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CHANGES IN NORMAL AND APOPTOTIC PYRAMIDAL NEURONAL CELL DENSITIES IN PREFRONTAL CORTEX OF THE MALE RAT FOLLOWING CHRONIC KHAT USE

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ABSTRACT

Long-term Khat consumption is associated with significant neuro-cognitive changes, which have been elucidated in behavioral studies. However, correlation of these neurophysiological changes with experimental structural changes in prefrontal neurons has not been described adequately. Young adult male Winstar rats, aged 2-3 months, weighing 200-300 grams were randomized into four groups of 11 each to correspond with those used as controls, those that received 500mg/kg, 1000mg/kg and 2000mg/kg body weight khat extracts respectively. The control rats were fed on normal diet, while experimental groups were fed on normal diet and khat extracts using oral gavage for 6 weeks. The animals were sacrificed, and their brains removed. Toluidine blue staining was used to elucidate the Nissl substance. Image-Fiji was used to analyse densities of normal and apoptotic pyramidal neuronal densities across the 4 experimental groups. Normal pyramidal neurons were identifiable by their characteristic uniform size and prominent nucleoli, while apoptotic cells displayed prominent eosinophilia and pyknotic nuclei. There was a statistically significant increase in apoptotic pyramidal neurons across the three khat-fed groups compared to controls, and a decrease in normal pyramidal neuronal population from controls with increasing doses of khat. This decrease in normal pyramidal neurons and increase in apoptosis could be a potential surrogate for neurocognitive perturbations associated with chronic khat use.

Keywords: Pyramidal neurons; khat; chronic; densities.

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INTRODUCTION

The leaves and bark of Khat has been consumed for centuries to alleviate fatigue, reduce hunger, enhance energy, stay alert and induce euphoria (Brenneisen *et al.,* 1990, Carrier, 2018).

The euphoriant and amphetamine-like effects of Khat are due to the phenylalkylamines cathinone and cathine, with cathinone being more potent. Cathinone closely resembles amphetamine in chemical structure and function, but in cathinone, there's an oxygen atom on the first carbon of the amphetamine sidechain instead of two hydrogen atoms (Muche, 2004). Cathinone is responsible for the main stimulant effects of khat. It is an unstable compound, which is rapidly metabolized to cathine, a less potent compound. Fresh khat leaves contain higher levels of cathinone compared to dried leaves, hence more psychoactive effects.

Evidence suggests that the PFC is involved in a dynamic neural network which controls intelligent, self-regulating behaviours (Fuster, 2001, Miller and Wallis, 2008). The primate PFC

is made up of medial, orbital and lateral areas. The rat PFC, like the primate one, is organized in medial, ventral/orbital and lateral areas. There is significant functional correlation as well between primate and rat PFC, with the rat medial PFC (mPFC) being involved in attentional processing, working memory, goal-directed behaviour and behavioural flexibility (Fusijawa *et al.,* 2008; Totah *et al.,* 2013). Moreover, anatomical comparison studies have found similar connectivity patterns of thalamo-cortical, PFC-basal ganglia and cortico-cortical pathways between primates and rats. Strong projections originating in the mediodorsal (MD) nucleus of the thalamus have been reported in the primate PFC and the rat mPFC (Dalley *et al.* 2004; Goldman-Rakic 2011).

The prefrontal cortex is a target of drugs and substances through dopaminergic, serotonergic and catecholaminergic pathways. A number of studies indicate that mPFC function is involved in drug abuse behaviors related to impulsivity and cognitive impairment. Impulsive choice has been associated with both serotonin release and DA receptor tone in mPFC (Winstanley *et al.,* 2006). Further, prefrontal lesions increase impulsive choice on the delay discounting task (Weissenborn *et al.,* 1997).

Despite the central role of the prefrontal cortex in neurobehavioural regulation, there's paucity of studies documenting the changes in neuronal and cortical architecture with administration of khat which is a widely used mild stimulant

MATERIALS AND METHODS

Experimental animals: young adult male Winstar rats (Rattus Norvigecus) aged 2-3 months and weighing 200-300 grammes were purchased from an accredited breeding institution and housed within the department of Veterinary Anatomy and Physiology of the University of Nairobi. They were housed in cages, with adequate ventilation and provided with a normal light and dark circadian cycle and given adequate and free access to food and water (ad libitum) and allowed to acclimatize for 7 days before beginning the experiments.

