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Research Article

Chronic Caffeine Ingestion Improves Memory and Learning and Increases Neuronal Population and Dendritic Length in the Hippocampus of Adult Mice

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Summary: Caffeine is the most widely consumed psychoactive drug in the world, ingested as a natural component of chocolate, coffee and tea, and as an added component to soda and energy drinks. Here we assessed behavioral changes caused by chronic caffeine administration as well as morphological changes within specific regions of the adult mice brain: the hippocampus and amygdala. Twenty-four adult male albino mice were randomly divided into three groups. Caffeine was administered daily by gavage for 8 weeks at a dosage of 20 mg/kg for the low dose (LD) group and 60 mg/kg for the high dose (HD) group while the third group served as control (CNT). After the period of administration, neurobehavioral tasks were carried out; Morris water maze for learning and memory open field test and elevated plus maze test for anxiety. The mice were sacrificed; their brain tissues were harvested and processed for H&E, Cresyl violet, and Golgi staining, and assessed qualitatively and quantitatively. Quantitative data from the neurobehavioural tests and neuronal cell counts were expressed as means ± standard errors of means and compared across the groups using analysis of variance (ANOVA). Significance was set at $p< 0.05$. Mice in the high dose group learned faster and had a significantly increased number of platform crossings in the Morris water maze test. There was, however, a slightly increased level of anxiety in the caffeine-treated mice, compared to controls. Histo-morphometric analysis revealed a significantly increased number of pyramidal neurons in the hippocampus in the low dose group, but a decreased neuronal count in the amygdala of the low dose and high dose groups compared to controls. The pyramidal neurons in the hippocampus of the caffeine-treated mice had increased apical dendritic length compared to the controls. Our findings strengthen the available data suggesting that prolonged caffeine intake improves cognition, and this process could be mediated by promoting the growth of dendrites and an increased number of neurons. However, this is coupled with an increased tendency to be anxiogenic.

Keywords*:* Anxiety, Amygdala, Caffeine, Cognition, Hippocampus

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INTRODUCTION

Caffeine, a non-selective adenosine receptor antagonist, is the most widely used psychoactive agent in the world. Adenosine is considered a 'fine-tune' neuromodulator that has a generally inhibitory effect on neuronal activity including synaptic transmission and plasticity (Ribeiro et al., 2003). As an adenosine receptor antagonist, caffeine tends to increase neuronal excitability which results in heightened arousal and attention (de Mendonca and Ribeiro, 2001). The A1 receptor subtype is the primary target of caffeine and is most prevalent in the hippocampus, a region of the limbic system that has been closely linked to learning, memory, and emotion (Fredholm et al. 1999). The hippocampus plays roles in memory formation and consolidation. Some studies have clearly shown that caffeine improves learning abilities, memory, and spatial orientation in various tests. The effect of caffeine on memory consolidation has recently been reported in human recognition memory whereby a single dosing of 200 mg caffeine administered to healthy adults after training was sufficient to improve the detection of subtle changes made to the training items 24h later (Borota et al. 2014). Watanabe and Ikegaya (2017) showed that caffeine reversibly increases Sharp Waves (SW; sharp waves being a biomarker for memory) in acute hippocampal slices in a dose-dependent manner. Caffeine induced about a 100% increase in the event frequency of SWs at concentrations of 60 and 200 μM. This effect was likely mediated via adenosine A1 receptors inhibition in either the Cornus Ammonis 3 (CA3) region or the dentate gyrus. The effects of caffeine on cognition are mediated primarily by blockade of the A_1 adenosine receptor (A_1R), as antagonists of A_1Rs enhance induction and stability of long-term potentiation (LTP) in hippocampal CA1 (Dunwiddie and Masino, 2001).

Studies have reported that higher levels of caffeine consumption in children (Ruxton, 2014) and young adult males (Trapp et al., 2014) correlates with increased anxiety. Rats administered acute caffeine (Ardais et al., 2014) also display increased anxiety while consuming caffeine during adolescence. Ample evidence suggest that caffeine consumption can exert anxiety in children, adolescent and adults, although sparse studies have examined the long-term

effects of chronic caffeine consumption on anxiety-related behaviors (Mahdi *et al.*, 2019).

