NEURONAL AND ASTROCYTIC STRUCTURAL CHANGES IN THE PREFRONTAL CORTEX OF THE MALE WISTAR RAT FOLLOWING CHRONIC KHAT USE

By Dr Paul Bundi Karau

H80/56794/2020

A thesis submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy in Human Anatomy of the University of Nairobi

May, 2023

DECLARATION

I hereby solemnly affirm that this thesis is my original work, and to the best of my knowledge, has not been submitted elsewhere for examination.

Candidate:

Sign: _____   Date : **20th May 2023**

       Dr. Paul Bundi Karau (BSc, MBChB, MMed)

       Department of Human Anatomy & Physiology, The University of Nairobi

This thesis has been submitted with our approval as the University supervisors:

Sign: Date: **20th May 2023**

       Dr Paul Odula, BSc.(Anat), MBChB, MMed (Surgery), PhD

       Department of Human Anatomy & Physiology, the University of Nairobi

Sign: Date: **20th May 2023**

       Prof. Moses Obimbo Madadi (MBChB, MSc, MMed, PhD)

       Department of Human Anatomy & Physiology, the University of Nairobi

DEDICATION

With love and gratitude, I dedicate this work to my dear wife, Dr. *Winnie Mueni Saumu- Bundi*, and our three boys; *Bundi Karau Junior*, *Maluki Bundi* and *Muriira Bundi.*

For the strength of our union, for the great fellowship, for the battles we fight together, for this boat we sail in.

May the good Lord bless you.

"Any man could, if he were so inclined, be the sculptor of his own brain."

Santiago Ramón y Cajal

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

Karau, PhD Thesis, Uon, 2023

Karau, PhD Thesis, Uon, 2023

LIST OF TABLES

LIST OF FIGURES

Karau, PhD Thesis, Uon, 2023

Karau, PhD Thesis, Uon, 2023

LIST OF ABBREVIATIONS

SUMMARY

Background

Chronic and Long-term use of khat may cause neuro-cognitive changes, which have been elucidated in behavioural studies. These may correlate with structural changes in the cytoarchitecture and histomorphometry of neuronal cells. With current research showing the centrality of astrocytes and other glial cells in neuronal signalling, there is possibility that these cells are also affected by chronic khat use. There's little literature on the structural changes in the prefrontal cortex neuronal and astrocytic cytoarchitecture and morphometry in chronic khat users.

Objective

To describe the changes in neuronal architecture and density, as well as astrocyte morphology in rats after long-term use of khat (miraa).

Study design

Randomized experimental study design.

Materials and Methods

Young adult male Wistar rats, aged 2-3 months, weighing 200-300 grams were used in this study. They were randomized into four groups of 11 each (control, K500, K1000 and K2000) to

correspond with those used as controls, those that received 500mg/kg, 1000mg/kg and 2000mg/kg body weight khat extracts respectively. We purchased fresh khat leaves from Mikinduri market in Meru and prepare crude extract using a validated method.

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. They were processed with Haematoxylin and Eosin and Toluidine blue for general histology. We performed immunohistochemical visualization of individual neurocellular populations across the four animal groups as follows: Glial Acidic Fibrillary Protein for astrocytes, 2', 3' cyclic nucleotide phosphodiesterase for oligodendrocytes and doublecortin for immature neurons.

Data Analysis

Photomicrographs of the stained sections were transferred to Image J-Fiji software to study normal and apoptotic pyramidal neuronal cell density, astrocyte density, oligodendrocyte density and the density of immature neurons. Data was entered into SPPS, IBM version 28.0 for analysis. We used Kruskal-Wallis H test to correlate the four animal groups in terms of neuronal densities and astrocyte densities.

Results

The mean body weight, average brain weight and maximum cortical length of the rat brains demonstrated a significant decrease with increasing khat doses compared to controls. We observed a significant increase in the density of apoptotic pyramidal neurons in experimental

groups compared to controls (p=0.027), while a decrease in normal pyramidal neurons was noted with increasing doses of khat. Further, we observed a non-significant increase in immature doublecortin staining neurons in khat-fed rats compared to controls.

There was observed a significant increase in immunoreactive astrocytes with high khat doses (2000mg/kg), coupled with increase complexity of astrocytic processes and gliosis. No group differences were noted in immunoreactive oligodendrocyte density with khat use although there was discernible reduction in myelination with increasing doses of khat.

Conclusions

This study has demonstrated a reduction in gross cortical indices, an increase in pyramidal neuronal apoptosis, a reduction in pyramidal neuronal density with increasing khat doses. This underscores the potential neurotoxic effects of high khat doses. The increase in astrocyte complexity, reduction in myelination and increase in immature neuroblasts in high khat doses compared to controls adds insights into the potential mechanisms involved in khat-induced brain changes, as well as the role of adult neurogenesis in substance use.

Recommendations

We recommend that a longitudinal study should be designed to identify the histological and immunohistochemical changes in the prefrontal cortex along the continuum of khat use so as to identify the time when changes start occurring. To eludicate the process of neuronal loss, ki-67 and caspase staining should be performed.

CHAPTER 1: INTRODUCTION, LITERATURE REVIEW, CONCEPTUAL FRAMEWORK AND RESEARCH OBJECTIVES

1.1   INTRODUCTION

The use of Khat (Miraa) is prevalent in East Africa and the Arabian peninsula. The plant is mainly used for its central nervous effects, especially euphoriant and stimulating effects (Mwenda et al., 2003). Recently, concerns have been raised on the long-term effects of Khat on cognitive function among the users.

While not many studies have been done on the exact mechanism of action of khat on the central nervous system, the general analogy between the effects of cathinone and amphetamine as well as the chemical similarity suggests that the two substances have a similar mechanism of action (Kalix, 1996). The effects of amphetamine are chiefly produced by activating the release of dopamine and catecholamines in the synaptic vesicles of neuronal cells in the brain. Cathinone also releases dopamine from synaptic terminals in the central nervous system, and recent evidence suggests that it also activates serotonergic synapses (Patel et al., 2000).

A great amount of research has been conducted on the behavioural changes associated with acute and subacute administration of khat in experimental animals. Further, some researchers have examined behavioural changes in khat users. While khat use results in a stimulating effect and a burst of energy, it is also associated with deficits in concentration, excessive talking and insomnia(Al Motareb et al., 2002). There is recorded evidence of hallucinations and even overt psychosis among khat users.

These effects point to structural and functional alterations in the prefrontal cortex, the part of the cerebral cortex responsible for controlling intelligent and self-regulating behaviours(Miller, 2000). In rodents, the medial prefrontal cortex is involved in attentional processing, modulation

of working memory, goal-directed behaviour and behavioural flexibility. The ventral medial prefrontal cortex corresponds with the limbic system in humans.

Initially, researchers thought that astrocytes played only physical supportive function to the neurons. However, recent research has shown that astrocytes are at the core of neuronal homeostatic, metabolic and protective functions (Ota et al., 2013). They play critical roles in supporting brain metabolism (Panov et al., 2014), formation and maintenance of synapses, synaptic transmission and information processing (Newman, 2003). This implies that any disease processes or substances affecting neuronal function could also affect astrocyte structure and function.

Despite the importance of Khat in the socio-economic construct of the people in East Africa, its effect on the brain call for more research to better understand it's effects. There is little or no recorded literature elucidating the changes in neuronal and astrocyte morphology and density on khat users. The aim of this study is to determine the changes in pyramidal neurons, astrocytes and oligodendrocytes in the prefrontal cortex of the male rat after chronic khat use.

1.2 LITERATURE REVIEW

1.2.1 Introduction to Khat and its use

Khat, commonly known as Miraa (*Catha edulis*) is a green plant that thrives in the highlands, and is found in East Africa especially Kenya, Middle East, Ethiopia and Somalia. The plant belongs to the sub-order Rosidae and family Celastraceae (Kalix, 1996). The main Khat-growing areas of Kenya is Meru County, especially Tigania and Igembe regions, around Nyambene hills. To a lesser extent, it is also grown in Mbooni, Makueni and some parts of Central Kenya. In Koibatek area of Baringo, Rift Valley, some wild Khat trees do grow, though no formal farming takes place(Mwenda et al., 2006).

To preserve its potency, khat is harvested in the morning and wrapped under moist conditions in banana leaves. This helps prevent dessication and loss of cathinone. High moisture content however makes it less potent, so that leaves harvested during the dry season have more active components, thus more potent than those harvested during the wet months of the year(Abebe et al., 2015; Samue Kindie, 2015).

Figure 1: Khat (Catha Edulis)

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 & Klantschnig, 2018). In Kenya, it also used culturally in payment of dowry, especially among the Meru ethnic group. There is no reliable data on the global prevalence of khat use, but reports indicate that approximately 5-20 million people worldwide chew miraa/khat on a daily basis (Corkery et al., 2011; Thomas & Williams, 2013).

Chewing of Khat is a social and past-time activity especially among the male folk in which the participants are engaged in heated discussions and other social activities. During khat chewing, the bark is peeled off and stuffed in the side of the cheek over several hours(Mwenda et al., 2006). The slow chewing allows intermittent release of the active cathinone, whereupon the

residues are then discarded from the mouth. The half-life of khat is approximately 4 hours, but this may vary depending on the amount of chewed barks(Feyissa & Kelly, 2008). Depletion of the acute energy-giving effects gives way to feelings of loss of energy, insomnia, malaise, depression and mental tiredness(Al-Motarreb et al., 2002; Wabe, 2011).

Figure 2: Bunches of freshly harvested khat. The barks are stripped and chewed for their stimulant effect

Figure 3: The Khat chewing process. Khat is stuffed on the sides of cheeks, sometimes for several minutes or hours.

1.2.2 Chemical composition of Khat

The leaves of Khat contain many different chemical substances such as alkaloids, sterols, terpenoids, glycosides, tannins, sterols, flavonoids, vitamins, amino acids and mineral elements(Feyissa & Kelly, 2008; Wabe, 2011). The major alkaloids are phenylalkylamines and cathedulins. Up to 62 types of cathedulins have been extracted and their chemical structures elucidated. Khat has potent phenylalkylamines such as [S-(−)-cathinone], and its diastereoisomers cathine [(+)-norpseudoephedrine] and norephedrine [1R, 2S-(−)-norephedrine]. The amount of cathinone in khat varies depending on variety and area its grown. On the average, 100 g fresh khat may approximately give about 36 mg cathinone and 8 mg norephedrine(Kalix, 1996).

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 side-chain instead of two hydrogen atoms(Al-Motarreb et al., 2002; Wabe, 2011).

The main method of consumption of Khat is by chewing, and less often brewing as tea or smoking. The bark is peeled off, as well as leaves removed from branches and chewed as a ball in the mouth until a thick macerated residue remains and is later expelled from the mouth. Chewers stuff their mouths with tender leaves, shoots and barks and wait as the active chemicals are released intermittently from the Khat(Feyissa & Kelly, 2008), whereupon active cathinone and cathine are dissolved in saliva. Among the methods of intake of khat, chewing khat has been shown to be an efficient way of releasing cathinone and cathine(Omar et al., 2015).

In a typical chewing session that takes two to ten hours, an average of 100-500 grammes are chewed. The juice of the thoroughly chewed barks and leaves is swallowed, and the solid residues expelled. The absorption of the soluble components of khat occurs in two phases, oral phase through the mucosa of the buccal cavity and a second phase after swallowing of the khat, at the gastric and small intestine level (Balint et al., 2009). The stimulant, energy-giving burst occurs almost immediately the chewing begins, as the active compounds are absorbed rapidly from the buccal mucosa. Cathinone's stimulant and amphetamine-like effects are short-lived, taking about 15–30 min(Thomas & Williams, 2013).

The main reason for chewing Khat is its effects on the central nervous system. Khat contains various chemicals that affect various body systems. Cathine was the first chemical compound to be isolated from Khat, and is a modest psychostimulant. Cathinone, a more potent compound, was isolated in 1962, and is chemically known as 1(s)-(-) alpha aminopropiophenone. It possesses amphetamine-like characteristics. Both compounds increase the release of dopamine, serotonin and adrenaline from the brain, hence are known as 'natural amphetamines.' Adrenaline stimulates glycolysis, which leads to release of glucose, providing energy for the flight response. A N-methyl analogue of cathinone, Methcathinone, has been isolated and is more potent than cathinone(Cox & Rampes, 2003).

Cathinone has been found to produce quantitatively similar locomotor stimulation in mice compared to amphetamine. Thus, the effects of cathine and cathinone on neurotransmitters is homologous to amphetamine effects; both stimulate the central nervous system and suppress hunger centres in the brain(Kalix, 1981).
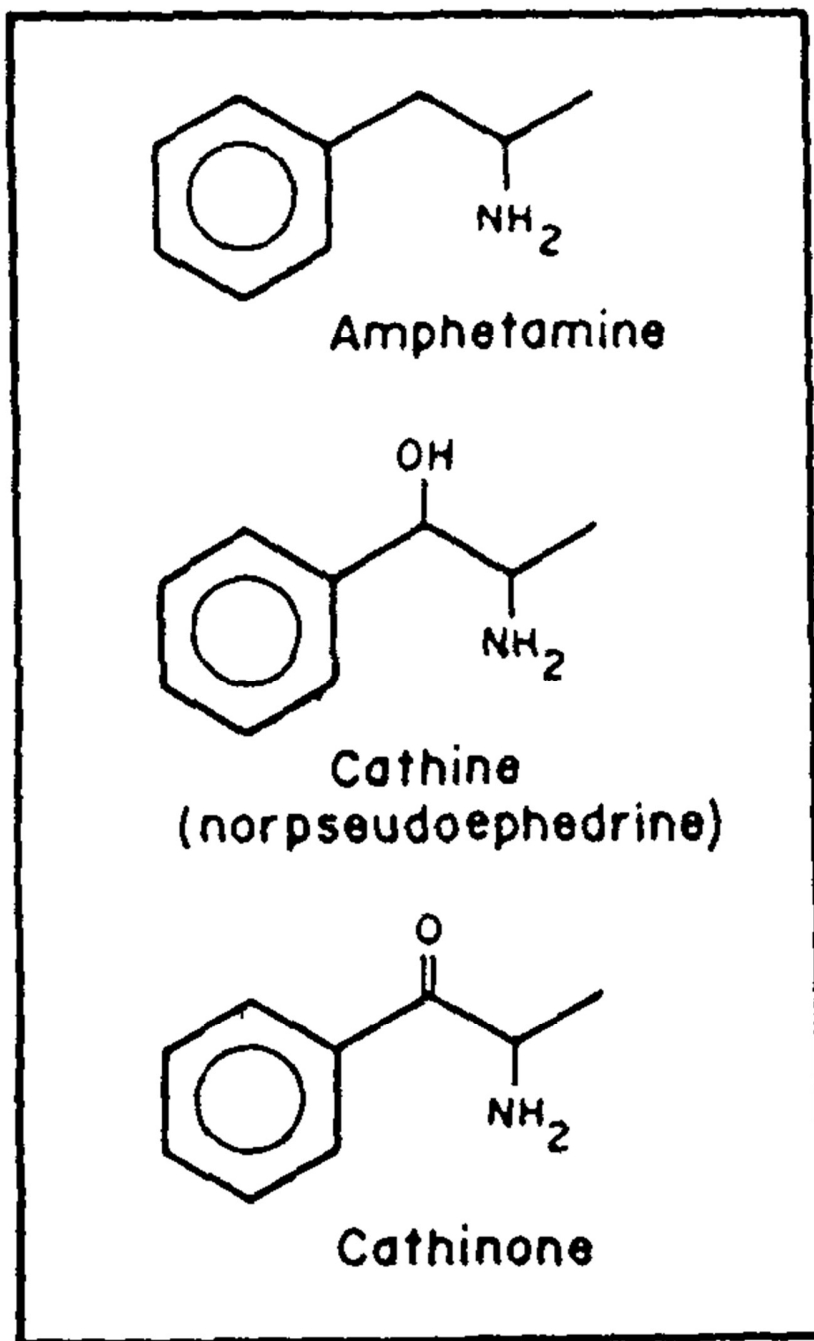
Figure 4: The chemical structure of amphetamine showing its close resemblance with cathine and cathinone, the main alkaloids in khat.

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(Ahmed et al., 2021). Fresh khat leaves contain higher levels of cathinone compared to dried leaves, hence more psychoactive effects. After oral administration, cathinone is rapidly absorbed through the oral mucosa and intestines. Its effects occur more rapidly compared to amphetamine. It acts at the neuronal level at the catecholaminergic synapse by delaying dopamine reuptake and enhance release. This replenishes the stores of adrenaline, hence the initial burst of energy seen in khat consumers (Milella & Nencini, 2010)

Table 1: Table showing chemical and functional similarity between cathinone and amphetamine (Graziani et al., 2008).

Model	Result
Feeding behaviour	Inhibited
Motor activity	Increased locomotor activity
Self-administration	Maintained
Drug discrimination	Amphetamine-like
Nociception	Analgesia
Cardiovascular	Pressure and heart rate stimulation
Metabolic	Lipolysis; increased oxygen consumption
Endocrine & Neurotransmission	NE and DA release; ACTH and corticosterone release

1.2.3 Chronic Khat use

Previous studies have compared rat years to human years. According to the National Institutes of Health (NIH) guidelines, the conversion of rat years to human years varies from pre-pubertal, pubertal, and adult stages ((Sengupta, 2013). During adolescence, it has been calculated that 10.3 rat days is equivalent to 1 human year (Quinn, 2005). In the current study, it is estimated that 6 weeks (42 days) of khat-feeding among young adult Wistar rats is equivalent to 4-5 years of almost daily khat use (more than 4 days per week) among khat users. This is corroborated by previous studies which have characterized habitual use of khat for more than 2 years as chronic khat use (Al-Habori, 2005).