The rats were proportioned into four experimental clusters of 11 each. The first group served as the control and were fed on a normal diet and 10ml/kg normal saline water as control. The other 3 groups were fed on a once-daily Khat extract at three different doses: 500mg/kg (K500), 1000mg/kg (K1000) and 2000mg/kg (K2000) for a period of 6 weeks, by oral gavage.

Ethical consideration: This study was carried within the guidelines of the University of Nairobi's animal handling guidelines. The research protocol with animal experimentation was approved by the University of Nairobi's Faculty Biosafety, Animal use and Ethics committee (REF **FVM BAUEC/2020/276).** Animals were handled in a humane way and euthanized using intraperitoneal ketamine and xylazine.

Khat extraction: Khat samples were collected from the farm and transported in a cooler box to the lab within 4 hours of harvesting. After weighing, each bunch was chopped to homogenize the sample and blended with 125ml of sterile distilled water. The blended mixture was then transferred to 40ml falcon tubes and centrifuged at 7000rpm for 6min.The supernatant was then transferred into 100ml bottles covered with aluminium foil to minimize exposure to light and stored at refrigerated conditions of 2°C awaiting lyophilisation. Supernatant from Khat extract was then dispensed in volumes of 3ml into vials for lyophilisation. The vials were first frozen at -80°C for 2 hrs then freeze dried under vacuum at 0.103mBar for 24hrs.

Preparation of brain sections: Animals were euthanized by intraperitoneal ketamine, followed by transcardiac perfusion with normal saline. The brains were fixed by cardiac perfusion with 0.9% normal saline followed by 4%

paraformaldehyde in 0.1M Phosphate buffer. Brains were quickly removed from the skulls and placed in buffered formaldehyde and embedded in paraffin blocks.

Histological sections 1mm thick were cut in the region between A and B as shown in *figure 1*, with the olfactory tract intact. (mPFC; Medial Prefrontal cortex). One out of 20 sections were randomly selected for staining from each animal group.

The tissue sections were dewaxed in two changes of xylene, each taking 5 minutes. The sections were then rehydrated in descending grades of alcohol starting from xylol 50:50, each taking 3 minutes. Slides were then placed in running tap water for 15 minutes. They were then immersed in 1% aqueous toluidine blue for 6 hours. Tissues were then rinsed in distilled water and differentiated in 95% ethyl alcohol. They were then dipped in absolute alcohol and xylol 50: 50 before being cleared in xylene. Mounting was then done using a DPX mountant for light microscopic study.

Staining (Toluidine blue)

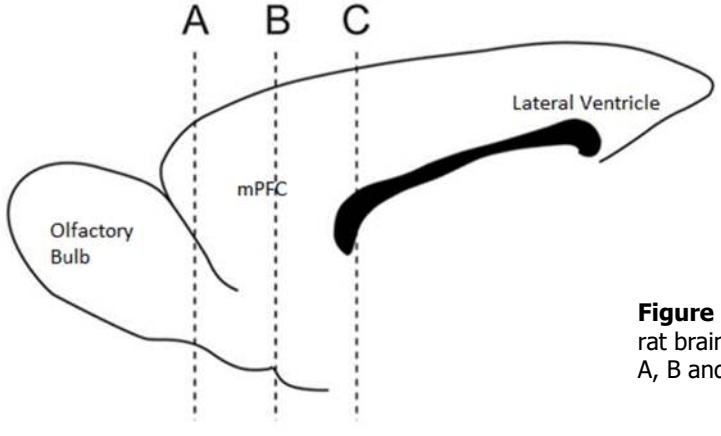

Figure 1: showing a cross-sectional representation of the rat brain and the areas representing the prefrontal cortex A, B and C (mPFC represents medial prefrontal cortex)