With regards to memory, caffeine has been found to improve attention on personalized acute consumption in humans (Lanini *et al*., 2015), and moderate doses of caffeine (20 mg/kg) in rodents resulted in enhancement of memory (Almosawi *et al.*, 2018). Chronic consumption caffeine intake reportedly prevented the cognitive decline associated with aging by promoting the growth of dendrites and spines in the hippocampal neurons of the adult mice brain (Vila-luna *et al*., 2012). Chronic administration of caffeine is thought to be a better model for evaluating the consequences of regular coffee intake than acute administration of caffeine (Ribeiro et al, 2003).

The morphological changes occurring in the normal (non-diseased) brain following long term administration of caffeine has been so far, overlooked. This is important in order to determine if the caffeine effects will be long-lasting or easily reversible following termination of administration. In this study therefore, we examined the effect of chronic caffeine administration in normal adult mice, on learning, memory and emotionality, as well as the morphological changes that occur in regions of the brain that bring about these changes that is, the hippocampus for learning and memory, and the amygdala for emotions/anxiety.

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MATERIALS AND METHODS

Animals and groupings: Thirty young adult male albino mice, about 3 months of age and weighing between 22 – 27g, were obtained from the animal house of the University of Ibadan and left to acclimatize for a week. Ethical approval for the study was obtained from the University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC/19/0006). The animals were then randomly assigned into three groups consisting of two experimental groups: low dose (n=10) and high dose (n=12) respectively and one control group (n=8). The mice in the low and high dose groups received 20 and 60 mg/ kg/ day of caffeine respectively (Olopade et al., 2021), obtained from Extra Pure Caffeine Anhydrous 98% powder (Laba Chemicals, USA) and dissolved in 0.3ml of tap water while the mice in the control group received the same volume of tap water only. The caffeine was administered daily by gavage, for eight weeks. Food and water were provided *ad libitum.*

Preliminary measurements: The body weights of the mice were measured weekly and they had a general physical examination, for general health, activity, and emotionality until the end of the study. Neurobehavioural assessments for cognitive functions - Morris water maze (MWM), and emotionality - Elevated plus maze (EPM) and Open-field tests, were carried out at the end of 8 weeks of caffeine administration. The tests were commenced 4 days before the termination of the caffeine administration because the Morris Water maze was carried out over 4 days. The sequence of neurobehavioural testing is given as follows: On the first day, the open field test was carried out, followed by the first day of the Morris water maze test. The next day, the Elevated Plus maze was carried out, followed by the Morris water maze test. On the third and fourth days, only the Morris water maze test was carried out.

Open Field Test: The open field is a white painted wooden box measuring 72cm by 72cm with black lines drawn on the floor to divide it into 18 cm by 18 cm squares. There is a centre square also measuring 18cm by 18cm. The mice were tested separately in the open field for a period of 10 minutes each to assess the following parameters of emotionality: grooming (that is, sets of heterogeneous constituents comprising face washing, body licking, paw licking, head and body shaking, scratching and genital licking), length of time spent in the centre square, length of time it spent freezing (i.e., staying in one position and not making any movements at all) and number of faecal boluses passed. These parameters were used to determine their anxiety level. On completion of the test for each mouse, the box was cleaned with 70% alcohol to prevent the subsequent mice from bias due to olfactory cues. (Olopade and Shokunbi, 2016).

Modified Morris Water Maze Test: The Morris water maze (MWM) tests hippocampal-dependent spatial learning and memory in rodents. The MWM consists of a circular pool of opaque water (120 cm in diameter, 30 cm in height) with a hidden circular escape platform (12 cm in diameter, 1 cm below the water level) which the mice must learn its location using contextual cues. The pool was marked North, South, East and West and the hidden platform was placed on the center of one of the four imaginary quadrants of the tank and maintained in the same position during all trials. Each mouse was dropped into the tank with its head facing the wall, allowed to swim freely and expected to search for the platform; the length of time it took to find the platform (in seconds) was recorded. If it did not find the platform after 120 seconds, the mouse was guided to the platform and allowed to stay there for 15 seconds. Each mouse went through four trials per day for three consecutive days. This test is a measure of the learning ability of the mouse. On the fourth day, a single probe trial was given to test the mouse's spatial memory retention in the water maze while the platform was removed. The mouse's memory of the initial location of the escape platform was measured by the time spent as well as its average speed in the target quadrant and the number of times it crossed the island zone where the platform was initially located. This was recorded as a test of memory retention/ability. (Angelucci *et al.*, 2002)