1.2.3 Anatomy of the prefrontal cortex (PFC)

In primates, the prefrontal cortex (PFC) is defined as the most anterior cortical structure in the frontal lobe separated by premotor and supplementary motor areas from the rest of the cerebral cortex. It constitutes almost one third of the cortical surface, and is highly evolved in primates. Evidence suggests that the PFC is involved in a dynamic neural network which controls intelligent, self-regulating behaviours(Fuster, 2001; Miller & Wallis, 2009). The primate PFC is made up of medial, orbital and lateral areas.
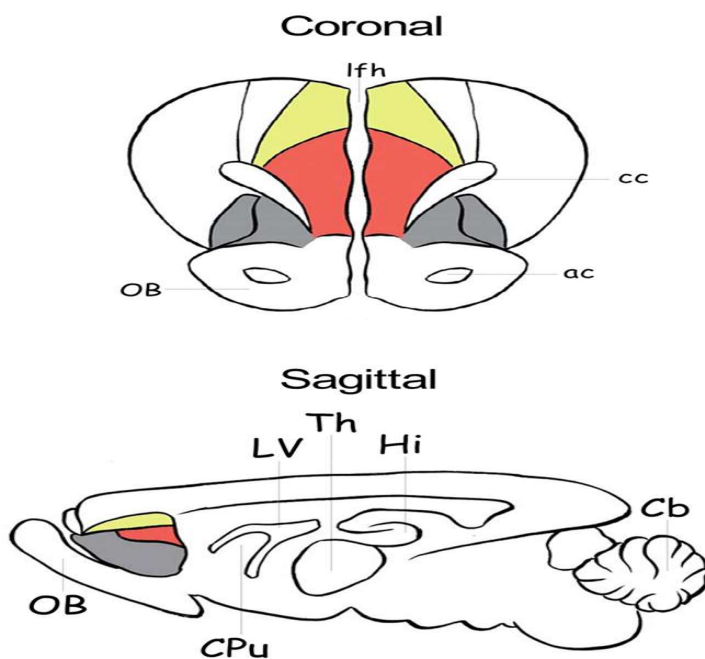
Figure 5: Diagram showing the medial prefrontal cortex (red), orbitofrontal cortex (grey), and anterior cingulated cortex (yellow), in sagitall and coronal sections of rat brain. OB, olfactory bulb; Cb, cerebellum; lfh, longitudinal fissure of hemisphere; cc, corpus callosum; ac, anterior commissure; CPu, caudate putamen; LV, lateral ventricle; Th, thalamus; Hi, hippocampus.

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(Fujisawa 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, 1987).

The PFC has also been defined as the cortical area that connects reciprocally with the mediodorsal thalamic nuclei (Uylings et al., 2003). The mPFC of the rat consists of three areas cytoarchitectonically;: infralimbic, prelimbic and anterior cingulated cortex. These areas have distinct and specific connections with cortical and subcortical structures of the brain, and serve distinct functions based on the connections. The anterior cingulated cortex modulates motor behaviour, while the Infralimbic and prelimbic areas serve emotional, cognitive and higher executive functions(Heidbreder & Groenewegen, 2003). Past studies have linked the acd of the rat to the primate supplementary motor and premotor areas in terms of structure and functional homology (Condé et al., 1995), while the ventral aspects of the PFC are homologous to the limbic system.

The frontal cortex has also been found to have hemisphere-specific functions. It is well accepted that cortical areas that serve motor and sensory functions are well lateralized, but less so for other cortices and locations(Hutsler & Galuske, 2003). However, language and speech were among the first to be found have a strong lateralization. Uylings et al., (2003)reported an

asymmetrical relationship between volume and total neuronal number Broca's area (BA) 44 in both male and female human brains. Similar evidence of asymmetry in the pyramidal neurons of left and right prefrontal cortex in the rat is inadequate. Higher brain functions such as emotional processing, which are partly served by the prefrontal cortex of the rat have been found to be asymmetrical in regulation. Davidson et al., (2000)reported that the right hemisphere takes a pre-eminent role in processes related to emotional processing. For instance, left frontal lobe strokes display a higher incidence of depression or catastrophic reactions compared to strokes affecting the right frontal lobe which caused indifference and manic responses (Robinson et al., 1984).

The rat PFC is a heterogenous structure, and it is better delineated into different subdivisions based upon their distinctive laminar features. Both the anterior cingulate cortex (ACd), and the prelimbic (PrL) region have clear laminar hallmarks, and their cytoarchitecture is characterized by a clear transition from layer 1, which is acellular, to a densely cellular layer 2. Layer 3 is broad and lightly stained with cells. Layer 5 in the ACd region is significantly broader compared to the PrL region. This sudden broadening of layer 5 is considered the transition point from the PrL to the ACd region(Krettek & Price, 1977; Van Eden & Uylings, 1985). Unlike the primate PFC, the rat PFC lacks a granular layer 4.

Neurons in the PFC are predominantly pyramidal, and have cholinergic, dopaminergic and serotoninergic connections. There are inhibitory neurons that are predominantly gabaergic, employing Gamma AminoButyric Acid (GABA) as their main neurotransmitter.

The PFC is at the centre of the Papez circuit, which is involved in memory and sorting and processing of emotions. The Limbic system serves as the centre of the brain's emotional processing. Paul Broca was the first to coin and use the term "limbic lobe" referring to a part of

the cerebral cortex that had a rim shape (Limbus = rim, in Latin) around the corpus callosum on the medial aspect of the hemicortex. Scientists for a long time thought that the "limbic lobe" was a primary cortical structure, but latter studies showed that many other major nuclei, not necessarily cortical, were part of the limbic system.

Previous studies by Heinrich Klüver and Paul Bucy (Klüver & Bucy, 1937, 1938, 1939) began to describe and expound on the complex motivational and emotional functions associated with the limbic lobe. For instance, monkeys subjected to bilateral temporal lobectomy demonstrated marked deviations in their emotional behaviour, with bursts of psychic loss of vision, blunted anger response, fear, oral tendencies and sexual aggressiveness.

These studies provided evidence that the medial temporal lobe was indeed a critical cog in the emotional circuit. (Papez, (1937) provided concrete evidence of the anatomical presumption of the limbic system, and showed that some brain regions in the medial temporal lobe are dedicated to emotional experience and expression. He argued that the hypothalamus has an effect on emotional expression, and that these emotions become consciously expressed and can be affected by higher inhibitory functions. According to Papez, there are reciprocal connections between the cingulate cortex and hypothalamus, as well as relay in the anterior dorsal thalamus. The cingulate gyrus sends projection fibres to the hippocampus which in turn connects to the hypothalamus via a large fibre bundle called fornix. These circuitous connections, henceforth christened the Papez circuit, provide a pathway for connecting brain regions involved in expression of emotions.

Improvements on the model proposed by James Papez were undertaken by Paul D. MacLean. It was found that other parts of the brain like the orbital and medial prefrontal cortex, ventral parts

of basal ganglia, mediodorsal nucleus of thalamus and the amygdala, a large nucleus in the temporal lobe anterior to hippocampus are also part of this complicated circuit. These brain parts, together with the hippocampus, hypothalamus and cingulate gyrus, is generally referred to as the "limbic system." All these structures interconnect intensively and none of them is solely

responsible for any specific emotional state.
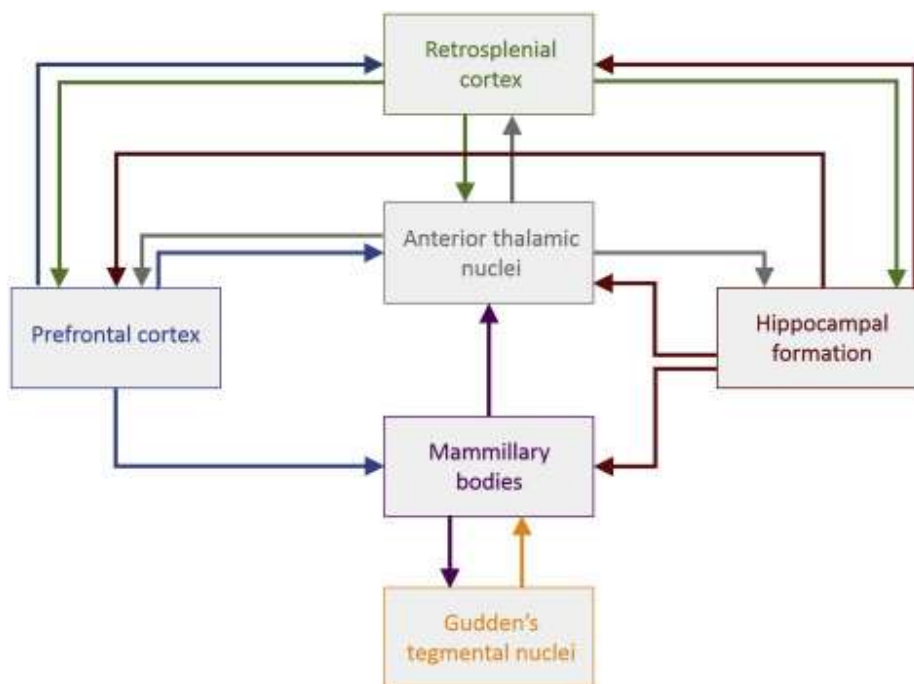
Figure 6: Schematic diagram showing the main connections within the wider Papez circuit. (Adapted from Handbook of Behavioral Neuroscience, 2018 (Nelson et al., 2018))

1.2.5 Prefrontal cortex in disease and substance abuse

The medial prefrontal cortex takes part in processing of attention, memory of work, and flexibility of behavioural aspects (Fuster, 2001). The dorsal Acd area of the medial prefrontal cortex plays critical roles in emotional and cognitive processing. (T. G. Bush et al., 1999).

The anterior cingulate area has been shown to play specific roles in different scopes of emotion such as handling stimuli related with emotions (Hadland et al., 2003; Morris et al., 1999; Rolls et al., 2003) decision making taking into account emotions and emotional experience (null Bush et al., 2000; Damasio et al., 2000).

Human studies have demonstrated striking personality changes with lesionectomy of the anterior cingulate cortex for management of affective disorders, and this may include emotional lability(Phan et al., 2002). Studies of emotional processing in normal healthy humans shows activation of anterior cingulate area, and this has also been evidenced in symptom provocation studies in mood disorders such as anxiety, phobia, and obsessive-compulsive disorders(Phan et al., 2002; Weissman et al., 2003).

Studies in rodents indicate that dendritic spines and dendrites in medial prefrontal cortex especially the anterior cingulate cortex are shaped in a big way by various emotional experiences such as brief and chronic maternal separation, social isolation and prenatal stress (Bock et al., 2005; Minzenberg et al., 2008; Murmu et al., 2006). Similar observations have been reported in other negative or neutral experiences such as stress or injection of corticosterone, which causes major changes in neuronal structures in prefrontal cortex (

Moreover, other types of negative or neutral experiences such as stress or injection of (Uher et al., 2004)cortex (Arnsten et al., 2014; S. M. Brown et al., 2005; Radley et al., 2004; Wellman, 2001).

In rats, medial prefrontal cortex is known to serve executive and supervisory roles such as attention to characteristics of stimuli and chore exigencies, behavioural flexibility and attention set-shifting (Dalley et al., 2004). Hence lesions in this area produce disorders of poor attention to stimulus. The medial prefrontal cortex is also useful in learning tasks which need increase cognitive ability, such as those involving complex discriminations and working memory (Euston et al., 2012). It also plays critical roles in extinction (Uher et al., 2004), and this may involve reciprocal connections with the hippocampus.

The prelimbilic and infralimbic parts of the medial prefrontal cortex play distinct roles in learning and impulse-oriented action. For instance, prelimbic area maintains representations of goals and the relationships between goals and actions, while the infralimbic area influences habitual responding by inhibiting goal-directed actions (Coutureau & Killcross, 2003).

Lesionectomy studies show that damage to infralimbic area results in impulsive action with untimely responses, whereas prelimbic lesions causes compulsive action resulting in perseverative responses (Chudasama et al., 2003). When both the infralimbic and prelimbic areas of the medial prefrontal cortex are damaged, there's resultant loss in goal-directed responses and perseverative habitual responding (Ragozzino et al., 1999).

There's near overwhelming evidence that the medial prefrontal cortex plays crucial role in drug abuse behaviours related to impulsivity, and this has been linked with the release of serotonin and dopamine(Winstanley et al., 2006).

It is therefore apparent that the prefrontal cortex is implicated extensively in abuse of drugs through self-administration and in impulsivity and compulsivity.

1.2.6 Astrocytes and other glial cells

Astrocytes are at the centre of brain function at the cellular and holistic brain level, and there exists as many astrocytes as neurons in the mammalian brain. They have been known to regulate the homeostatic and ionic balance of neurotransmitters, provide metabolic nourishment to neurons and monitor and regulate synaptic activity. They therefore moderate synaptic plasticity and cause changes in behaviour, placing them at the centre of physiological tasks, progression and outcome of neurological diseases.

Intermediate filament proteins, Glial Fibrillary Acidic Protein (GFAP) and vimentin which are expressed in astrocytes are crucial in astrocytic control of neurogenesis, neuronal remodelling and neuronal regeneration. Ablative experiments of GFAP and vimentin in mice (Colucci-Guyon et al., 1999; Pekny et al., 1995; Pekny & Lane, 2007; Pekny & Pekna, 2004) creates more permissive environment to transplantation of neural grafts or neural stem cells(Kinouchi et al., 2003; Widestrand et al., 2007) and increases axonal and synaptic regeneration(Bao et al., 2012; Menet et al., 2003; Wilhelmsson et al., 2004).

Astrocytes also regulate the blood-brain barrier operations and characteristics, and may modulate the neurogenic niche indirectly by determining the accessibility of blood-derived factors/molecules that modulate neurogenesis (Barres, 2008; Guérit et al., 2021). They are thought to release signals that stimulate synaptogenesis, as well as signals that initiate removal and pruning of redundant synapses (Nägler et al., 2001).

They also help maintain contacts between synapses after neuronal injury. Hence, mice with ablation of GFAP and vimentin have a more pronounced loss of synapses in the hippocampus in the acute phase (4 days) after the entorhinal cortex lesion. Remarkably, synaptic recovery of

these mice was also increased, reaching the levels on the uninjured side on day 14 after lesion (Wilhelmsson et al., 2004).

In summary, astrocytes both directly and indirectly control the neurogenic niche and integration and survival of newly formed neurons. They therefore form a good potential target for drug modulation for generation and to promote survival of newly formed neurons in diseases such as stroke and degenerative conditions.

Positioned at the crossroads between the neuro-vasculature and neurons, astrocytes can modulate exchange of molecules between neurons and the extracellular space and the vascular compartment. Due to their high numbers and dense packaging in the brain, peripheral astrocyte processes may constitute half of the glial cell volume and about 80% of its surface(Liu et al., 2018). The perisynaptic processes, which ensheath synapses, display lamellar structure; their functions are multi-faceted, e.g. isolation of individual synapses from their environment, uptake of neurotransmitters, and involvement in synaptic transmission and plasticity. Interaction of neuronal, glial, and vascular elements results in the formation of specialized functional territories, which may range in size from a few nanometers up to centimeters.

Astrocytes constitute a large percentage of the brain volume. Their microscopic and fine architecture in the human brain is complicated and elaborate with a winding network of fine processes (Oberheim et al., 2006). Because of this complicated and intricate strictire and network, they are able to modulate and monitor a large number of synapses and participate efficiently in homeostatic regulation of neurotransmitters within the brain. (T. G. Bush et al., 1999; Newman, 2003a).

Having been now known to release neurotransmitters such as glutamate in a calcium-dependent mechanism, scientists now consider astrocytes indispensable in signaling processes in concert with neurons (Parpura et al., 1994; Parpura & Zorec, 2010).