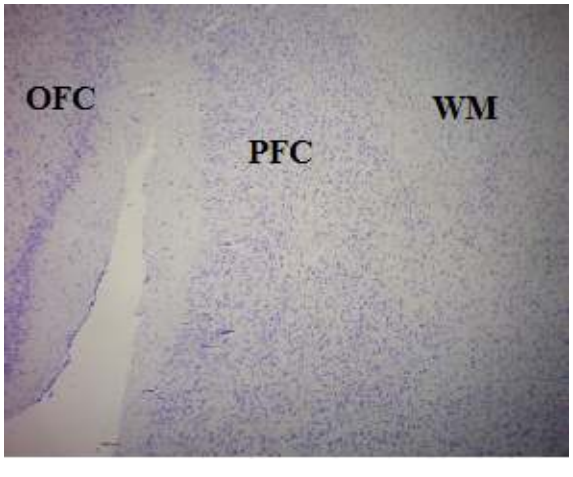

***Figure 2**:* shows the histological appearance of the prefrontal cortex, with the adjacent olfactory cortex present.

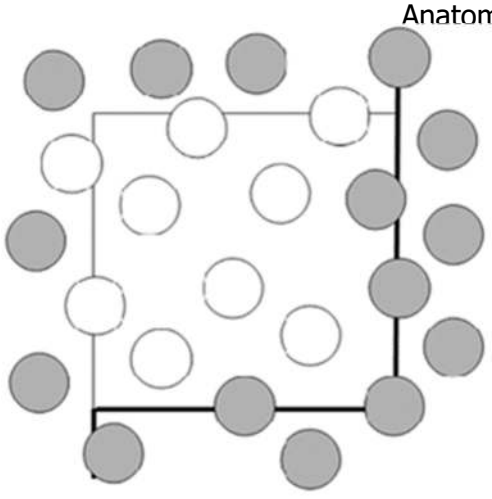

Figure 3: An illustration of the cell counting method. The bold lines represent the exclusion zone while the lighter lines represent the inclusion zone. All cells within the area bounded and touching the inclusion zone were counted.

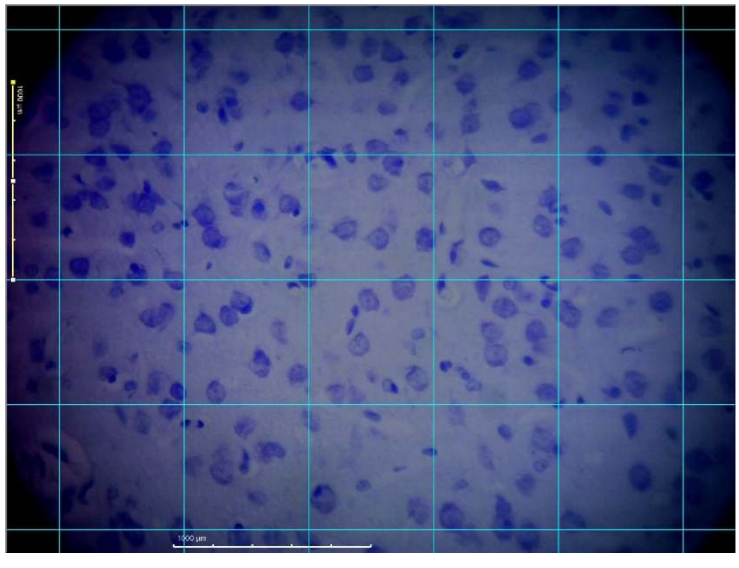

Figure 4: Figure displaying the grids used to determine the pyramidal neuronal densities

Determination of neuronal cell densities: Cell counts were taken at different distances from the midline. Photomicrographs were loaded onto Fiji image J software. Next, a network of grid squares was superimposed onto the images and the neurons found in that region; neurons within the square as well as those crossed by the inclusion line were counted. Both the normal healthy pyramidal neurons and the apoptotic neurons that fell within the grid squares were counted. The counts were taken from 5 squares chosen at random. Each square had an area of 400,000 μm^2. Figures 3 and 4 further illustrates the method used in neuronal analysis by image-fiji.

Cell densities per field ($n/\mu m^2$) = Number of cells counted (n) / grid area (μm^2).