Elevated Plus Maze: The elevated plus maze tests anxiety-like behavior in rodents; it consists of two open arms (25 cm × 5 cm) and two enclosed arms of the same size at opposite sides of each other. The enclosed arms are surrounded by 15 cm high walls. The edges, 3 mm high, surround the open arms, minimizing the likelihood of animals falling from the apparatus. Both arms are 1m above the floor. Between the arms is a central square area (5 cm × 5 cm) where the mouse was placed. The entire apparatus was cleaned using 70% ethanol between subjects in order to eliminate olfactory clues. Each mouse was individually placed in the central square of the maze and allowed to freely explore the apparatus. The mouse behavior was recorded for the test period of 5 min and then analyzed. The number of entries per arm and the time spent in the open arms was recorded. An entry is recorded when all four paws enter the arm. The numbers of entries and time spent in the open arms reflect the general emotionality of the mice. A decreased number

of entrances into the open arms of the maze, as well as the decreased amount of time spent in them, are considered as a measure of anxiety-like behavior. (Almowasi *et al*., 2018).

Tissue processing and Stereology: The animals were euthanized at the end of eight weeks of caffeine administration after the neurobehavioural tests had been performed. Each mouse was anaesthetized with ketamine/xylazine (90/10 mg/kg) and transcardiac perfusion was done with 10% neural buffered formalin.

The mice brains were removed, post fixed in the same fixatives for 48 hours, processed, sectioned to reveal the *cornus ammonis* (CA) 1 region of hippocampus and amygdala, and stained using Haematoxylin and Eosin, and Cresyl stains. Random samples of framed regions of interest with an area of 417 µm by 459 µm in the hippocampus and amygdala were selected for neuronal counts, using two sections per animal and three animals per group.

A subset of three mice brains per group was selected for Modified Golgi stain according to the protocol previously described (Olopade et al., 2021), and used to study the dendritic arborization of the neurons in the *cornus ammonis* (CA) 1 region of hippocampus and amygdala. Silver impregnated neurons with well-defined cell body and processes were selected for qualitative morphological assessment. Their dendritic lengths were measured with the aid of computerized image analysis (ImageJ software version 1.46). This was done by placing a transparent grid with concentric rings of 10-mm spacing, over the picture of the neuron with its dendrites, the estimate of the total dendritic length was then taken by the software.

Statistical Analysis

Quantitative data from the scores in the behavioural test as well as data from the neuronal count and dendritic lengths from the histological slides are presented as mean ± SEM. D'Agostino-Pearson omnibus normality test was used to determine normality of the distribution of the means after which, the analysis of variance (ANOVA) test was used to compare the means. Turkey's multiple comparison post-hoc test was used to compare within the groups. The statistical significance was set at $p < 0.05$.

RESULTS

A total of 30 adult male mice were acquired for this experiment, 6 of which died before the end of the study - mainly from fight wounds. Two rats from the low dose group and four from the high dose group died before the end of the study, therefore 24 adult males were used in the analyses, eight in the low dose, and eight in the high dose group while eight served as controls. The mice in the control group were generally calmer than those receiving caffeine, which were hyperactive especially immediately after the caffeine administration.

Behavioural Assessment

Open field test: The parameters assessed in the open field task, related to emotionality were the time spent in center square and grooming.

