most aberrant being the MDMA treated groups. These observations are indications that the agents, especially MDMA affected GFAP expressions. In the MDMA treated groups, the GFAP is expressed in a relatively diffused pattern. Since the GFAP expression demonstrates the relative spatial distribution of the ganglion cell; thus, the relatively diffused pattern would indicate spatial diffusion or elaboration of the ganglion cells in response to the treatment or exposure to MDMA. This observation is not an indication of any major established pathological observation in determining retinal functional and structural integrity. However, it is an indication that the agents had effects on these retinal cells. There is also, little known about the possible pattern of progression or the implication of such effects over longer period of time.

While the status the retinal pigment epithelium is being used as a very vital marker in several degenerative diseases and processes of the retina; the integrity of the ganglion cells layer is considered to be an important indicator in certain other diseases such as the glaucoma [25,26,27,28]. In the instance of glaucoma, the reasons for ganglion cells later damage is largely attributed to increased intraocular pressure. While the case in the instance of the current investigation is not glaucoma, it is still important to note how MDMA effects on the inner retinal layer is observed and to note the possible complications which might include visual impairment if these cells are eventually destroyed. This again emphasises why the effects of MDMA, or other psychoactive agents should be considered from multiple perspectives. MDMA is reported to cause retinal haemorrhage in a human case study, and this is being attributed to sudden rise in blood pressure [29]. Meanwhile, our current investigation did not model an acute but prolonged use; yet, the evidence that the MDMA use affected the inner retinal lining is observable. It is also important to emphasise that cell death is not the foremost early vital observation to predict glaucoma-linked vision impairment; rather, dendritic changes of the cell is a significant early change to note [30,31].

4.4 Caffeine, Nicotine and MDMA Effects on Rhodopsin Expression in the Retina

Rhodopsin expression is generally observed across the groups without a major observable aberration in the patterns of expression (Figure 5). The general expression of rhodopsin across the groups indicated that the rods are generally preserved in the retinas (Figure 5A compared with Figure 5B-G). To this end, photon absorption by the rods as a functional parameter would be generally preserved. Poor expression on the other hand would have been an indication that the rod photoreceptors are affected negatively or destroyed. On the other hand, overexpression of rhodopsin might be disruptive to the normal and adequate distribution of transducin, hence constituting a negative functional influence on the latter [32].

While retinal pigment epithelium degeneration is used as an indicator for actual or impending retinal damage; it is also known that the damage of ether would influence the damage of the other. Factors attributed to these include their high

oxygen tension as well as the rate and duration of photon flux [33]. It is also being suggested that rod degeneration, before the cone, is typical of retinal degeneration especially in the diabetic retinopathy [34]. Cone-rod dependence has been investigated and preservation of rods could actually help preserve the cones from degeneration [35]. Albeit, there are instances where the cones degenerate first and then the rods, such as in the cone-rod dystrophy [36].

In this instance, the integrity of the rod is being used to measure the state of the photoreceptors; noting that rods death typically has negative effects on the cones as well by promoting their deaths [37,38,39]. Therefore, the relative preservation of the rods is an indication of the relative preservation of the cones and other functions as well. Furthermore, these observations show that there is no evidence to suggest that the use of these substances of a moderate period of time could induce photoreceptors degeneration.

5. CONCLUSION

The experiment considered retina structural integrity considering its whole thickness, inner and outer layers thickness, rhodopsin and GFAP expression and found changes in retinal thickness and GFAP expressions that were attributable to the exposure. Each of caffeine, nicotine and MDMA had its peculiar effects on these parameters that increased with dose. MDMA affected ganglion cell's GFAP expression the most. This body of evidence might not be conclusive to indicate specific pathologies. However, they are pointers to changes that resulted from the effects of the psychoactive agents' use. This is what our findings could establish. These effects might on retina structural and functional integrity might have immediate and latter consequences vision. The latter should be a subject of further research endeavour.

ETHICAL APPROVAL

The experimental animals were housed in standard rat cages; welfare was ensured. Ethical approval was obtained from the Babcock University (Nigeria) Health Research and Ethics Committee (BUHREC872/19).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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