Data analysis and presentation: Morphometric data collected were entered into the Statistical Package for Social Sciences (SPSS) software (Version 22.0, Chicago, Illinois) for coding, tabulation and statistical analysis. The independent variables were control/experimental groups while the dependent variable was the pyramidal neuronal densities (cells/400,000um^2). Descriptive statistics such as mean, standard deviation, median, interquartile ranges, standard error of the mean were determined for each of the study groups. Due to the inequality in the control and experimental groups, non-parametric tests were run. The Kruskal Wallis H test was used for comparison across the four study groups (control, experimental 1 (500 mg/kg),

experimental 2 (1000 mg/kg) and experimental 3 (2000 mg/kg)). Pairwise comparisons were used to check for statistically significant inter-group differences. A p-value of ≤0.05 was considered significant at a confidence interval of 95%.

RESULTS

Pyramidal Neuronal Densities

An increase in the apoptotic Pyramidal Neuronal Density (PND) was noted on administration of the Khat extracts over the study period. PND increased progressively with the highest neuronal density being observed in experimental group 3 that received 2000 mg/kg of Khat extract. These intergroup differences in the apoptotic PND were statistically significant (p = 0.049). The photomicrographs revealed an increased number of apoptotic Pyramidal cells with an increase in the Khat dosages (*Figure 5*).

The pyramidal cells of the control group appeared to be uniform in size and some had prominent nucleoli, with most of them displaying limited signs of undergoing apoptosis. The experimental groups had numerous apoptotic pyramidal neurons identified by the characteristic cytoplasmic eosinophilia and pyknotic nuclei (*Figure 5*).

The general trend of increase in the apoptotic PND with an increase in dosage of Khat per body weight can also be derived from the bar graph (*Figure 6*) and box plots (*Figure 7*). Pairwise comparisons revealed that there were statistically significant differences noted between the groups. The experimental groups that received the Khat extracts displayed significantly lower apoptotic pyramidal neuronal densities (*Table 1*).

A decrease in the normal Pyramidal Neuronal Density (PND) was noted on the administration of the Khat extracts over the study period. The normal PND decreased progressively with the lowest neuronal density being observed in experimental group 3 that received 2000 mg/kg of Khat extract. These intergroup differences in the apoptotic PND were statistically significant (p = 0.027). The photomicrographs revealed fewer normal Pyramidal cells with an increase in the Khat dosages (*Figure 5*). The pyramidal cells of the control group appeared to be uniform in size and some had prominent nucleoli, with most of them displaying limited signs of undergoing apoptosis. The experimental groups had relatively fewer normal pyramidal neurons.

The general trend of the decrease in the normal PND with an increase in dosage of Khat per body weight can also be derived from the bar graph (*Figure 8*) and box plots (*Figure 9*). There were no statistically significant differences noted between the groups upon running pairwise comparisons and adjusting the significances for the multiple comparisons (*Table 2*).