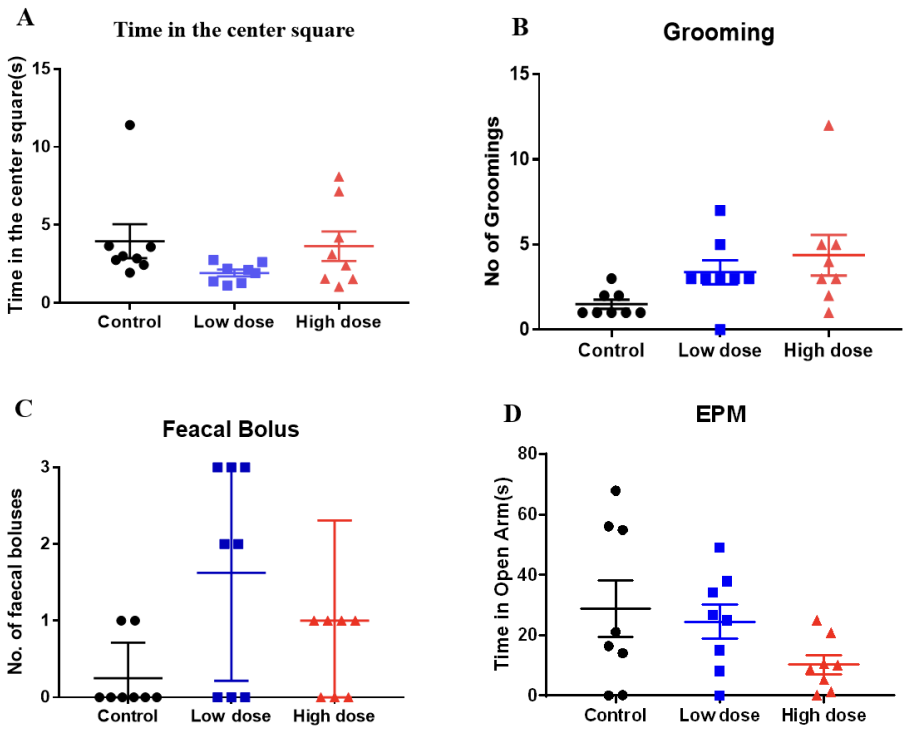

Figure 1:

Scatter plots showing the A) length of time the mice spent in the center square B) number of groomings and C) number of faecal boluses D) Time spent in the open arms of the elevated plus maze (EPM) across the groups.

There was no significant difference in the time spent by the mice in the centre square across the three groups ($F=1.699$; $p=0.20$ and $R^2=0.1393$). Similarly, no significant difference was observed in the number of faecal boluses passed by the mice across the groups. There was however a tendency towards more grooming in the experimental groups than in the controls, but it did not reach significant levels: control (1.5 ± 0.267); low dose (3.375 ± 0.706) and high dose (4.375 ± 1.194) ($F=3.20$; $p=0.06$ and $R^2=0.233$) (Fig. 1A-C).

There was no significant difference in time spent in the open arms of the elevated plus maze apparatus across the three groups (F=2.15, p=0.141 and R2 =0.169) (Fig. 1D).