It is notable that instead of neurons, astrocytes do synthesize glutamate via a de novo pathway, and also store glucose in the form of glycogen(Hertz & Zielke, 2004). There are many schools of thoughts and hypotheses on the contribution of astrocytes to metabolic and communication roles in the brain, but owing to their high oxidative metabolic activity, it is possible that they are actually at the centre of this activity. Further, astrocytes have abundant mitochondria, found even in their fine processes (Lovatt et al., 2007).
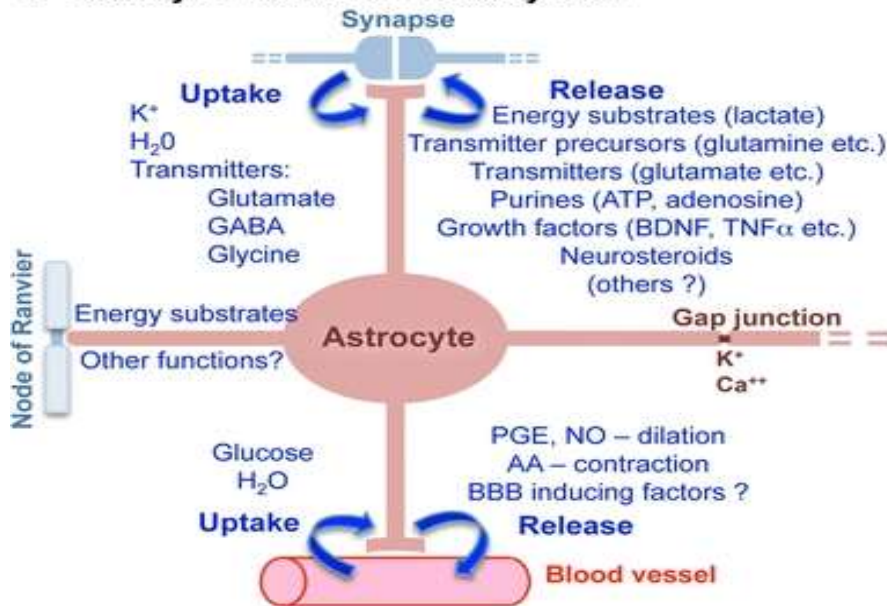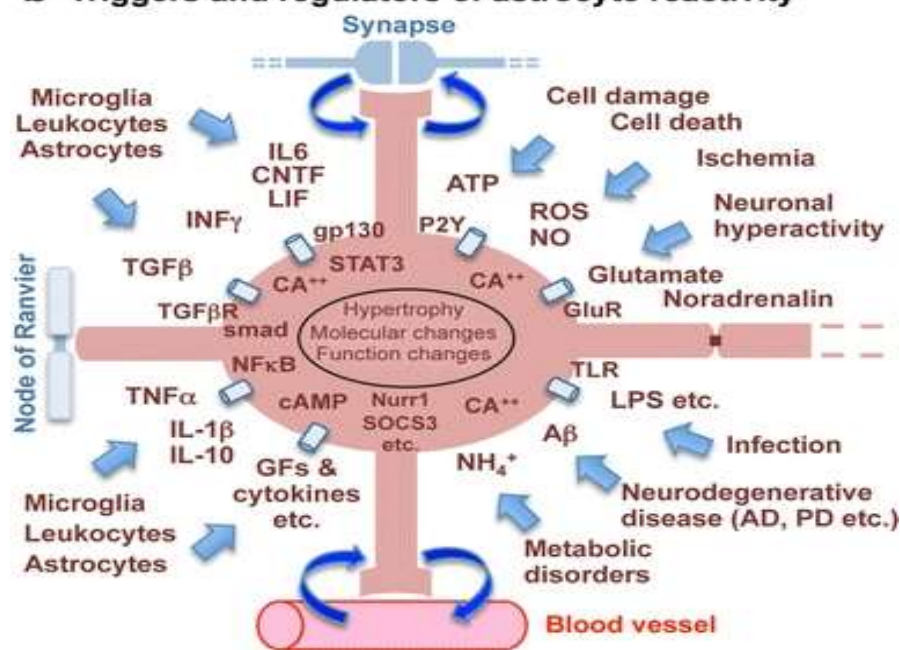
Figure 7: An illustration of astrocyte function in the healthy brain (Adapted from (Nelson et al., 2018).

1.2.7 Role of oligodendrocytes in drug and substance use

Oligodendrocytes are involved in the formation and conservation of myelin, which basically consists of packed layers of their cell membrane wrapped around the axons of neurons(Baumann & Pham-Dinh, 2001; Butt & Dinsdale, 2005). This acts as insulation against dissipation of ionic gradients, allowing fast conduction of action potentials between consecutive nodes of Ranvier, ensuring efficient exchange of neural impulses between brain centers (Nave & Werner, 2014).

It appears that oligodendrocytes don't only produce myelin; they also produce signals and growth factors that support axons of the neurons and astrocytes in their vicinity(Du & Dreyfus, 2002; Nave & Trapp, 2008; Simons & Nave, 2016). A lot has yet to be unravelled on the complete functions of oligodendrocytes.

Oligodendrocyte function has been found to be affected by substances such as alcohol. For instance, prolonged and repeated alcohol intake causes damage and adaptations in the myelin that ensheaths the gray and white matter. Discernible loss of myelin both in neuroimaging and postmortem histochemical studies has been observed. This has also been explained in neurological disturbances such as Wernicke-Korsakoff syndrome, hepatic encephalopathy, alcohol cerebellar degeneration- all these demonstrate myelin loss in the cortex and subcortical areas(Zahr & Pfefferbaum, 2017).

In alcohol use disorders associated with nutritional deficiencies, the main mechanism of brain perturbations is disruption of myelination, hence hampering neuronal communication and other functions. (Alexander-Kaufman et al., 2007; Baydyuk et al., 2020). The high prevalence of cigarette smoking that goes hand in hand with ethanol abuse may form a destructive partnership that destroys myelin proteins (Tong et al., 2015).
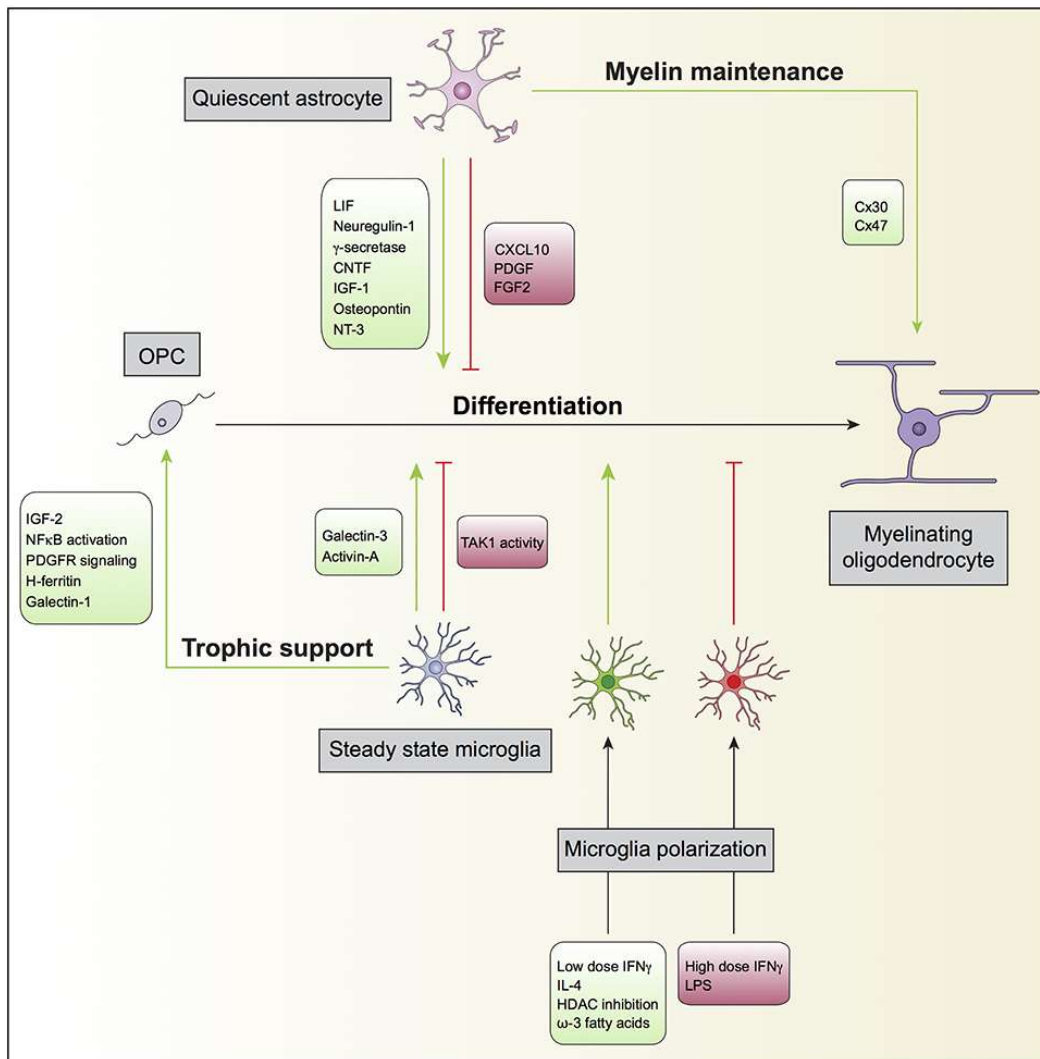
Figure 8: An illustration of the role of oligodendrocytes in myelination and trophic support of other brain cells (Adapted from (Nelson et al., 2018).

Oligodendrocytes precursors are oligodendrocyte progenitor cells (OPC). These cells have the

potential to multiply, migrate and differentiate into mature oligodendrocytes capable of

myelination. New OPCs are usually recruited in case of demyelination to differentiate into

myelinating oligodendrocytes to restore myelin and hence ensure proper neuronal function and structure. However, substances of abuse, and their attendant nutritional deficiencies and toxic metabolites hamper this recapitulation process (Franklin & Goldman, 2015).

Precursor cells for oligodendrocytes form about 8% of the cellular population in the central nervous system (Levine et al., 2001) and are found in high numbers and density in areas such as the optic nerve(Shi et al., 1998), motor cortex, corpus callosum(Clarke et al., 2012), and cerebellum(Zezula et al., 2001). Here, they ensure a steady cource of new oligodendrocytes, sustaining remyelination as and when needed. Adult OPCs have limited potential for myelin formation (Bradl & Lassmann, 2010), but can be quite efficient during development or injury(Shi et al., 1998).

Oligodendrocytes are produced at a steady rate in the healthy adult brain. In human brain, although the oligodendrocyte turnover is very stable, with an annual change of 0.3% (Yeung et al., 2014), new studies reveal that myelination and remodelling of myelin is a life-long process, not just during childhood and adolescence. Actually, it was shown that inhibiting the formation of new oligodendrocytes during adulthood, without compromising preexisting oligodendrocytes and myelin, prevented mice from learning new motor skills(McKenzie et al., 2014), suggesting that the formation of new myelinating oligodendrocytes during adult life is an important mechanism for neuroplasticity.

Given that astrocytes and oligodendrocytes originate from the same precursor cell, and their extensive crosstalk and co-ordinated function, it is therefore plausible that use of substances such as khat may structurally affect both cells, and may explain the neurocognitive changes that occur in their use.

1.2.8 Preference for male rats in khat studies

Chronic khat use among communities in Kenya and the Horn of Africa is mostly practiced by men (Alele et al., 2013), and in most of these communities, it is considered reprehensible and shameful for women to use khat ((Abdulwaheb et al., 2007). Further, it has been found that estrogen and progesterone modulate the structure and function of neurons and neuroglial cells (McEwen & Milner, 2017). Estrogen has particularly been found to protect neurons from excitotoxicity and inflammation.

It is therefore plausible that use of mixed sex during this study would confound the effects of khat due to influence of reproductive hormones.

Consequently, the choice of male rats in their young adult stage corresponds to the human group that is most likely to abuse khat.

1.3   STATEMENT OF THE PROBLEM

Use of Khat remains a prevalent, albeit controversial practice in several countries in the world. Its effects on the brain have been demonstrated mostly through its close pharmacological similarity with amphetamine (Kalix, 1981). Several behavioural studies point at changes in cognition, behaviour and other executive functions, which are associated with the prefrontal cortex (Corkery et al., 2011). Current research links astrocytes with various functional neural pathways (Hertz & Zielke, 2004). It is therefore plausible that substances that affect neural function may affect the structure of astrocytes.

Despite the studied link between long-term khat use and neurocognitive perturbations, there are few studies describing the anatomic changes in the prefrontal cortex such as cortical volume, pyramidal neuronal density, immature neuronal architecture and quantification of apoptotic neurons. Additionally changes in the structure and distribution of astrocytes in the PFC have not been studied among long term khat users, despite strong evidence that these cells are central in neuronal homeostatic and communication function.

1.4   CONCEPTUAL FRAMEWORK

```
                        ┌─────────────────────┐
                        │  Male rats (4 groups)│
                        └─────────────────────┘
                           ↙               ↘
┌──────────────────────┐         ┌──────────────────────────┐
│ Fed on normal saline,│  ←→     │ Structure of astrocytes, │
│ and doses of khat for│         │ and immature and mature  │
│ 6 weeks              │         │ pyramidal neurons and    │
│                      │         │ oligodendrocytes         │
└──────────────────────┘         └──────────────────────────┘
```
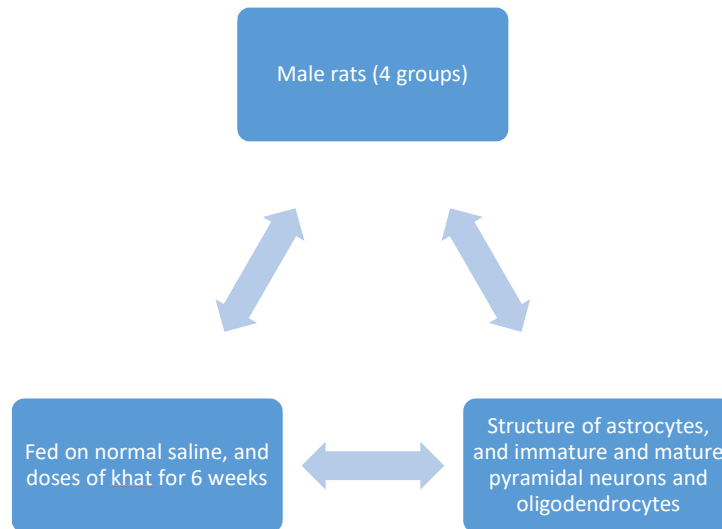
What is known/unknown

Khat is known to have behavioral and neurocognitive effects.

These functions are modulated in the PFC

The structural correlates are unknown

What will be done

- 4 groups of rats (Male) randomized on physiological saline and various doses of khat
- Fed on this diet for 6 weeks (chronic use)
- Brains harvested, PFC identified, light microscopy and IHC performed

Expected outcomes

- Differences in the PFC neuronal and astrocytic morphology visualized and analysed
- Data disseminated

HYPOTHESIS

Long term khat use does not result in changes in neuronal and astrocyte cell density, morphology and distribution in the prefrontal cortex of rats.

1.5   OBJECTIVES OF THE STUDY

1.5.1 Broad Objective

To evaluate the changes in neuronal architecture and density, as well as neuroglial morphology in rats after long-term use of khat (miraa).

1.5.2 Specific Objectives

a) To evaluate the gross morphometric indices of the rat brain following chronic khat use

b) To evaluate the changes in density and morphology of normal and apoptotic pyramidal neurons following chronic khat use

c) To determine the presence and density of immature migrating neurons following chronic khat use

d) To determine the changes in astrocyte and oligodendrocyte distribution, density and morphology following chronic khat use

Karau, PhD Thesis, Uon, 2023

CHAPTER 2:   MATERIALS AND METHODS

2.1 Study Design

This was a Randomized experimental study design using young adult male Wistar rats

2.2 Study Setting

Animal handling and khat experiments were performed at the animal house of the Department of Veterinary Anatomy and Physiology, the University of Nairobi. Thereafter animals were sacrificed at the histology laboratory of the Department of Human Anatomy, the University of Nairobi where fixation and light microscopy were performed. Paraffin-embedded blocks were shipped to the School of Anatomical Sciences, Section of Morphological Sciences, the University of the Witwatersrand, Johannesburg, South Africa where immunohistochemistry was carried out.

2.3 KHAT PROCESSING

2.3.1 Khat Extraction Procedure

Khat samples (consisting fresh shoots) were collected from a local farm in Mikinduri, Tigania Central Subcounty, Meru County, Kenya and transported in a cooler box to the lab within 4 hours of harvesting. For standardization, khat was purchased from the same trader, who in turn had harvested it from the same farm in all instances. This was done during the August dry season. Khat was harvested between 6am and 8am every day. Identification of the khat species and cultivar was done by a qualified botanist at the Kenya Bureau of Standards.

Upon arrival, each bunch of Khat was weighed and data recorded as shown in table 2 below. After weighing, each bunch was chopped to homogenize the sample and blended with 125ml of sterile distilled water.

Figure 9: Illustration of the processes used in sample preparation of khat before extraction by lyophilization

 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.

Figure 10: Crude extract in 100ml bottle

Table 2: *Weight of Khat bunches for extraction*

Sample Code	Weight (grams)	Sample Code	Weight (grams)
A	126.13	11A	153.47
B	145.66	N	183.39
C	155.39	O	141.66
D	112.47	P	159.16
E	158.16	Q	142.05
F	129.91	R	138.31
G	142.1	S	134.85
H	146.55	T	140.21
I	160.4	U	142.1
J	122.81	W	147.38
L	144.1	X	170.13
M	216.13	Y	127.74
		Z	169.34
1A	156.52	6A	143.73
2A	197.35	7A	179.54
3A	114.91	8A	122.29
4A	153.04	9A	102.71
5A	132.2	10A	169.93

∗Each bunch of Khat was blended in 125ml of distilled water

Karau, PhD Thesis, Uon, 2023

2.3.2 Lyophilisation Procedure

Supernatant from Khat extract was then dispensed in volumes of 3ml into vials for lyophilisation. The vials were first frozen at -80℃ for 2 hrs then freeze dried under vacuum at 0.103mBar for 24hrs.

Figure 11A: Extracts frozen at -80℃ before lyophilization, covered in aluminium foil to avoid direct contact with light.

Figure 11B: Lyophilization procedure taking place in a lyophilizer for 24 hours

Figure 11C: Khat extract sample (left) and the freeze-drying indicator (right) after lyophilization.

2.4 ANIMALS: HANDLING OF RATS, EXPERIMENTS AND TISSUE HARVESTING

2.4.1 Animal Procurement and Housing

Young adult male Wistar rats (*Rattus Norvigecus*) aged 2-3 months and weighing 200-300 grammes were used in the experiment (Figure 12). The animals 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*). Before any experiment, they were allowed to acclimatize in their respective cages for at least 7 days.

Figure 12: Photograph of a young adult male wistar rat, at 2-3 months, weighing 200-300g

2.4.2 Ethical considerations in animal handling

We carried out all animal handling and experiment procedures pursuant to provisions of the Guide for Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985). The animals were kept in animal houses under standard conditions, adequate ventilation and correct temperatures. They were fed on rat pellet feeds and provided with drinking water *(ad libitum)*.