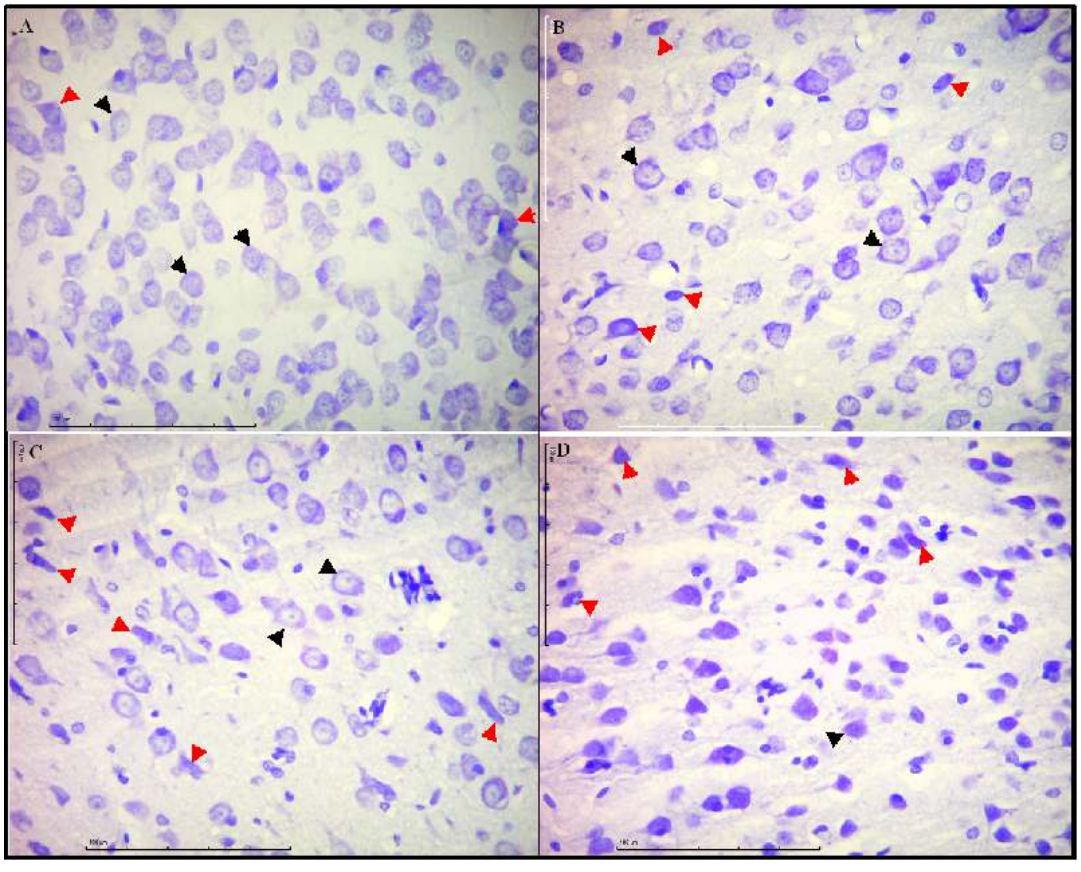

Figure 5: shows neuronal changes following Khat administration. Fig 5A: Photomicrograph of the prefrontal cortex of the control group. Note the abundance of normal Pyramidal neuronal cells (black arrowheads) with uniformly staining and rounded nuclei with prominent nucleoli. Note the relative scarcity of apoptotic neurons in this group. The apoptotic neurons (red arrowheads) displayed characteristic cytoplasmic eosinophilia and pyknotic nuclei. Note the relative increase in pyknotic and apoptotic pyramidal cells with increasing doses of khat Fig 5B(500mg/kg), Fig 5C (1000mg/kg) and Fig 5D (2000mg/kg) with decreasing density of normal pyramidal cells. (Toluidine blue stain). Magnification = x400

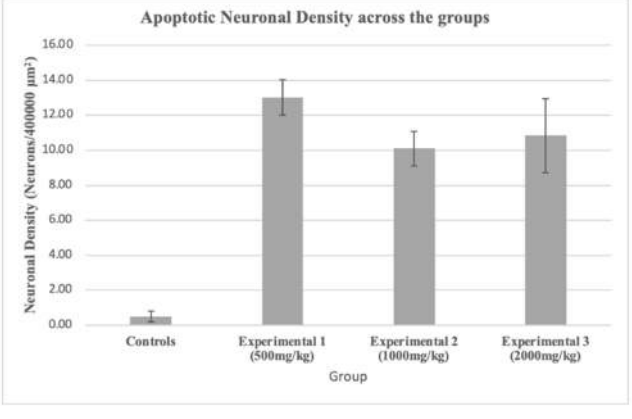

Figure 6: Graph demonstrating the general trend of apoptotic neuronal densities in controls and

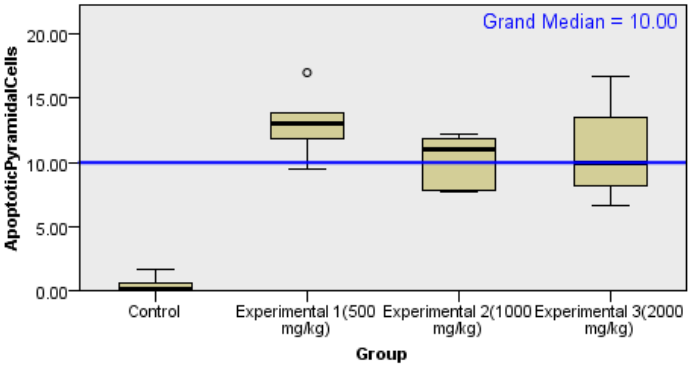

Figure 7: Box plots of the apoptotic pyramidal neuronal densities in controls and experimental rat groups