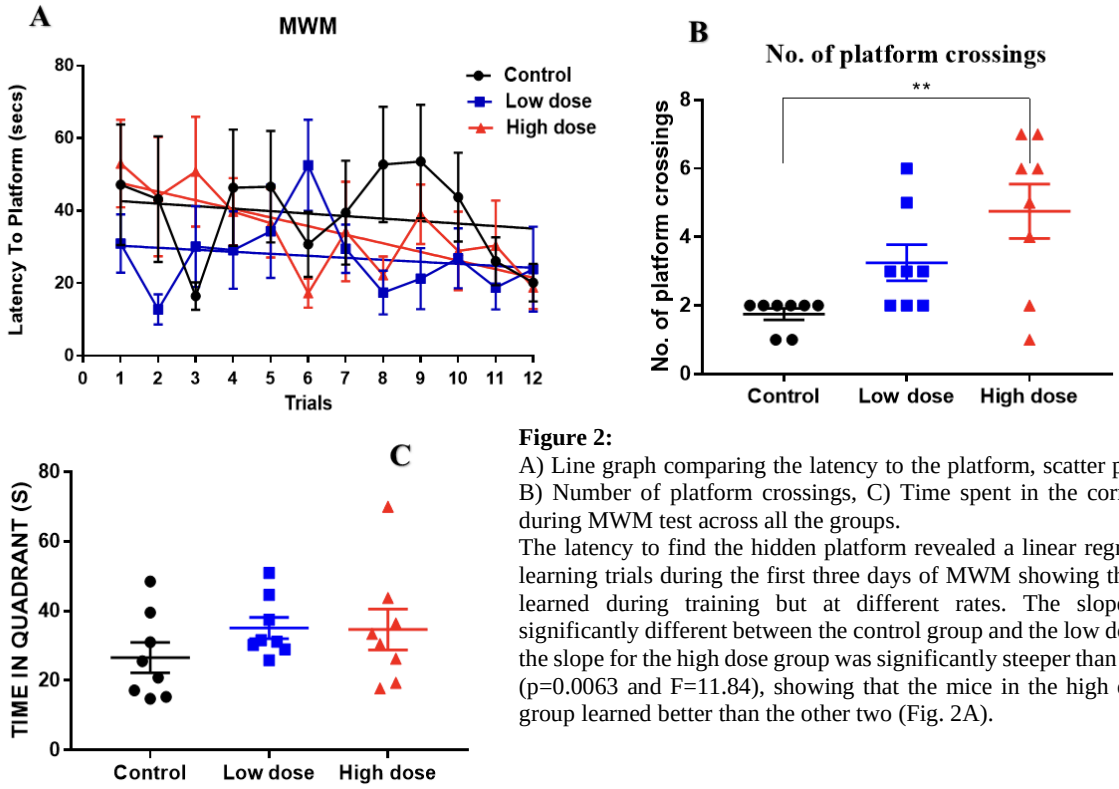

Figure 2:
A) Line graph comparing the latency to the platform, scatter plots showing B) Number of platform crossings, C) Time spent in the correct quadrant during MWM test across all the groups.
The latency to find the hidden platform revealed a linear regression in the learning trials during the first three days of MWM showing that all groups learned during training but at different rates. The slopes were not significantly different between the control group and the low dose group but the slope for the high dose group was significantly steeper than the other two (p=0.0063 and F=11.84), showing that the mice in the high dose caffeine group learned better than the other two (Fig. 2A).