This study was carried out pursuant to the University of Nairobi's animal handling guidelines. The research protocol with animal experimentation was approved by the Biosafety, Animal use and Ethics committee of the University of Nairobi (REF FVM BAUEC/2020/276).

2.4.3 Animal experiments

The rats were placed into four trial 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 experimental groups were fed on normal rat diet ad libitum, in addition to a Khat extract once a day at three increasing dosages: 500mg/kg (K500), 1000mg/kg (K1000) and 2000mg/kg (K2000) for a period of 6 weeks, by oral gavage.

The doses of Khat correspond to the approximate amount of Khat weight consumed by humans (chronic users) and the yield value of the lyophilized Khat extract (estimated at 1-2%). These are then converted to rat's dose based on the method by Reagan-Shaw et al., 2008).

Animals were weighed every week to determine the dose of khat to be fed, and the weights plotted in an Excel sheet.

2.4.4 Animal sedation and perfusion

The animals were sedated by inhalational chloroform by placing them in a jar with cotton wool soaked in chloroform. They were then euthanized with intraperitoneal ketamine (100mg/kg) and xylazine (10mg/kg).

The rats were then put in lithotomy position over an operating board and their limbs held out in a crucifixion position. The chest cavity was opened by midline skin incision, with reflection and retraction of ribs. An 18-gauge needle, which was attached to a clear plastic tube connected to the syringe with perfusate, was inserted into the left ventricle and tied with artery forceps. An

outflow needle was placed in the right atrium to drain blood and perfusate. Figure 13a and b

shows the perfusion procedure performed using the gravity method.

Figure 13a: Photograph showing perfusion by the gravity method
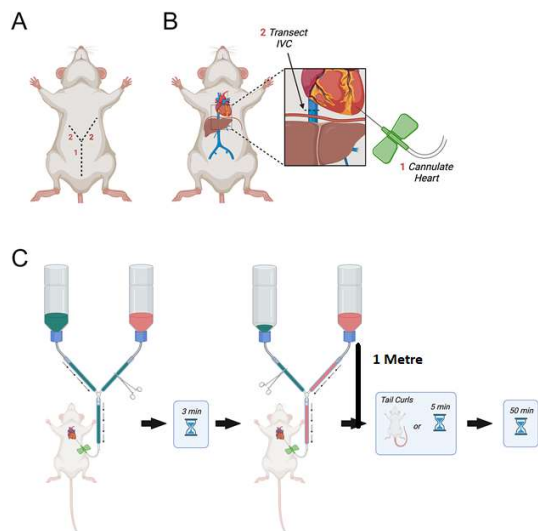
Figure 13b: A depiction of the gravity method of perfusion, adapted from (Rana et al., 2022)

The right atrium was nicked open using a surgical blade to drain the blood and fixative completely during perfusion. Using gravity, we expended perfusate equivalent of 15% of body weight for 20 minutes to drain blood and ensure only clear fluid came out of the rat (Rana et al., 2022).

The rat brains were fixed by cardiac perfusion with 0.9% saline followed by 200-300ml of 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Figure 13shows the perfusion procedure for the animals.

2.4.5 Dissection and brain harvesting

Using a scalpel blade, the head of the rat was severed at the atlanto-occipital junction and the skin gently removed over the head. Scissors and bone chip were used to chip off the skull bones until the brain was reached with an intact olfactory bulb extending towards the area of the nasal bone.

We stripped away the pachymeninges with great care not to damage the brain, starting from the frontal region to the occipital area, and dissected out the tentorium cerebelli. The falx cerebri butting in between the olfactory bulbs was gently removed, and the cranial nerves at the base of the brain cut off to free the brain.

We examined the gross structure of the brain visually with the help of a lens, scalpel and forceps. Figure 14 shows an extracted whole brain of the rat with intact major structures.

The following gross parameters were measured

1. Body weight (g): Weight of the rat in grams

2. Absolute brain weight (g): Weight of the whole dissected brain without the meninges

3. Relative brain weight: Ratio of brain weight to the body weight

4. Maximum cerebral cortical width (mm): maximum length across the most lateral portion of the parietal lobes of the cerebral cortex

5. Maximum cortex length (mm): maximum length from the tip of the frontal lobe to the caudal most portion of the occipital lobe of the cerebral cortex
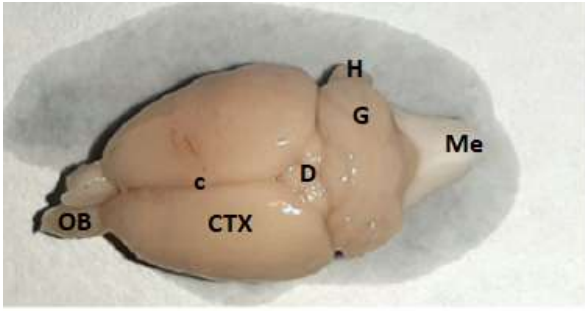
Figure 14: An extracted rat brain showing a lissencephalic cortex and major structures. OB-Olfactory bulb, CTX; Cortex, c; Cerebral longitudinal fissure, D; Caudal colliculus, G; Flocculus, H; Paraflocculus, Me; Medulla oblongata.

Figure 15: Measurement of the antero-posterior diameter of the rat brain

Figure 16: Measurement of the vertical diameter of the rat brain

2.5 Light Microscopic Techniques

2.5.1 Preparation of tissues for staining

The brains were then rapidly removed from the skulls, put in buffered formaldehyde and embedded in paraffin blocks. Coronal blocks were obtained as per the method described by Paxinos and Watson. The tissues were stained with haematoxylin and eosin for cellular detail. Light microscopy was performed in the histology section of the Department of Human Anatomy, the University of Nairobi.
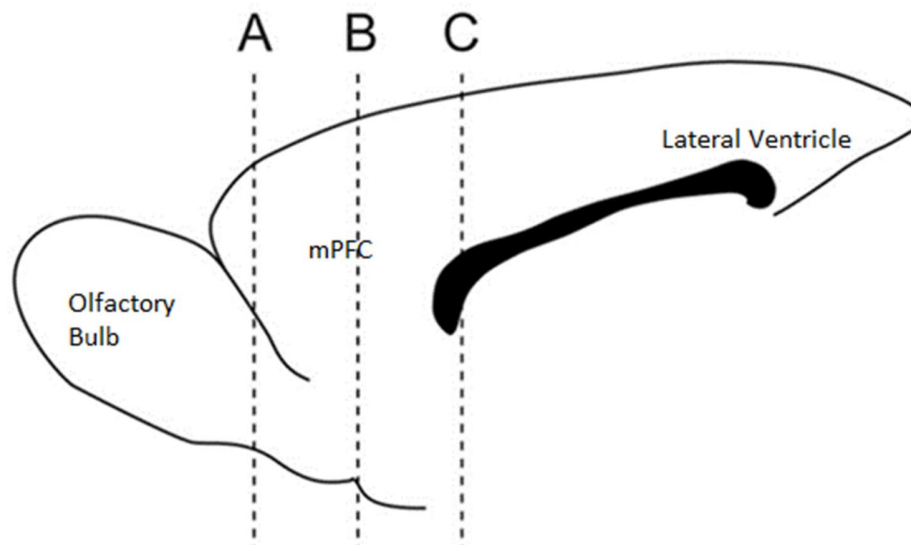
Figure 17: Sketch of the rat brain showing the area where histological sections 1mm thick were cut in the region between A and B, with the olfactory tract intact. (mPFC; Medial Prefrontal cortex)

2.5.2 Haematoxylin and Eosin method

The harvested sections of the prefrontal cortex, randomly selected from both hemispheres for khat-fed rats and control groups were fixed in formaldehyde by immersion method for 24 hours afterwards with dehydration in rising concentrations of alcohol (70%, 95% and 100%) each for 30 minutes. Xylene was used to clear, followed by wax infiltration for 24 hours.

The tissues were then embedded in paraffin wax. The embedded tissues were serially cut into 5 micrometre thick sections with a microtome, starting from the outer gray matter to the deep cortical tissues in order to have a view of all the layers. Every 5th section was sampled and

picked on a glass slide. It was dried in an oven, dipped in xylene to remove paraffin wax, and rehydrated in descending strengths of ethanol (100%, 95% and 70%).

After rehydration the sections were placed in a jar containing Haematoxylin for 10-15 minutes then flushed in tap-water for 2 minutes to remove any excess stain. The sections were stained in 1% eosin solution for 3 minutes, followed by dehydration in ascending grades of ethanol from 70% to absolute alcohol. The sections were finally cleared in xylene before being covered with cover slides. Slides are then examined under light microscope and photomicrographs taken.

2.5.3   Toluidine blue staining for Nissl substance

The tissue sections were dewaxed in two cycles of xylene, each taking 5 minutes, and then rehydrated in descending grades of alcohol starting from xylol 50:50, each taking 3 minutes. Slides were placed in running tap water for 15 minutes. They were then immersed in 1% aqueous toluidine blue for 6 hours. Stained tissues were 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.

2.6   Immunohistochemistry

Paraffin-embedded blocks were transported to the University of the Witwatersrand, Johannesburg, Republic of South Africa, where immunohistochemistry and photography was performed at the School of Anatomical Sciences of the university.

2.6.1   Preparation of buffer solutions

a) 10Mm Sodium Citrate Buffer PH 6

Anhydrous tri-sodium citrate 2.9 g was added to 1000mls of distilled water, and mixed in a magnetic stirrer to dissolve and PH adjusted to 6 with 1M hydrochloric acid. When required, we added 0.05@ Tween 20.

b) 1M Phosphate buffer PH 7.4 (PBS)

Sodium chloride 16g, disodium hydrogen phosphate (anhydrous) 3.5g, potassium chloride 0.4g, potassium dihydrogen phosphate 0.4g were added to 2000mls distilled water, and mixed in a magnetic stirrer. PH was adjusted to 7.4 using 1M hydrochloric acid.

c) Diamino-Benzoic (DAB) working solution

In a clear Bijou bottle, we measured out 1mg DAB (0.001g). To this, we added 2mls Tris HCl and mixed well. This formed solution (a). In a separate tube, we added 29μL cold distilled water, 1 μL hydrogen peroxide (100%). This formed solution (b). The final DAB working solution was formed by adding 20 μL of solution (b) to DAB solution (a). The combination of solution a and b was only done 30 minutes before the DAB solution was used for IHC.

2.6.2 Immunohistochemical methods for paraffin-embedded sections

Day 1

Sections were deparaffinized by immersing in xylene twice for ten minutes. They were then rehydrated through graded alcohols, starting with absolute alcohol to 95% alcohol. This was followed by washing the slides in running tap water for 5 minutes. Antigen retrieval was performed by incubating the slides in citrate buffer at pH 6 overnight in a water-bath set at $60^{\circ}C$.

Day 2

On the second day, sections were removed from the heated water-bath citrate buffer and allowed to cool for 20 minutes. They were then rinsed with PBS buffer once. We blocked endogenous peroxidase with 1% hydrogen peroxide in methanol. This solution was made by simply adding 2mls hydrogen peroxide to 198mls of 100% methanol. Slides were immersed in this solution for 20 minutes. After this treatment, they were again washed in PBS buffer three runs of 5 minutes each. This was followed by incubation in a moist chamber in 5% normal goat serum for 30 minutes. We prepared 5% normal goat serum by adding 100 µL of normal goat serum to 1900mls of PBS buffer. Since each slide required approximately 100 µL of 5% normal goat serum to completely cover the tissue, we prepared enough based on the number of slides.

After incubation, the normal goat serum was tapped off slides, and then primary antibody was used to incubate tissues overnight at $4^{0}C$.

NB: Following manufacturer's instructions and from literature review, we optimized primary antibodies as follows:

- o Doublecortin (DCX) using dilutions of 1:100, 1:200 and a negative control. After viewing initial trial slides, a dilution of 1:100 was found to be optimally staining and was selected.

- o 2', 3'- Cyclic-3'-Nucleotide Phosphodiesterase (CNPase) using dilutions of 1:1000, 1:1600, 1:2000 and a negative control. A dilution of 1:1600 was finally selected.

- o GFAP using dilutions of 1:200, 1:300 and a negative control. After optimization, a dilution of 1:300 was selected.

Day 3

After overnight incubation in a cold chamber with the primary antibody, slides were washed in PBS thrice, each wash lasting 5 minutes. They were then incubated in secondary antibody (biotinylated goat anti-rabbit, Vector Labs, 1:1000), for 30 minutes.

The tissues were washed in PBS for three runs of 5 minutes each. They were incubated for 30 minutes with Avidin-Biotin Complex (ABC). ABC solution was prepared 30 minutes before use by adding 25 µL Avidin + 25 µL Biotin to 1.25ml of PBS buffer. This was followed by three washes of 5 minutes each in PBS buffer. The tissues were then incubated with DAB working solution for 5 minutes, rinsed in running tap water. They were counterstained with Haematoxylin for 1 minute, washed again in tap water for 5 minutes and hydrated in graded

alcohols. Last clearing was done in xylene and slides were mounted with Entellen. Stained tissues were then viewed and imaged as necessary.

2.7   Determination of Cell Densities

2.7.1 Pyramidal 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.

Cell densities per field (n/μm^2) = Number of cells counted (n) / grid area (μm^2).
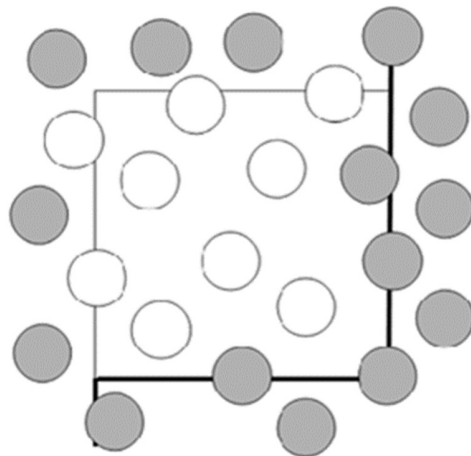
Figure 18: An illustration of the cell counting method.

FIGURE LEGEND: 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.
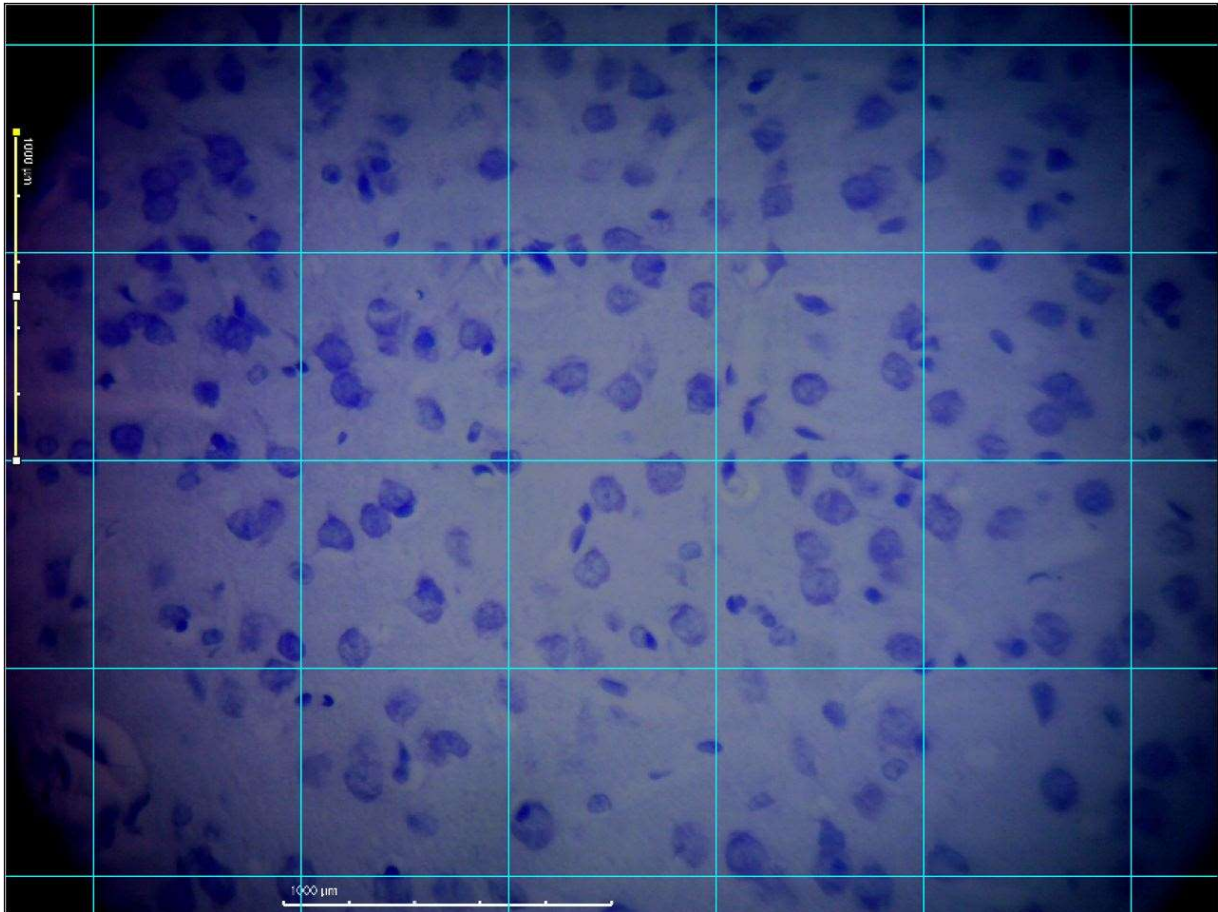
Figure 19: Figure displaying the grids used to determine the pyramidal neuronal densities

2.7.2 Immature Doublecortin staining neurons

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. Only the immature pyramidal neurons that had taken up the DCX stain and that fell within the grid squares were counted. The counts were taken from a random square grid after which every fifth grid was chosen. A total of 6 grids per rat were assessed for the neuronal density and subsequently averaged. Each square grid had an area of 1,000 μm^2.