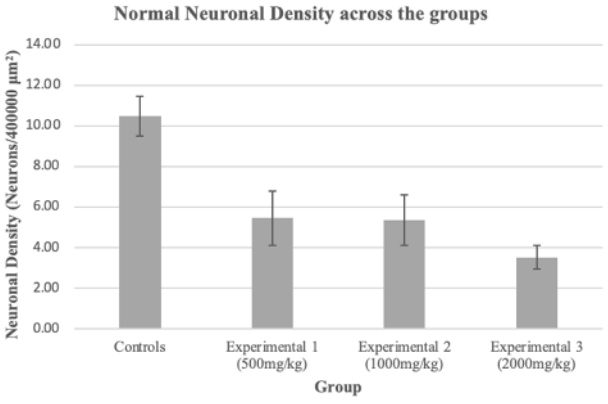

Figure 8: General trend of normal pyramidal neuronal densities in controls and experimental rat groups

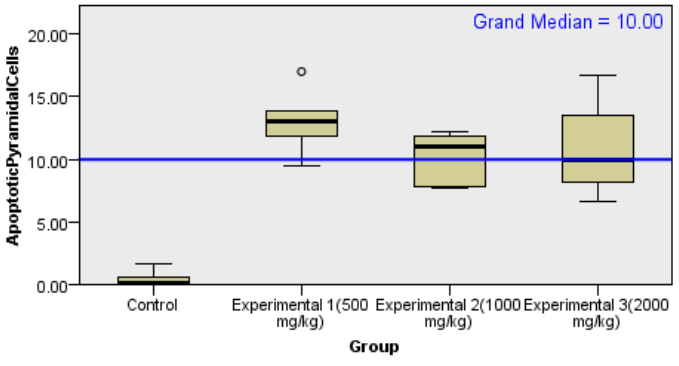

Figure 9: Box plots of the normal pyramidal neuronal densities in control and khat-fed rats

Table 1: Apoptotic pyramidal neuronal densities in control and khat-fed rat groups

Group	Apoptotic Neuronal Density (cells/400000 µm^2)		p value against:
	Mean ± SD	**Median ± IQR**	**Control**
Control	0.50 ± 0.71	0.17 ± 1.17	-
Experimental 1 (500 mg/kg)	13.03 ± 2.47	13.00 ± 3.38	**0.034***
Experimental 2 (1000 mg/kg)	10.10 ± 2.19	11.00 ± 4.25	**0.009***
Experimental 3 (2000 mg/kg)	10.84 ± 4.20	10.00 ± 7.67	**0.016***

Table 2: Normal pyramidal neuronal density in control and experimental rat groups after consumption of khat

Group	Normal Neuronal Density (cells/400000 µm^2)		*p value against:
	Mean ± SD	**Median ± IQR**	**Control**
Control	10.50 ± 2.18	10.50 ± 3.50	-
Experimental 1	5.47 ± 3.29	6.17 ± 6.05	0.214
Experimental 2	5.37 ± 2.80	4.67 ± 5.62	0.347
Experimental 3	3.54 ± 1.18	3.34 ± 2.21	0.098

DISCUSSION

The main objective of this study was to document the changes in normal and apoptotic pyramidal neuronal densities in the PFC of winstar rats with increasing doses of khat. Our study has demonstrated a dose-dependent decrease in the density of normal pyramidal neurons and an increase in apoptotic neurons with increased doses of khat.

Behavoural studies have documented impairment of working memory of khat-fed winstar rats in a dose-dependent manner. A similar observation has been made in other cognitive behavioural functions (Echoru *et al*., 2018). Lesions in the PFC of humans and non-human primates have been shown to cause impairment of working memory (Gregoriou *et al*., 2014). Cathinone, the main psychostimulant alkaloid in khat, exerts amphetamine-like effects on neuronal and glial cells, stimulates dopaminergic action, leading to excitotoxicity and eventual neuronal loss.

Previous studies among khat-fed rats demonstrated major histopathological changes in the PFC, with remarkable vacuolation, gliosis, focal necrosis and hemorrhage within the PFC (Echoru *et al*., 2018). Damage or reduction in density of PFC pyramidal neurons may translate to impaired cellular communication which in turn causes impaired movement of nutrients within and across cells, further fueling neuronal apoptosis and necrosis (Fernandez & Rodriguez, 2011).