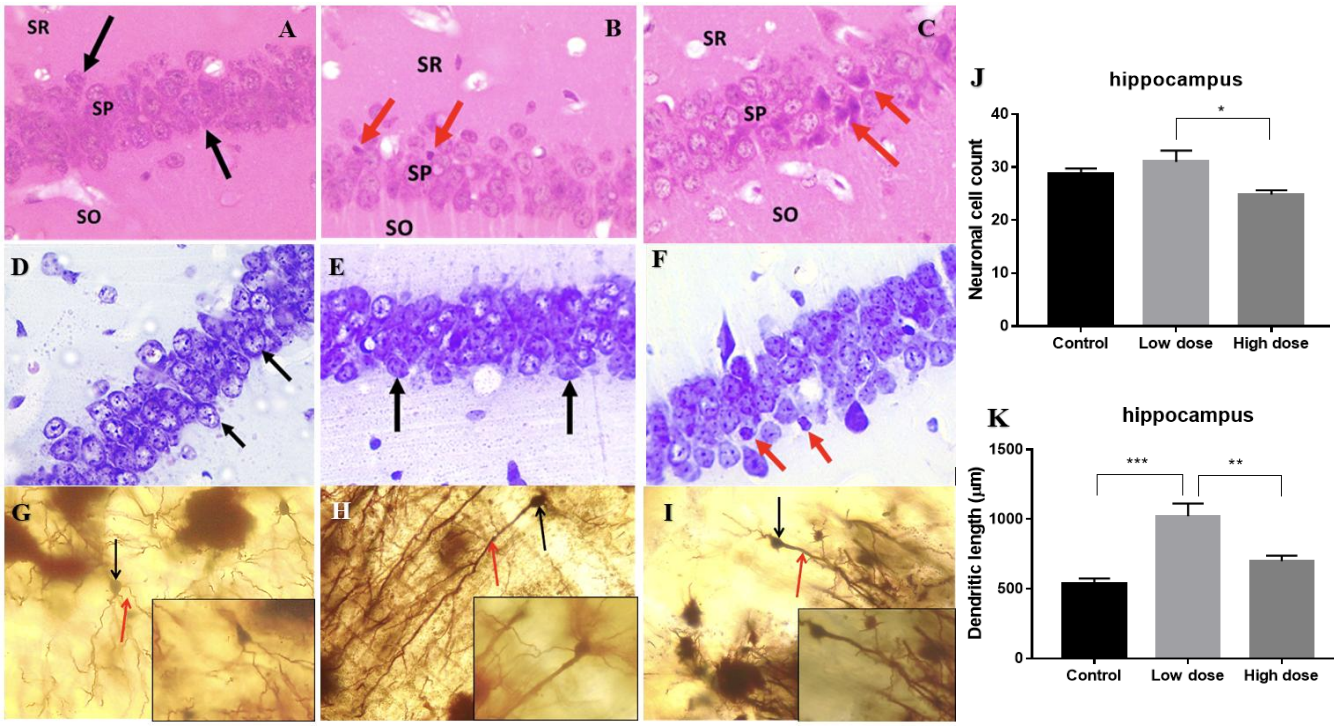

Figure 3:
Representative stained sections of CA1 region of the hippocampus of the mice brains. Top row: H&E stain (A) CNT; (B) LD; (C) HD. Middle row: Cresyl violet stain (D) CNT; (E) LD; (F) HD; Black arrow: normal pyramidal neurons; Red arrow: shrunken/darkly neurons undergoing pyknosis. Bottom row: Golgi stain (G) CNT (H) LD (I) HD. The black arrow shows the soma of the neuron while the red arrow shows the dendrites. (J) Bar chart representing the neuronal cell count across the three groups. (K) Bar chart representing the dendritic length across the three groups. Magnification: ×100(A-F); ×40 (G-I) SO: stratum oriens; SP: stratum pyramidialis; SR: stratum radialis.

The number of platform crossings is the measure of memory consolidation after the training trials in the Morris Water Maze test. The number of platform crossings was significantly higher in the high dose (4.75 ± 0.796) than the control (1.75 ± 0.164) and low dose (3.25 ± 0.523) groups. (F=7.2; p=0.004 and R^2 =0.407) (Fig 2 B). This showed that

memory retention of the mice in the high dose group was better than that of the mice in the low dose and control group. There was, however, no significant difference in time spent in the correct quadrant among animals in the three groups ($F=1.102$, $p=0.35$ and $R^2=0.09$) (Fig. 2C).

Histological Plates: The effect of caffeine on the cytoarchitecture of the hippocampus (CA 1) and amygdala were assessed. The brains of mice in the control and low dose groups had more compact, tightly packed neurons in the stratum pyramidialis of the CA1 region in the hippocampus than the ones in the high dose group which were sparse and appeared shrunken. Neuronal count was slightly increased in the low dose, but reduced in the high dose group, compared to the control. Thus, statistical analysis revealed a significantly decreased number of neurons in the high dose group compared to the low dose group ($p=0.03$) (Fig. 3).

The hippocampal pyramidal neurons in the control group had more arborization in the basal dendrites than the caffeine-treated groups, while the low dose caffeine group had longer apical dendrites (Fig. 3). The dendritic length of the hippocampal neurons of the mice in the low dose group was significantly greater than those in the control and high dose groups ($p<0.0003$).

Histological sections of the amygdala showed the intercalated cells especially in the control group with a lot of normal neurons. There was no visible difference in the structure of the cells across the groups. Cresyl violet photomicrograph shows black arrows that indicate normal neurons. The neuronal count in the amygdala of the mice in the low dose caffeine group was significantly less those in the control group ($p=0.014$) (Fig. 4).

DISCUSSION

In this study, we administered two different doses of caffeine to young adult male albino mice by oral gavage, as seen in Pechlivanova *et al,* (2012) and Olopade and Shokunbi (2018), for eight weeks. The high dose caffeine group exhibited significantly improved indices of learning and memory, and tended towards more anxious behaviour, compared to the control and low dose caffeine groups.

The high dose caffeine group learnt better with a steeper slope period than the other groups, and had better memory consolidation, shown by increased number of platform crossings. This contradicts previous studies like Angelucci *et al.,* (2002), who administered 10mg/kg of caffeine and Almosawi *et al.,* (2018), who gave 20mg/kg, however these two instances were in acute administration. Mahdi *et al.,* (2019) administered 20 mg/kg caffeine for one month and showed caffeine selectively increasing memory, but, in this study, only the high dose group showed significantly improved learning and memory. According to Dunwiddie and Masino (2001), it is very intriguing that unlike receptors of most of neurotransmitters, which once antagonised have pathological consequences, adenosine receptor antagonism apparently induces improvement in mental function and performance.