In figure 20 below, 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.
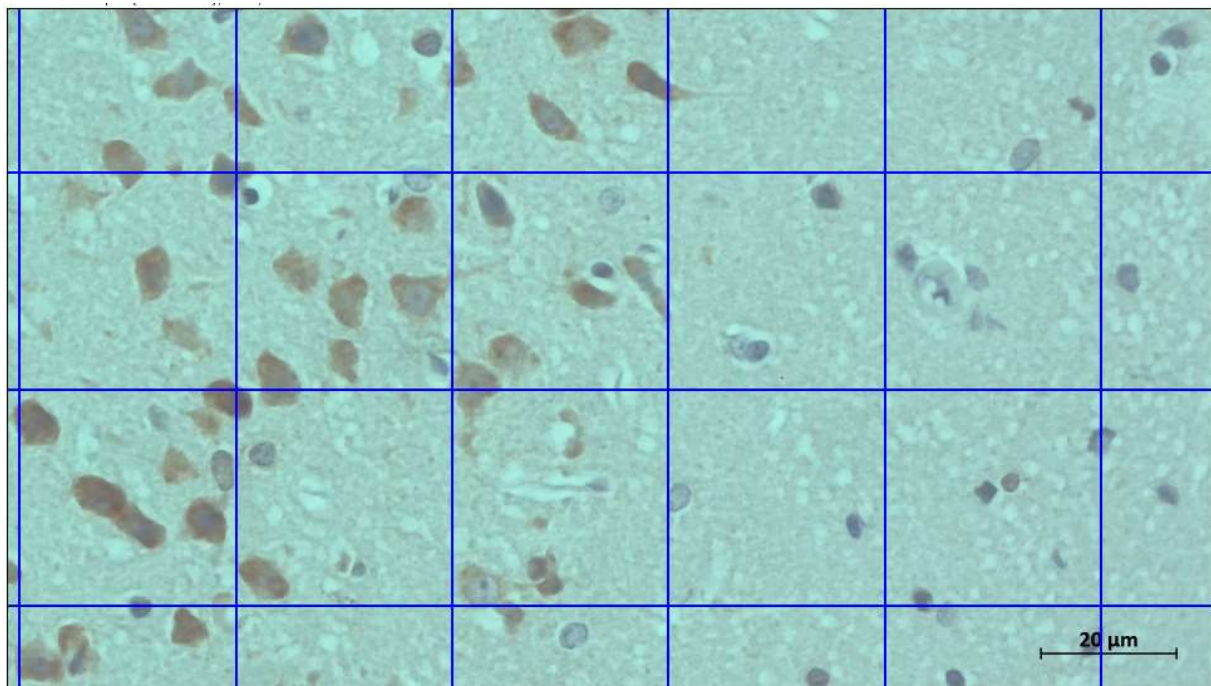
Figure 20: Figure displaying the grids used to determine the immature neuronal densities

2.7.3 GFAP immunoreactive astrocytes

The photomicrographs (X40 magnification) were analyzed using Fiji image J software. Grids lines were centered on the photomicrographs making a total of 15 complete square boxes, each with an area of 1000um^2 (Figure). Complete astrocytes (with cell body and processes) were counted in all the fifteen boxes. The average number of astrocytes per group was entered onto Excel for purposes of generating a bar graph.
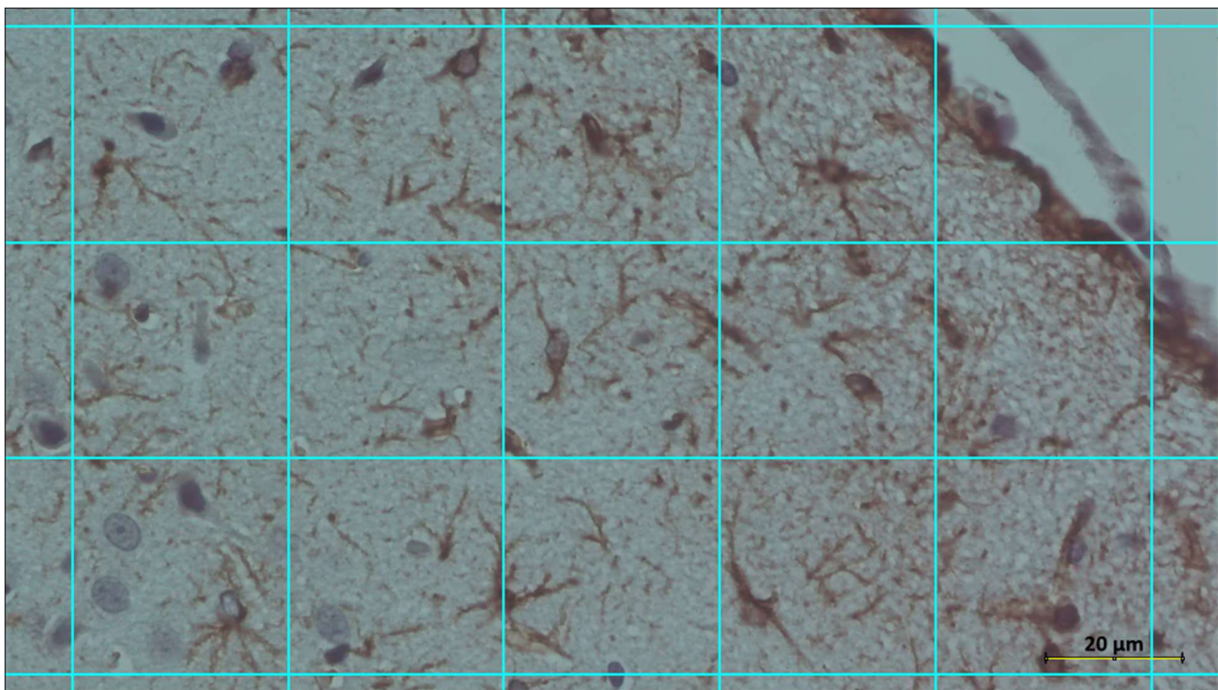
Figure 21: Figure displaying the grids used to determine the immature neuronal densities

2.7.4 Determination of oligodendrocyte densities

Photomicrographs were loaded onto Fiji image J software. The oligodendrocytes that had taken up the CNPase stain in the entire field of view were counted. A total of 2 field of views per rat were assessed for the oligodendrocyte and subsequently averaged. Each field of view had an area of 18,000 μm^2.

Oligodendrocyte densities per field ($n/\mu m^2$) = Number of oligodendrocytes counted (n) / field of view area (μm^2).

2.8   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), immature DCX positive neurons and GFAP immunoreactive astrocytes. 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. Further, we were unable to control the normal distribution of all variables among the rats, and the population was small, justifying the need for non-parametric tests instead of parametric tests.

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%. Tables, graphs and photomicrographs were then used to demonstrate histological findings.

CHAPTER 3: RESULTS

3.1 Gross Morphometric indices of rat brains

The adult brains displayed putative lissencephaly, with prominent olfactory bulbs, an oblong cerebral cortex and prominent cerebellum and medulla as shown in Fig 22 below.

The mean body weight of the control rats was 414.36g, and there was a decrease in body weight with increasing doses of khat. The mean body weights in group 2 (fed 1000mg/kg khat) and group 3 (2000mg/kg khat) were significantly lower than controls ($p < 0.002$ and $p < 0.0018$) respectively. Table 3 below and Figure 23 illustrates the body weights of the controls and experimental groups.

Control rats had an average brain weight of 2.04g, and showed a decrease across the experimental groups, with group 3 (2000mg/kg) having a significantly lower brain weight (mean 1.88g) ($p < 0.024$). However, the relative brain weight was lowest in controls (0.5%) and highest in group 2 rats (0.69g), and the difference was statistically significant ($p < 0.04$).

The maximum cortical length was largest in controls (16.72mm) and smallest in group 3 rats fed on 2000mg/kg khat (15.83mm) and the difference was statistically significant ($p < 0.03$). The cerebral cortical width, and supero-inferior thickness of brains of controls were significantly higher than group 2 and 3. In all brain indices except the relative brain weight, there was a gradual decrease across the groups.

Karau, PhD Thesis, Uon, 2023
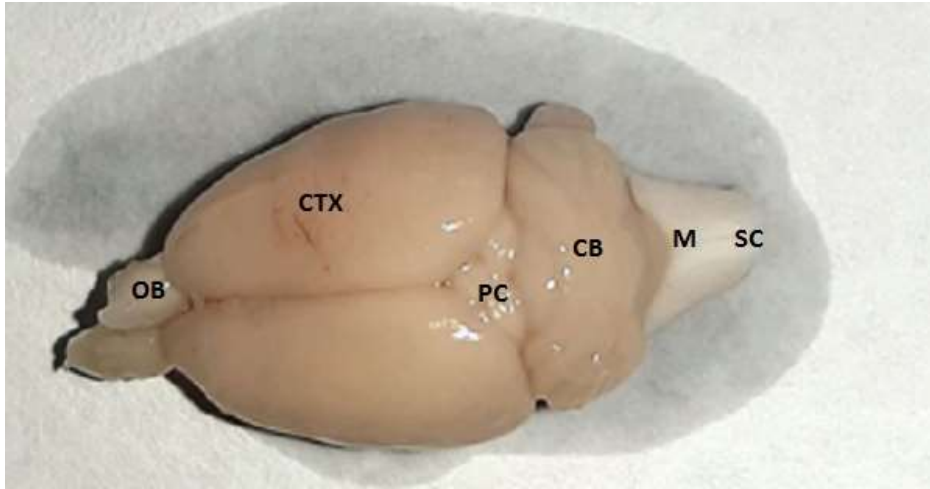
Figure 22: Extracted rat brain showing a lissencephalic brain and major structures like cerebral cortex (CTX), olfactory bulb (OB), posterior colliculi (PC), medulla (M), spinal cord (SC) and cerebellum (CB)

Table 3: Mean body weight and fixed brain weights of controls and experimental rat groups after 6 weeks of khat feeding

Parameters	Body weight (g) ±SEM	Brain weight (g) ±SEM	Relative brain weight (%) ± SEM
Controls	414.36±16.32	2.04±0.05	0.5
Group 1 (500mg/kg)	314.64±10.98	1.97±0.06	0.63
Group 2 (1000mg/kg)	305.55± 21.18[a]	1.92±0.03	0.69[c]
Group 3 (2000mg/kg)	290.64±19.39[a]	1.88±0.03[b]	0.68

[a] The average body weight of group 2 (1000mg/kg) and group 3 (2000mg/kg) were significantly lower than controls, [b] the absolute brain weight of group 3 rats (2000mg/kg khat-fed) was significantly lower than control rats, and [c] the relative (%) brain weight of group 2 (1000mg/kg khat-fed) was significantly higher than control rats.

Table 4: Mean cerebral cortical parameters of controls and experimental rat groups after 6 weeks of khat feeding

Parameters Rat groups	Maximum cortical length (mm)	Maximum cortical width (mm)	Supero-inferior brain thickness (mm)
Controls	16.72	15.77	10.02
Group 1 (500mg/kg khat)	16.43	15.42	9.85
Group 2 (1000mg/kg khat)	16.03	15.38	9.66
Group 3 (2000mg/kg)	15.82	15.23	9.53
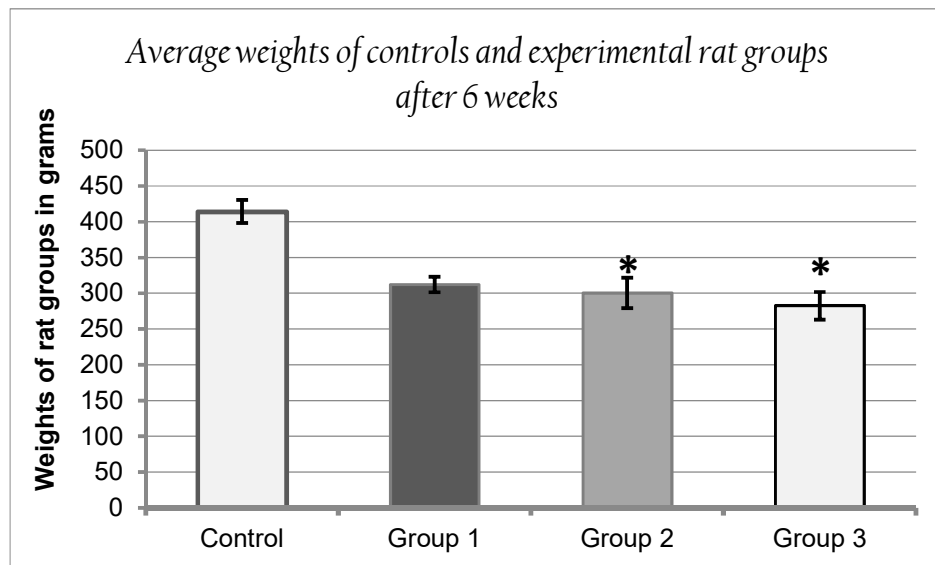
Figure 23: Average weights of controls and experimental rat groups after 6 weeks

*

The weights in group 2 and 3 are significantly lower compared to control group at 6 weeks (p <0.002, p < 0.0018 respectively).
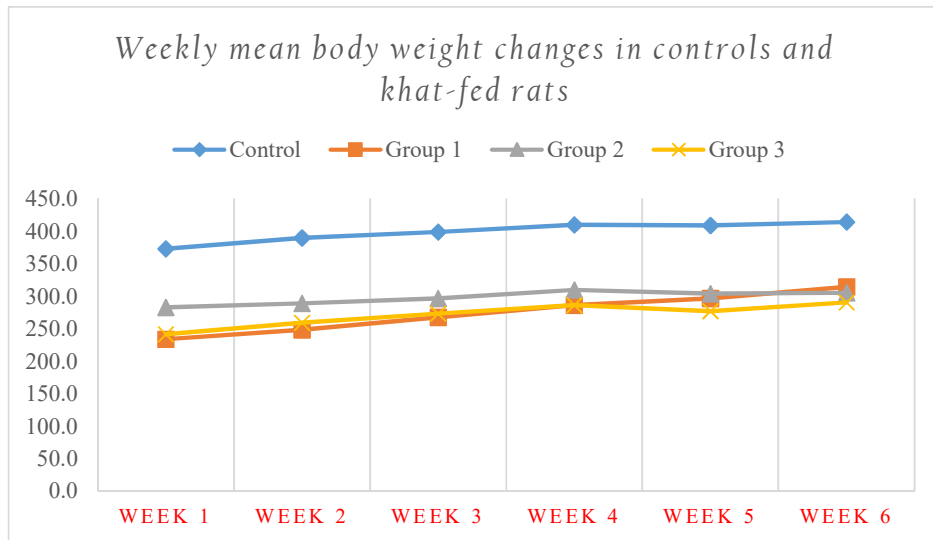
Figure 24: Weekly mean body weight changes in controls and khat-fed rats
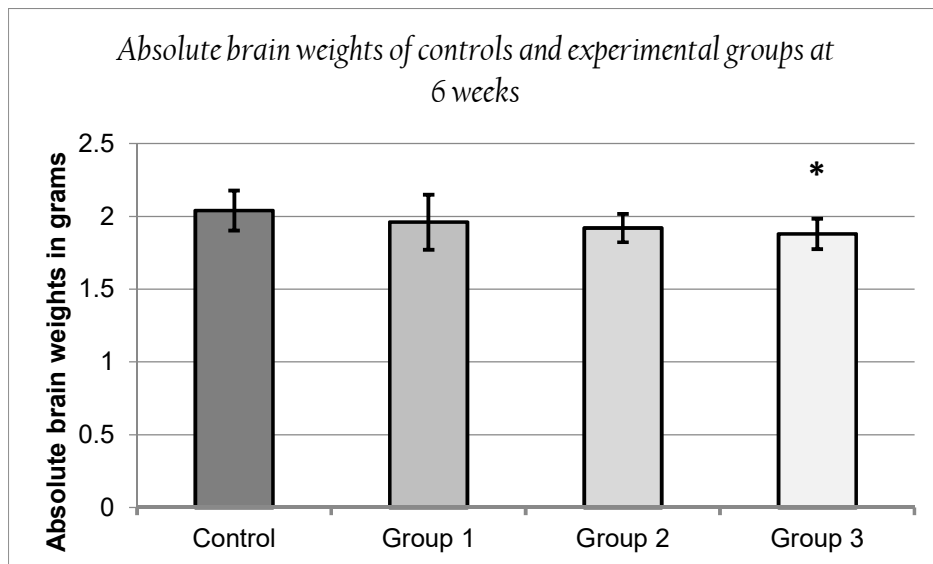
Figure 25: Absolute brain weights of controls and experimental groups at 6 weeks