These changes have been reported elsewhere in the brain. Muche $ Abdelwahub (2007) reported reduction in density and disruption of neuronal density in Purkinje cells of the cerebellum.

Another postulated mechanism of neuronal disruption and death is alterations in cerebral perfusion, leading to cerebral ischemia and ischemic neuronal necrosis (Echoru *et al*., 2018). Cerebral ischemia leads to increased intracellular calcium which triggers the cascade of processes leading to neuronal apoptosis and cell death as evidenced in our study.

The molecular neural-biochemical interactions of khat, a CNS stimulant during chronic exposure thus has profound neuronal injury effects and modulates decrease in neuronal cell populations in some brain regions (Blaesing *et al*., 2001).

In conclusion, our study has demonstrated decreased pyramidal neuronal density in the PFC, as well as increased apoptotic neuronal density in the PFC with increasing doses of khat. This adds to the armamentarium of evidence that khat, beyond certain doses could be detrimental to neurocognitive functions mediated by the PFC.

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REFERENCES

1. Brenneisen R, Fisch HU, Koelbing U, Geisshusler S, Kalix P. 1990. Amphetamine-like effects in humans of the khat alkaloid cathinone. British journal of clinical pharmacology 30(6), 825-828.
2. Carrier N, Klantschnig G. 2018. Quasilegality: khat, cannabis and Africa's drug laws. Third World Quarterly 39(2), pp.350-365.
3. Dalley JW, Cardinal RN, Robbins TW. 2004. 'Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates', Neuroscience & Biobehavioral Reviews 28 (7), 771-84.
4. Echoru I, Bukenya EM, Masilili G, Owembabazi E, Lemuel AM, Ahimbisibwe J. 2018. Commonly Consumed KHAT (Catha Edulis) distorts the Prefrontal Cortex Histology and Function in Adult Wistar Rats. Anatomy Journal of Africa 7: 1121 – 1132.
5. Fernandez-Espejo E, Rodriguez-Espinosa N. 2011. Psychostimulant drugs and neuroplasticity. Biochem Pharmacol 12(2): 188-195.
6. Fujisawa S, Amarasingham A, Harrison MT, Buzsáki G. 2008. Behavior-dependent short-term assembly dynamics in the medial prefrontal cortex. Nat Neurosci 11(7):823-33.
7. FusterJM. 2001. The prefrontal cortex—an update: time is of the essence. Neuron 1;30(2):319-33.
8. Goldman-Rakic PS. 2011. 'Circuitry of Primate Prefrontal Cortex and Regulation of Behavior by Representational Memory'. Comprehensive Physiology., (John Wiley & Sons, Inc.).
9. Gregoriou GG, Rossi AF, Ungerleider LG, Desimone R. 2014. Lesions of prefrontal cortex reduce attentional modulation of neuronal responses and synchrony in V4. Nat Neurosci 17(7), 1003–11.
10. Muche A, Abdelwuhab M. 2007. Does intake of Ethanol and Khat (Catha Edulis Forsk) Affect the Total Number of Purkinje Neurons in Early Postnatal Rats. Pharmaco 114:102-14.
11. Muche Abebe. 2004. "Effect of Ethanol and KHAT (Catha Edulis Forsk) on Cerebellar Cortex of the Rat." PhD diss., Addis Ababa University, 2004.
12. Totah NKB, Jackson ME, Moghaddam B. 2013. 'Preparatory attention relies on dynamic interactions between prelimbic cortex and anterior cingulate cortex', Cerebral Cortex 23 (3), 729-38.
13. Wallis JD, Miller EK. 2003. From rule to response: neuronal processes in the premotor and prefrontal cortex. Journal of neurophysiology 90(3):1790-806.
14. Weissenborn R, Robbins TW, Everitt BJ. 1997. Effects of medial prefrontal or anterior cingulate cortex lesions on responding for cocaine under fixed-ratio and second-order schedules of reinforcement in rats. Psychopharmacology 34(3):242-57.
15. Winstanley CA, Theobald DE, Dalley JW, Cardinal RN, Robbins TW. 2006. Double dissociation between serotonergic and dopaminergic modulation of medial prefrontal and orbitofrontal cortex during a test of impulsive choice. Cerebral cortex 1;16(1):106-14.