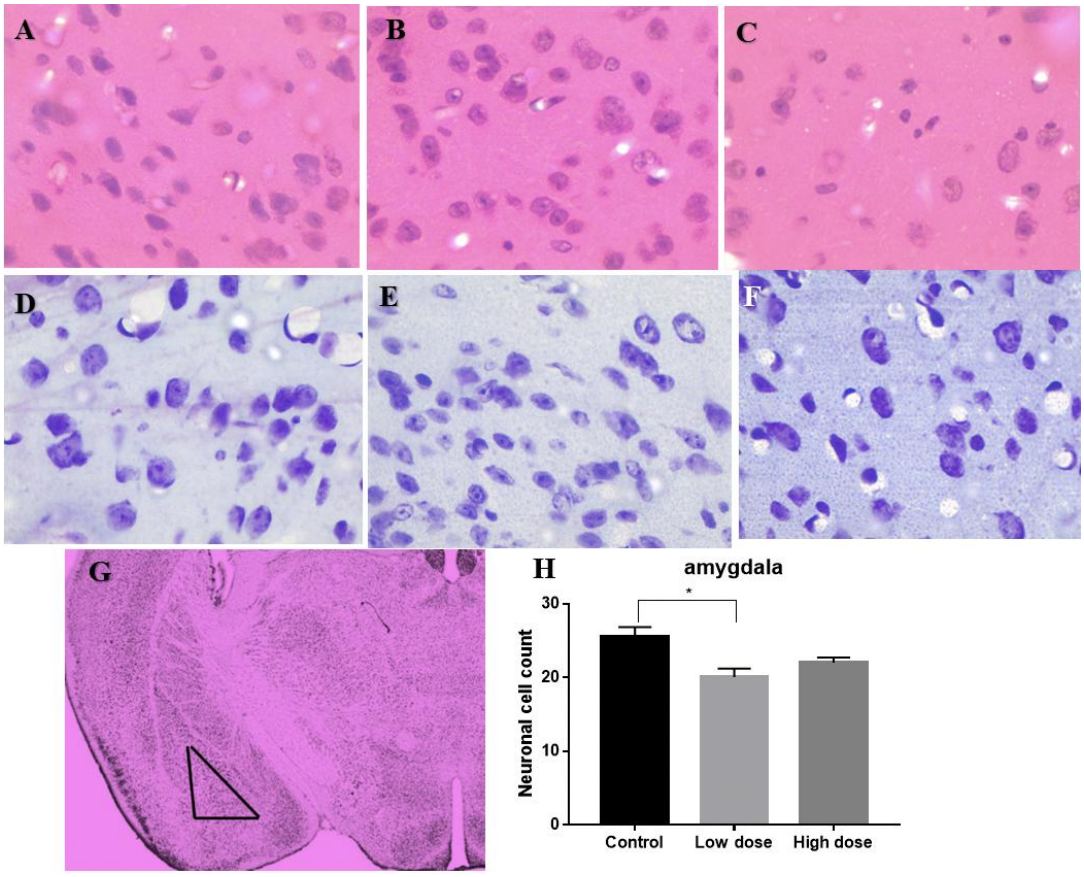

Figure 4:
Representative stained sections of the amygdala region of mice brain. Top row: H&E stain (A)CNT; (B) LD; (C) HD. Middle row: Cresyl violet stain (D) CNT; (E) LD; (F) HD; Black arrows indicate neuronal cells. Magnification ×100. Bottom row: H&E stain (G) Low magnification (x4) of the mouse brain, indicating the region of amygdala in a black triangle. (H)Bar chart representing the neuronal count across the groups.

The cognitive effects of caffeine are mostly due to antagonism of adenosine A1 receptors in the hippocampus and cortex, the brain areas mostly involved in cognition, however, caffeine also aids information processing and performance thus improving behavioural routines, arousal enhancement and sensorimotor gating (Fredholm et al., 1999).

The anxiety-like behaviours measured showed that experimental animals exhibited some degree of anxiety following caffeine administration, but its values did not reach significant levels. The mice in the low dose group spent the shortest time in the centre square of the open field, similar to the observations of Noschang *et al.,* 2009 and Vila-Luna *et al.,* 2012, supporting that subjects administered caffeine could develop anxiety in open spaces.