*Absolute brain weight of group 3 rats significantly lower compared to controls (p<0.0024)
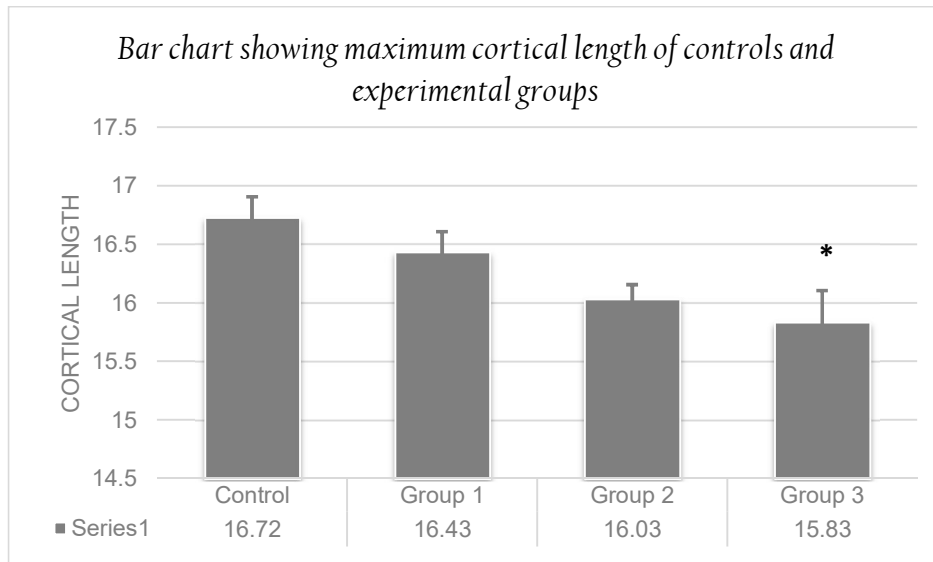
Figure 26: Bar chart showing maximum cortical length of controls and experimental groups

*Maximum cortical length of the rats fed on 2000mg/kg Khat was significantly lower compared to controls (p<0.03)
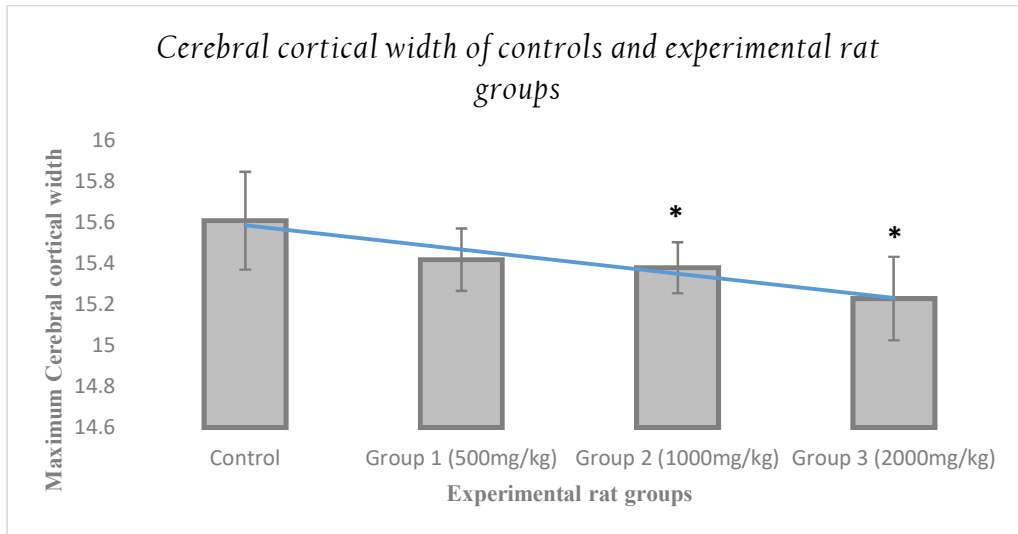
Figure 27: Cerebral cortical width of controls and experimental rat groups

*Cerebral cortex width of group 2 and group 3 rat groups was significantly less than rats that

were fed on normal diet ($p < 0.015$ and 0.012 respectively).
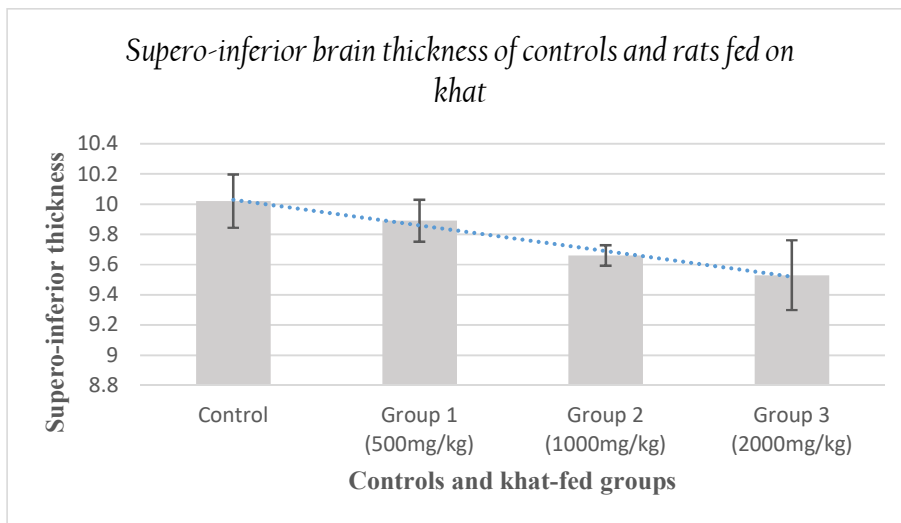
Figure 28: Supero-inferior brain thickness of controls and rats fed on khat

*The supero-inferior thickness of control rats was significantly larger compared to rats fed on

1000mg/kg khat ($p < 0.0026$) and those fed on 2000mg/kg khat ($p < 0.0018$)

3.2 Changes in normal and apoptotic pyramidal neuronal cell densities

Pyramidal neurons were identified by the presence of a visible cytoplasm around the empty looking nucleus, with the nuclear envelope displaying folds and/or indentations. They were also larger compared to the glial cells. There was also an abundance of astrocytes, with light/scanty cytoplasm that was not often visualized and lacked nuclear folds. Oligodendrocytes were noted as small, round dark staining cells.
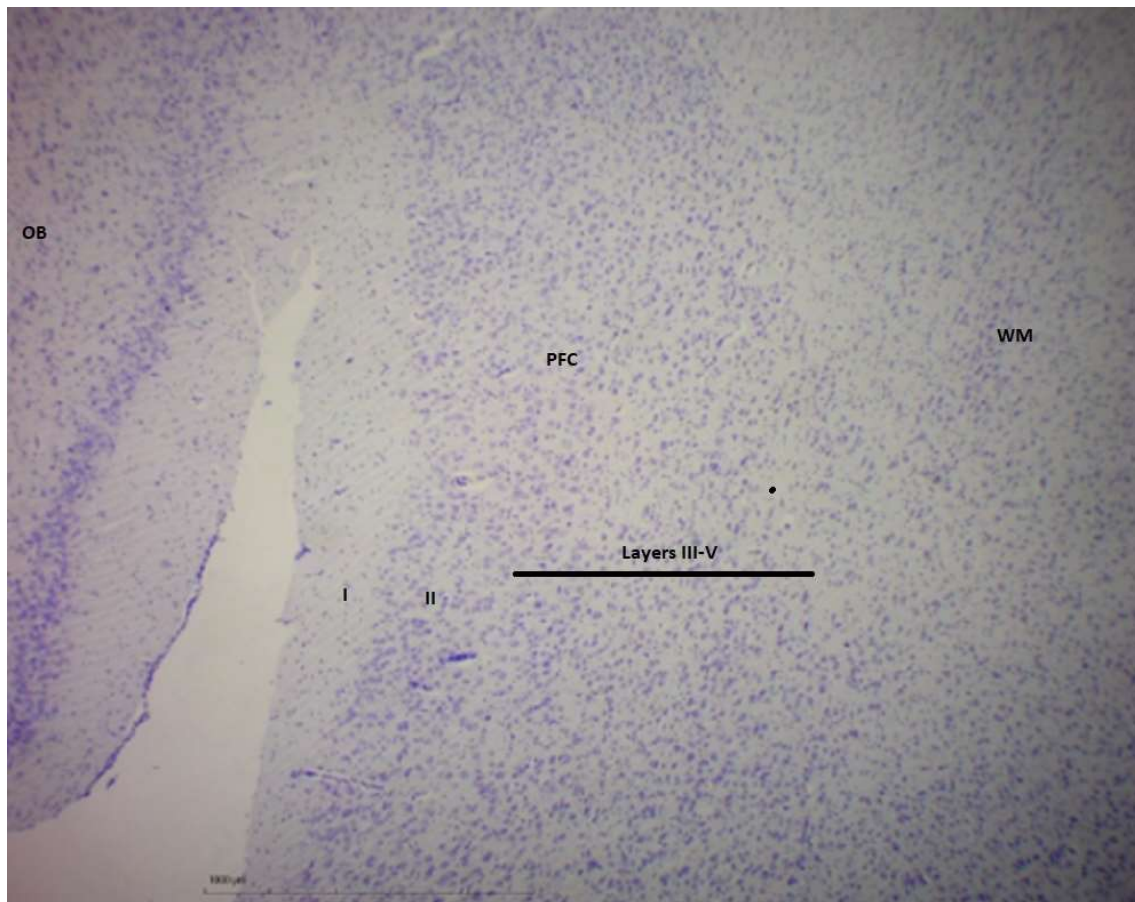
Figure 29: Photomicrograph of a coronal section through the frontal lobe of the right cerebral hemisphere. The prefrontal cortex (PFC) and its associated white matter (WM) is located adjacent to the olfactory bulb (OC). (Toluidine blue staining, X40).
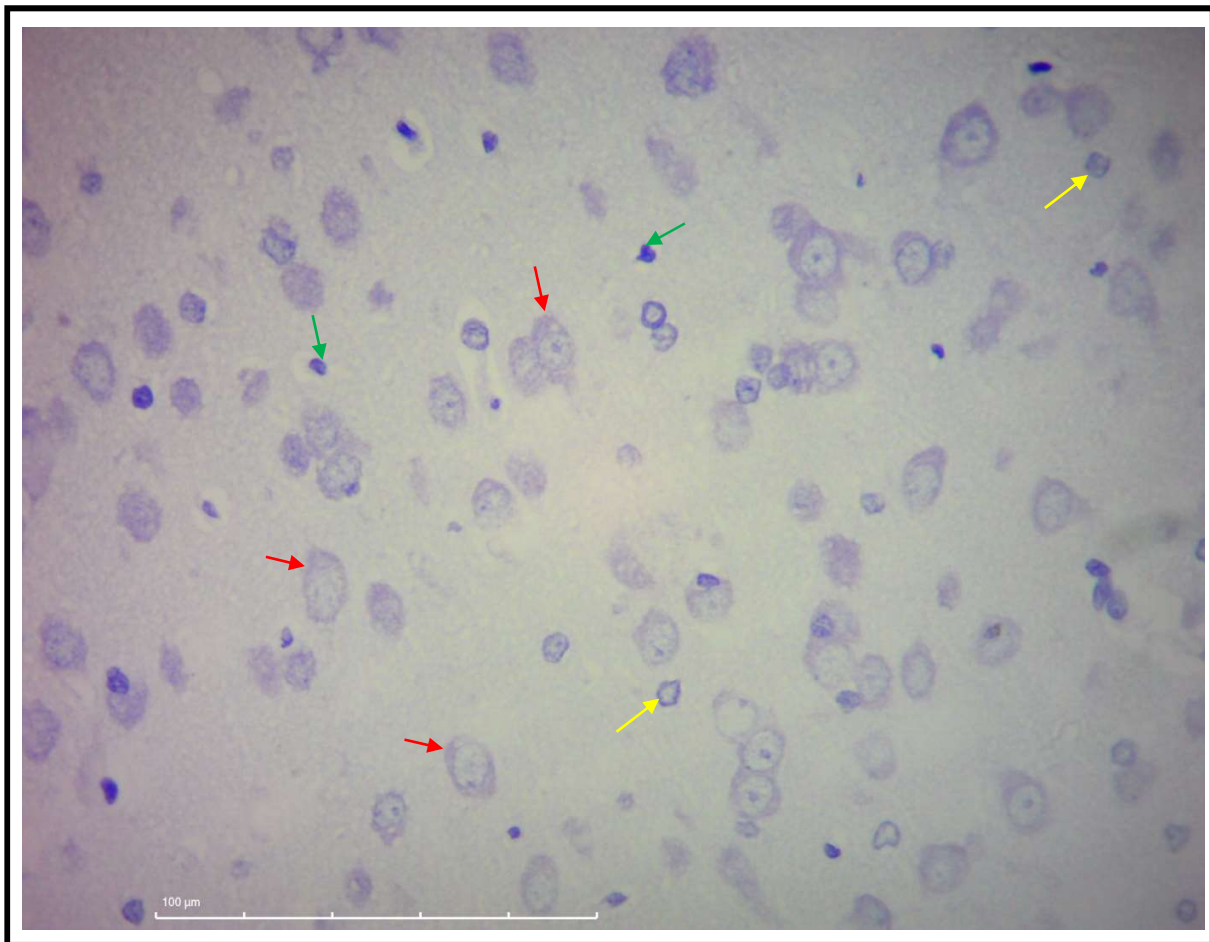
Figure 30: Photomicrograph illustrating the different cell types found in the PFC. Neurons (red arrows) are larger in size and have darker staining cytoplasm. Astrocytes (yellow arrows) are the smaller light staining cells. Oligodendrocytes (green arrows) are the small, round dark staining cells. (Toluidine blue staining, x400)

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 31*).

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 31*).

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 32*) and box plots (*Figure 33*). 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 5*).

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 a decreased number of normal Pyramidal cells with an increase in the Khat dosages (*Figure 31*). 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 34*) and box plots (*Figure 35*). There were

no statistically significant differences noted between the groups upon running pairwise comparisons and adjusting the significances for the multiple comparisons (*Table 6*).
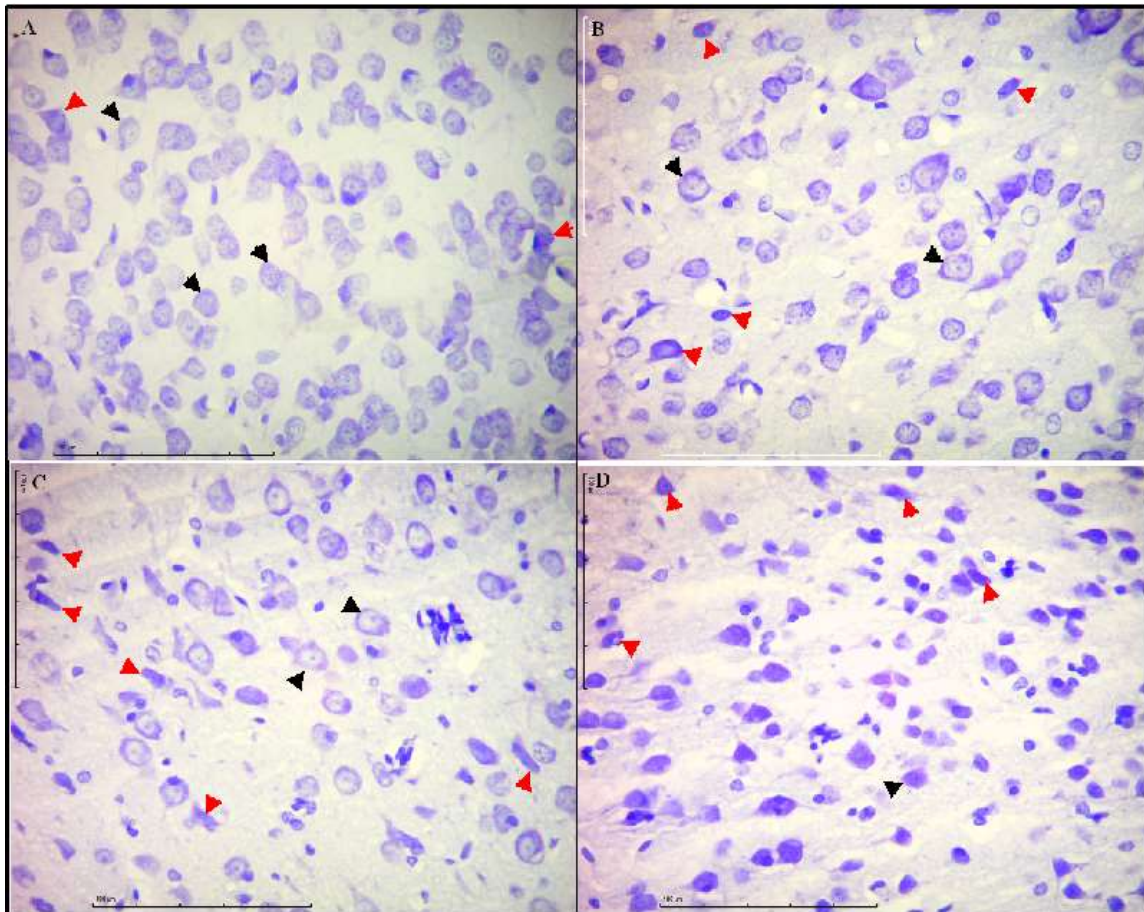
Figure 31: Photomicrograph showing neuronal changes following Khat administration. Fig 31A: Photomicrograph of the prefrontal cortex of the control group. Note the abundance of normal Pyramidal neuronal cells (black arrowheads) with uniformly staining and rounded nuclei with prominent nucleoli. Note 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 Figure 31B(500mg/kg), Fig 31C (1000mg/kg) and Fig 31D (2000mg/kg) with decreasing density of normal pyramidal cells. (Toluidine blue stain). Magnification = x400
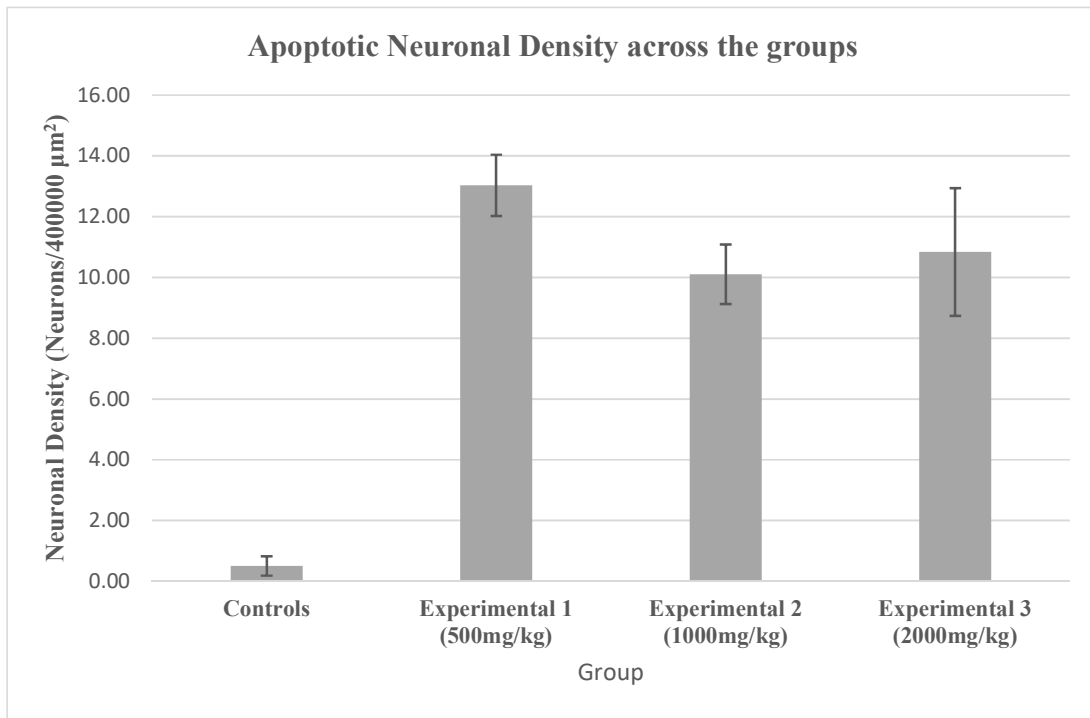
Figure 32: Graph demonstrating the general trend of apoptotic neuronal densities in controls and experimental groups.
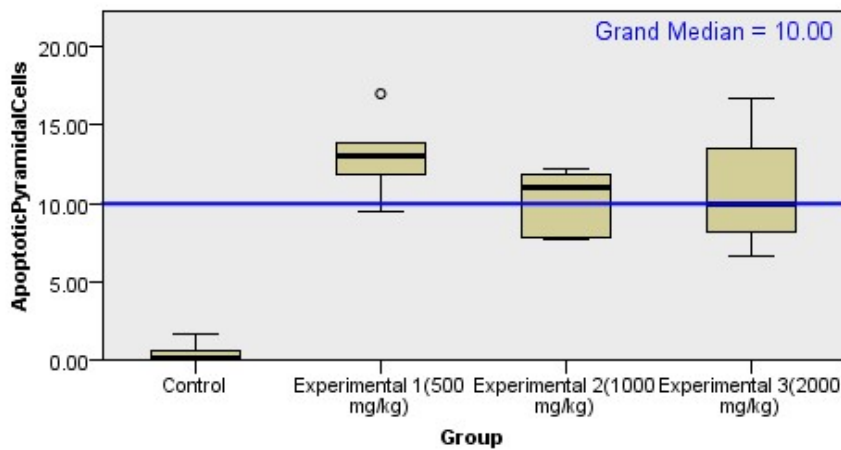
Figure 33: Box plots of the apoptotic pyramidal neuronal densities in controls and experimental rat groups
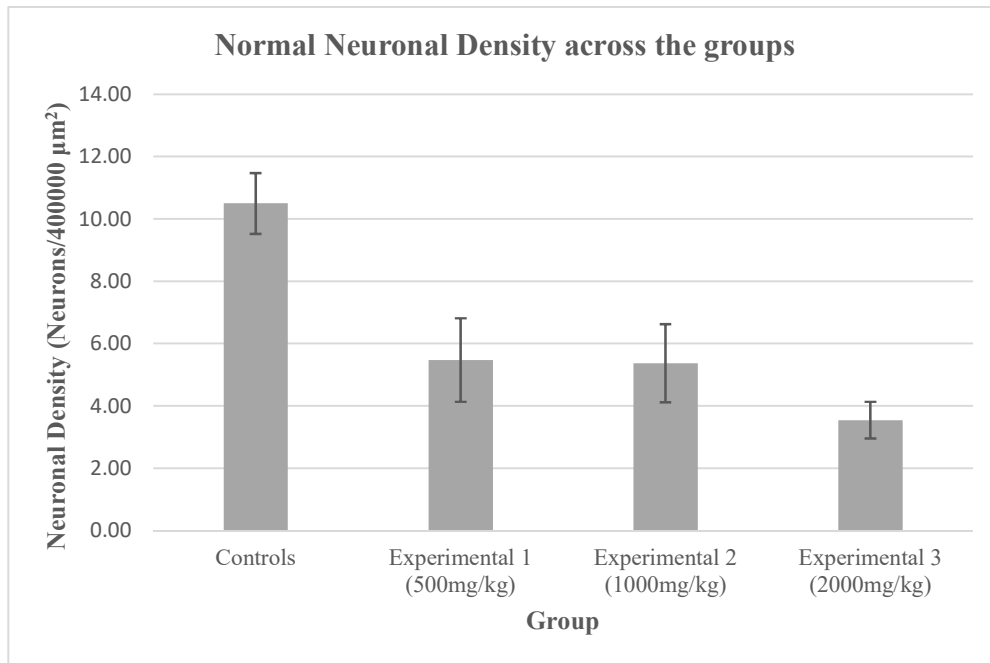
Figure 34: *General trend of normal pyramidal neuronal densities in controls and experimental rat groups*
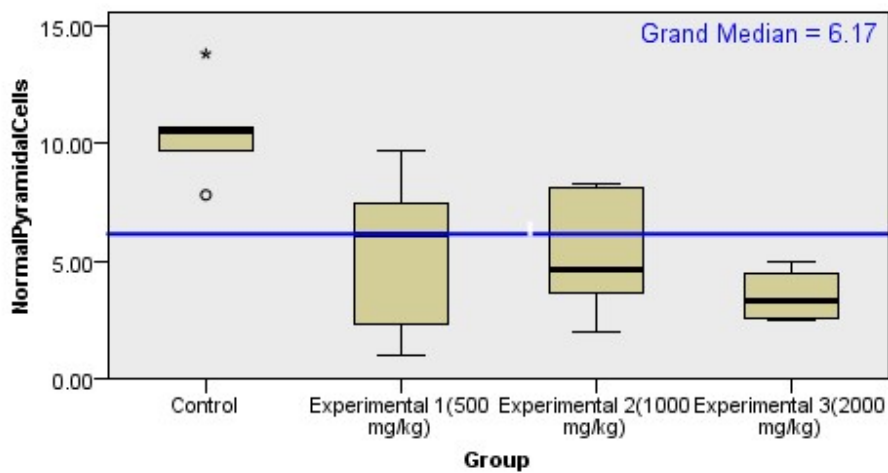
Figure 35: *Box plots of the normal pyramidal neuronal densities in control and khat-fed rats*

Table 5: 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 6: 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

3.3   Changes in immature Doublecortin immunoreactive neurons in the prefrontal cortex

Across the four rat groups, Doublecortin (DCX)-immunoreactive neurons were visualized with a preponderance of deeply staining cells in layers II and III of the prefrontal cortex. The DCX-positive cells exhibit pyramidal-like shape and characteristics.

Some cells had sturdy neuronal processes, with occasionally double processes from opposite ends. In some cases, the processes appeared to establish a relationship with distant cells.

An increase in the DCX stained immature pyramidal neuronal density was noted on administration of the Khat extracts over the study period with the highest neuronal density being observed in experimental group 1 that received 500 mg/kg of Khat extract after which a progressive decrease in the DCX+ neuronal density was observed with an increase in dosage of Khat.

The experimental groups that received the Khat extracts displayed higher DCX+ immature pyramidal neuronal densities. However, these intergroup differences were not statistically significant (p = 0.053) (*Table 7*).

The photomicrographs revealed that the pyramidal cells of the control group appeared to be uniform in size and most of them did not take up the DCX counterstain and only took up the primary Nissl stain. Few DCX+ neurons were noted in the control group. The experimental groups had numerous DCX+ pyramidal neurons identified by virtue of them taking up the characteristic brown DCX stain (*Figure 38*).

The general trend of the densities of the DCX+ neurons with an increase in the dosage of Khat per body weight can also be derived from the bar graph (*Figure 36*) and box plots below (*Figure 37*).

Table 7: DCX+ Immature neuronal density in experimental and control rat groups

Group	DCX+ Neuronal Density (cells/1000 μm^2)		p value against:
	Mean ± SD	Median	Control
Control	4.00 ± 1.41	4.00	-
Experimental 1 (500 mg/kg)	7.83 ± 1.61	8.50	0.053
Experimental 2 (1000 mg/kg)	6.00 ± 1.32	5.50	
Experimental 3 (2000 mg/kg)	4.17 ± 1.04	4.50	
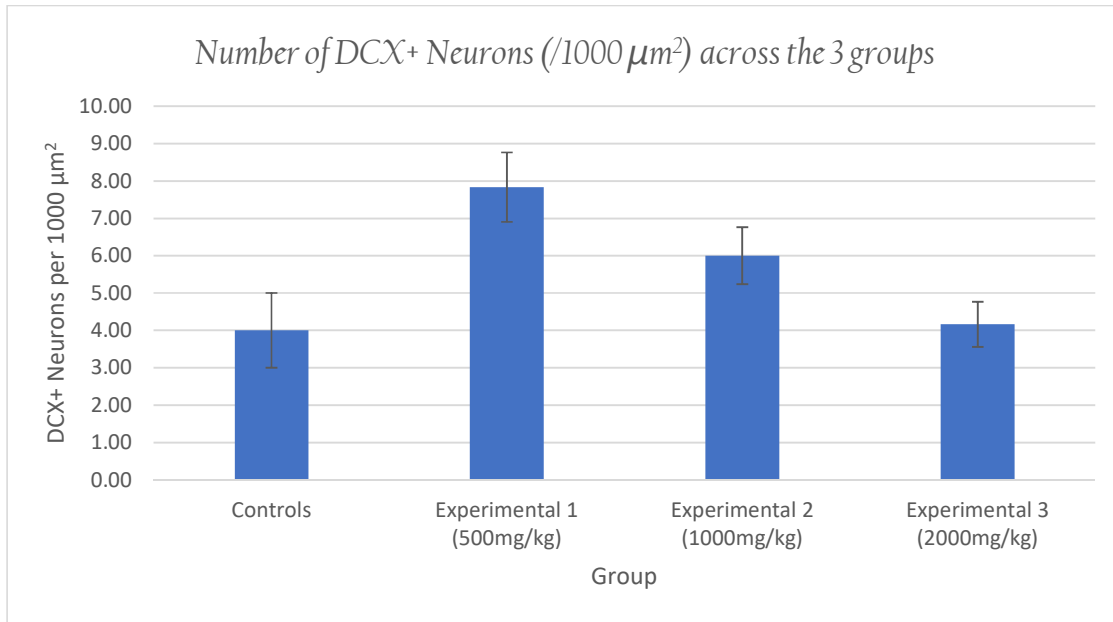
Figure 36: General Trend of DCX+ Neuronal Densities in each study group
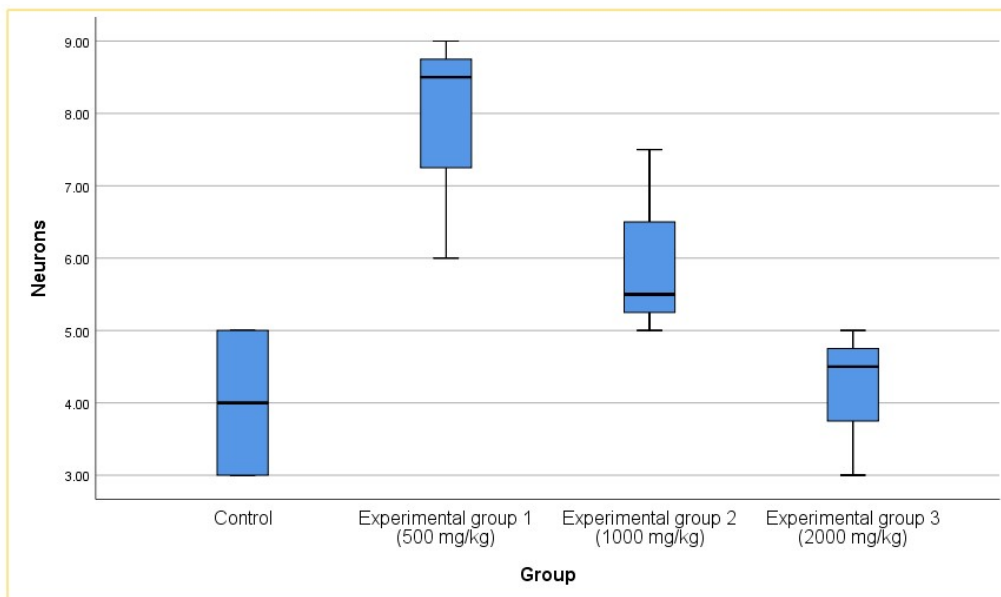
Figure 37: Box plots of the DCX+ Pyramidal Neuronal Densities in each study group

Legends for figure 38A-D

Figure 38A-D: DCX+ neuronal changes following Khat extract administration.

Figure 38A: Photomicrograph of the prefrontal cortex of the control group. Note the abundance of Pyramidal neuronal cells (black arrowheads) that do not take up the DCX stain. Note the relative scarcity of DCX+ neurons (red arrowheads) in this group when compared to the other groups (Nissl stain with DCX counterstain). Magnification = x400.

Figure 38B: Photomicrograph of the prefrontal cortex of the experimental group 1 (500 mg/kg). Note the increase in the DCX+ Pyramidal cells with brown staining of the cytoplasm within the cell body (red arrowheads). The number of the DCX- Pyramidal neurons (black arrowheads) is relatively lesser than what was seen in the control group. (Nissl stain with DCX counterstain). Magnification = x400.

Figure 38C: Photomicrograph of the prefrontal cortex of the experimental group 3 (1000 mg/kg). Note the reduction in number of the DCX- Pyramidal cells (black arrowheads) in comparison to the control group. There is a relatively lower density of DCX+ pyramidal neuronal cells (red arrowheads) when compared to experimental group 1 (Nissl stain with DCX counterstain). Magnification = x400.

Figure 38D: Photomicrograph of the prefrontal cortex of the experimental group 3 (2000 mg/kg). Note the reduced density of DCX+ cells (red arrowheads) as compared to the other experimental groups. The processes of the neuron can also be noted on some neurons (blue arrows) (Nissl stain with DCX counterstain). Magnification = x400.

Figure 38: DCX+ neuronal changes following Khat extract administration.
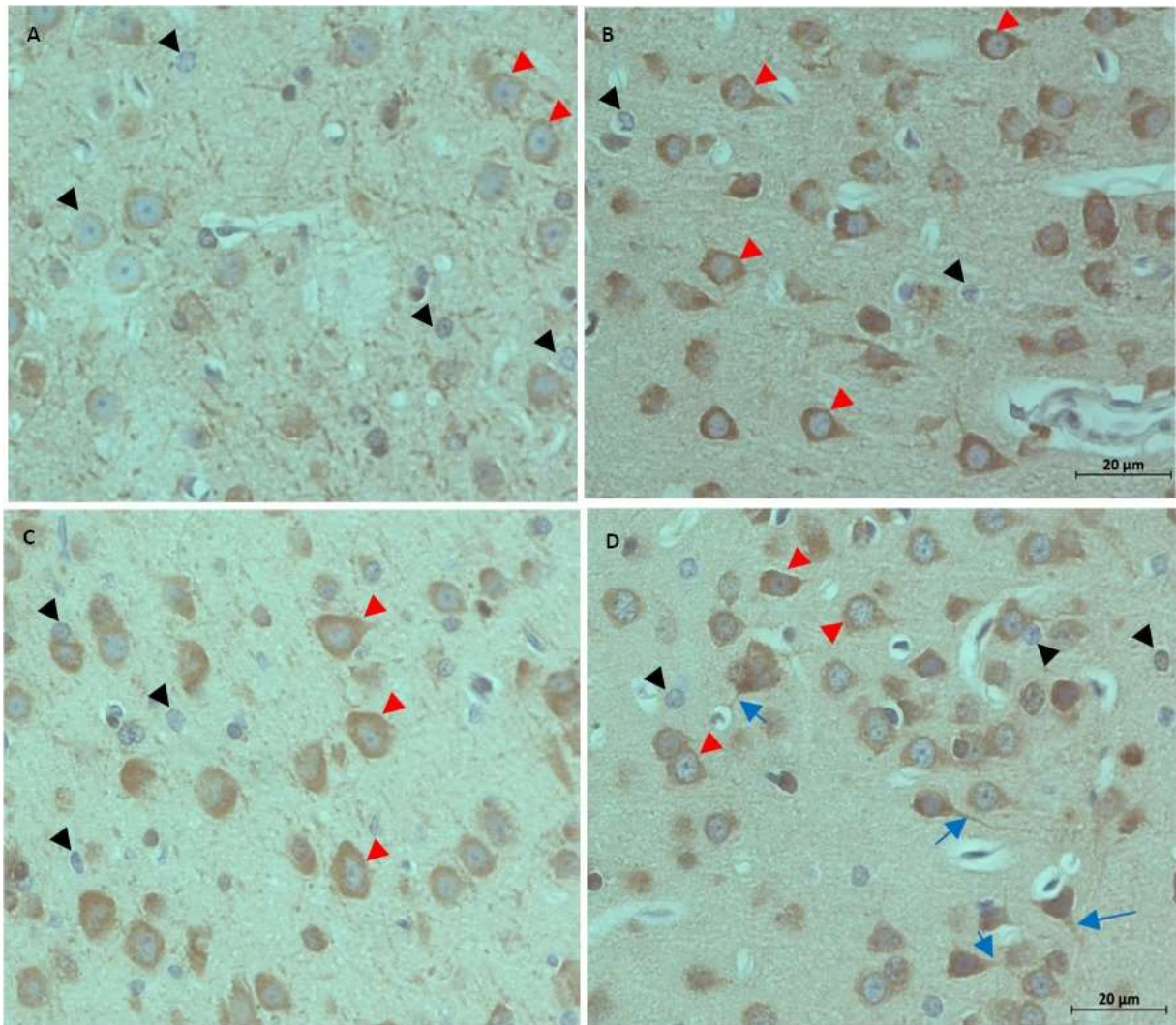
3.4 Histomorphometric changes in astrocytes in the prefrontal cortex