The Elevated Plus Maze (EPM) is considered sensitive to anxiety state of rodents, based on the principle that exposure to an elevated and open arm leads to an approach conflict that is stronger than that evoked by the closed arm (Pellow *et al.,* 1985). No significant differences were detected between control animals and those that had consumed caffeine in this study, but the high dose group spent the shortest time in the open arm of the EPM. This was similar to an earlier study which reported that chronic high dose caffeine treatment (50mg/kg) was associated with reduced time spent by mice in the open arms of an EPM in comparison with their controls (El Yacoubi *et al.,* 2000, Ardias *et al.,* 2014). It has been reported that long-term excessive caffeine intake can result in caffeine-induced anxiety disorders, which mimics organic mental disorders, such as panic disorder, generalized anxiety disorder, bipolar disorder, or even schizophrenia (Ribeiro et al., 2002).

The pyramidal cells in the CA1 region of the hippocampus were slightly increased in the low dose but reduced in the high dose group. The hippocampus is a region of the brain with high rate of expression of the adenosine receptors (Ribeiro *et al.,* 2002), which Ekong et al., 2017 reported had reduced hippocampal population cell count after chronic caffeine administration at 40mg/kg. The gradual loss of pyramidal neurons reported in the high dose group might be due inhibition of adult neurogenesis by caffeine as reported by Han et al., (2007).

In a human study carried out by Lanini *et al.,* 2016; acute caffeine dose of low to moderate consumption (25-300mg), was seen to sustain simple attention, improve subjective arousal and selectively enhance performance in executive updating (various cognitive abilities and behavior). Likewise, concerning chronic consumption, John-kozlow *et al.,* (2002) observed that elderly women (but not men) who consumed large amounts of caffeine throughout their lifetimes performed better on memory and other cognitive tasks than non-caffeine drinkers. These positive reports seen may be as a result of the increased cell numbers and increased length of the dendrites in the region of the brain concerned with memory such as we observed in the hippocampus in this study. Similar observations have been made in respect of the benefits of caffeine in prevention and alleviation of neurodegenerative disease such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) (Kolahdouzan and Hamadeh, 2017; Gökcen and Şanlier, 2019). Golgi-stained sections of the brains in this region revealed that hippocampal neurons of mice in the caffeine groups have significantly longer dendritic length compared to controls. This is similar to the report of Vila-Luna *et al.,* (2012).

The amygdala is a region of the brain concerned with emotions and anxiety symptoms (Vriend et *al*., 2016); recent discovery also links the amygdala to the sensorimotor cortex through functional circuits (Toschi *et al*., 2017). Examination of the amygdala in this study revealed normal neurons in both the control group and the experimental groups. However neuronal count revealed a reduction in the number of neurons in the low dose caffeine group. This reduction in neuronal count may be responsible for reduced oscillation stimulation pattern which may cause increased pathological functioning of the amygdala as seen in other antisocial personality disorder (Kolla *et al*., 2017; Henigsberg *et al*., 2019). In humans, high doses of caffeine have been shown to increase tension and symptoms of anxiety, nervousness and jitteriness (Stafford et al., 2007). Caffeine has also been reported to reliably decrease hand steadiness (Bovim *et al*., 1995) and hand tremors have been found to increase with caffeine users (Koller *et al.,* 1987). These behaviours may result from a reduction in the neuronal number as seen in this study, which thus alters structural and functional connectivity between the amygdala and other regions, like the medial prefrontal cortex (mPFC), as reported by Kim *et al*., 2011.

In this study, chronic caffeine consumption was shown to selectively bring about enhanced performance in learning and memory, but with a tendency towards anxiety-like parameters. When compared to acute administration of caffeine regardless of meal intake, caffeine of moderate doses (220-225 mg) decreased fatigue and improved simple and sustained attention and executive functions such as cognitive abilities responsible for the regulation of cognition and behavior (Lanini *et al*., 2016). Acute behavioural activities carried out by Almosawi *et al*., (2018) on mice with a similar concentration like this study but different administration route showed caffeine enhanced cognition and motor activities at low dose. Although the underlying mechanisms for the positive cognitive effect of chronic caffeine administration are not fully understood, Sallaberry *et al*., (2012) reported that modifications in brain-derived neurotrophic factor (BDNF) and related proteins in the hippocampus contribute to these effects on age-associated losses in memory encoding.

In conclusion, we have shown that the chronic administration of caffeine for eight (8) weeks promotes cognitive functions, learning and memory, but increased the level of anxiety in mice. These behavioural changes were associated with increased number and longer dendrites of hippocampal neurons but little structural change in the amygdala.

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