3.4.1 Astrocytes are arranged in layers

It was observed that GFAP reactive astrocytes have a cytoarchitectonic layer arrangement akin to that of neurons, with abundance of astrocytes in the second and third layers of the cortex. Figure 39 shows the layer arrangement of astrocytes.
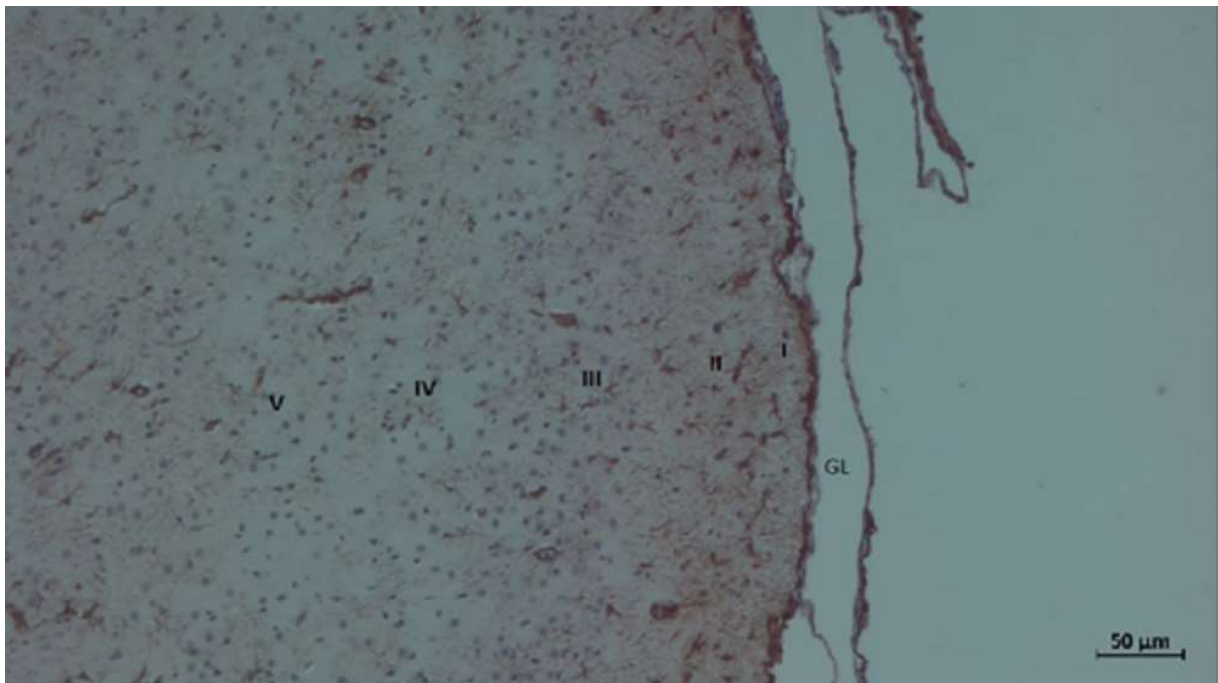
Figure 39: Photomicrograph showing arrangements of astrocytes in the layers of the prefrontal cortex, with predominance of astrocytes in layers II and III (Mag x 100, GFAP Stain).

3.4.2 Astrocyte density increases with increasing doses of khat

There was a general increase in the average number of astrocytes across the groups (Figure 40). The astrocyte density in groups 1, 2, 3 and 4 was 7, 8, 9.7 and 10 per $15000um^2$ respectively.

A similar trend for the astrocyte density is replicated in the respective box plots (Figure 41). Kruskal Wallis test reveals a significant difference in astrocyte count in group 3 (2000mg/kg) compared to control rat group (p<0.0025).

Figure 42 shows the increase in astrocyte numbers with increasing doses of Khat.
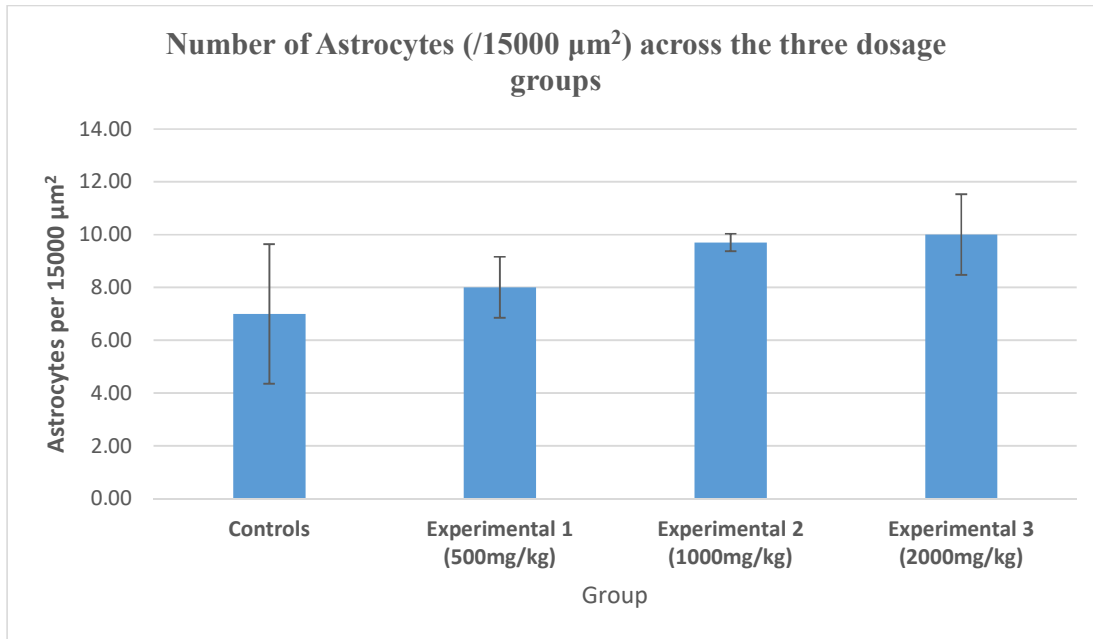
Figure 40: *Graph depicting the general trend of Astrocyte Densities in each study group*
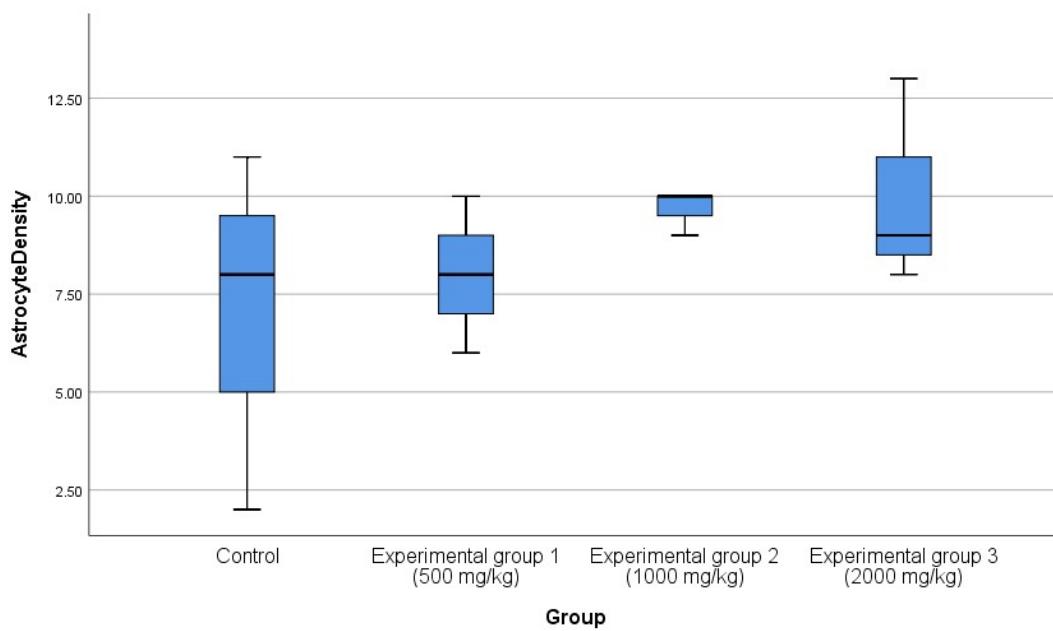
Figure 41: *Box plots of the Astrocyte Densities in each study group*
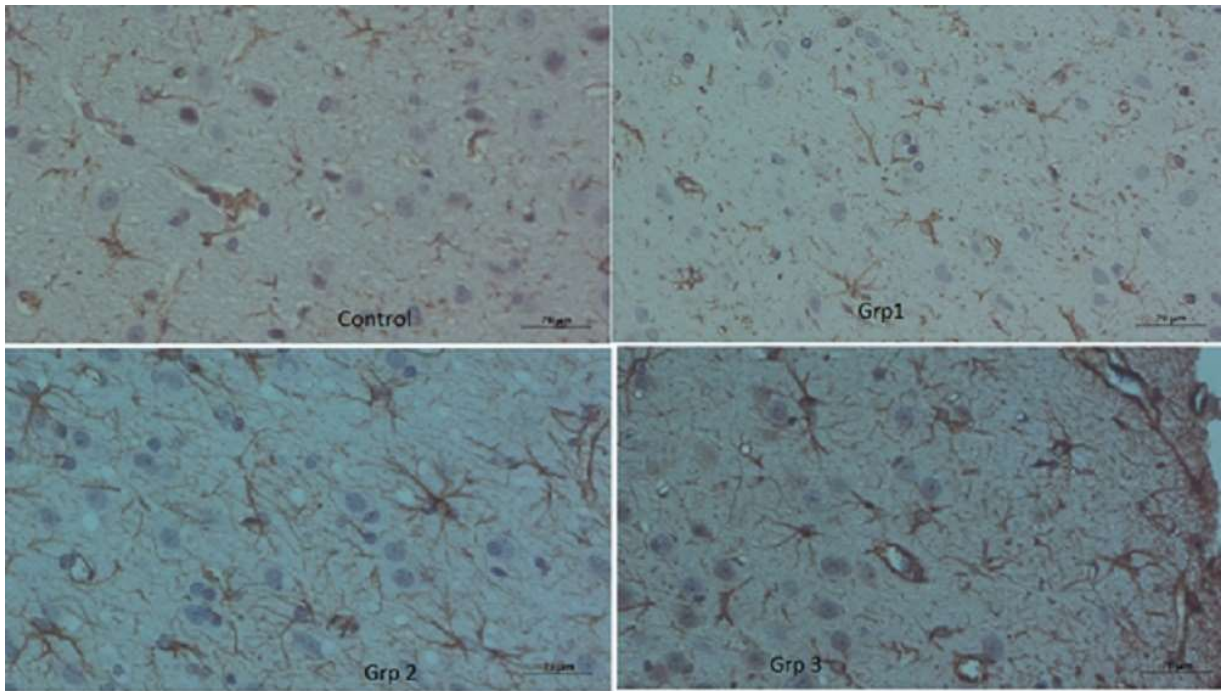
Figure 42: Representative photomicrograph of the prefrontal cortex showing an increase in GFAP immunoreactive astrocytes with increasing doses of khat with relatively more cells and more exuberant branching in group 2 (1000mg/kg) and group 3 (2000mg/kg). (Magnification X400, GFAP Stain).
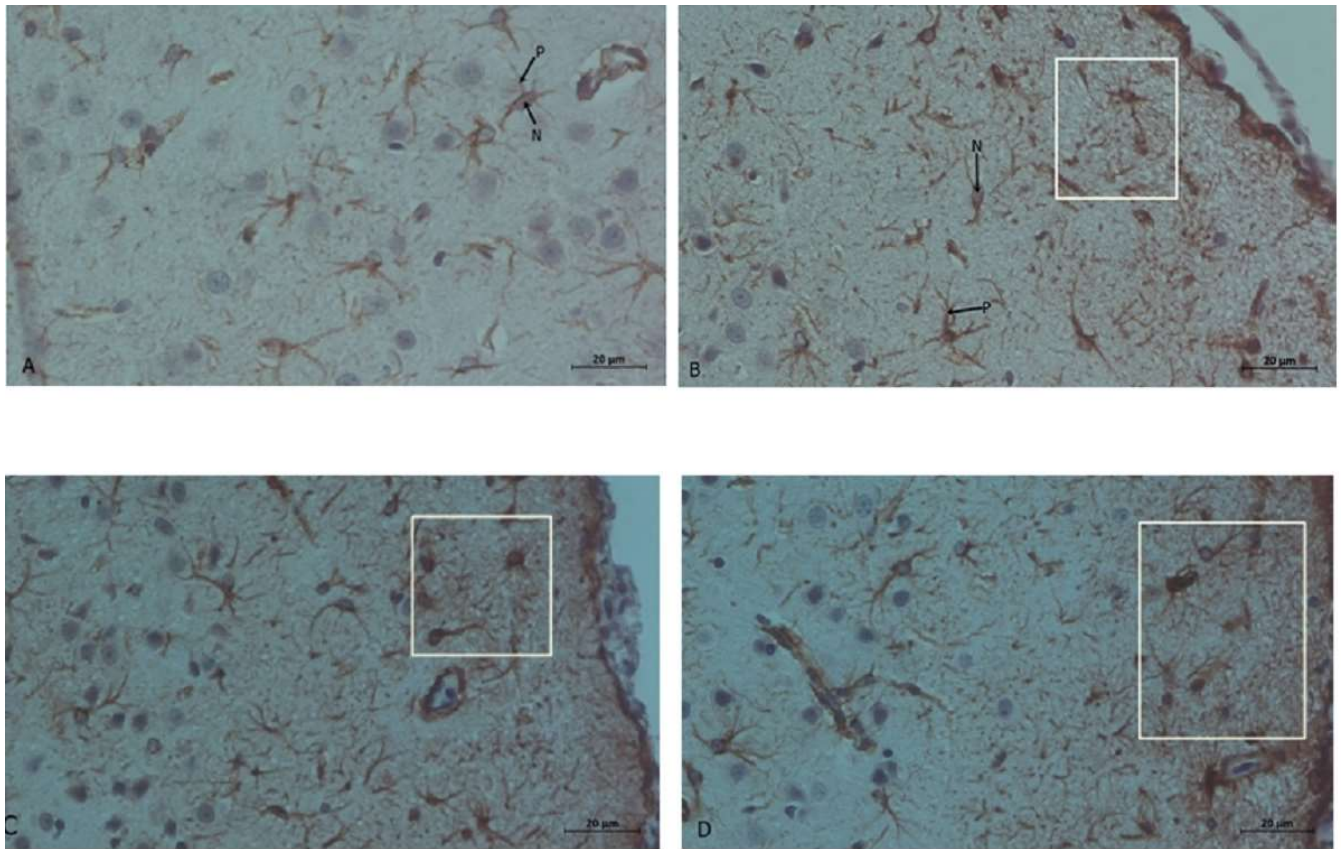
Figure 43: Photo micrograph of the prefrontal cortex of the control group. Note the appearance of a normal astrocyte having a relatively smaller nucleus (N) with indistinct nucleolus and fewer and shorter processes (P). In group 1 (500mg/kg), the numerous astrocytes with stout ramifying processes (P). Nucleolus (N) become noticeable and evidence of mild astrogliosis is seen in the area delimited by a rectangle. While in group 2 (1000mg/kg) an area of moderate gliosis is delimited by the rectangle. Note the densely staining cytoplasm of the astrocytes in the rectangle. In the experimental group 3 (2000 mg/kg), there is a greatly increased number of astrocytes with severe astrogliosis in the area delimited by the white rectangle. Magnification = x 400 (GFAP).

3.4.3   Increased GFAP reactivity and gliosis in experimental rat groups

It was noted that astrocytes in the prefrontal cortex of the experimental group had densely staining cytoplasm (Figure 43, C & D). Whereas the nucleolus in the control group was almost indistinct, it was very prominent in the experimental group.

The features exhibited by astrocytes in the experimental group characterize the reactive response of astrocytes to injury (gliosis).

Gliosis was noted in layer 1 and 2 of the medial prefrontal cortex. It was noted that gliosis was mild in group 1, moderate in group 2 and severe in group 3 (Figures 43B, C and D respectively).

3.4.4   Complexity of astrocytic processes increases in experimental rats

 In the control group, the astrocyte processes were noted to be fewer and shorter. On the other hand, the astrocytes in the experimental group demonstrated numerous, stout and ramifying process. This is depicted in Figure 44.
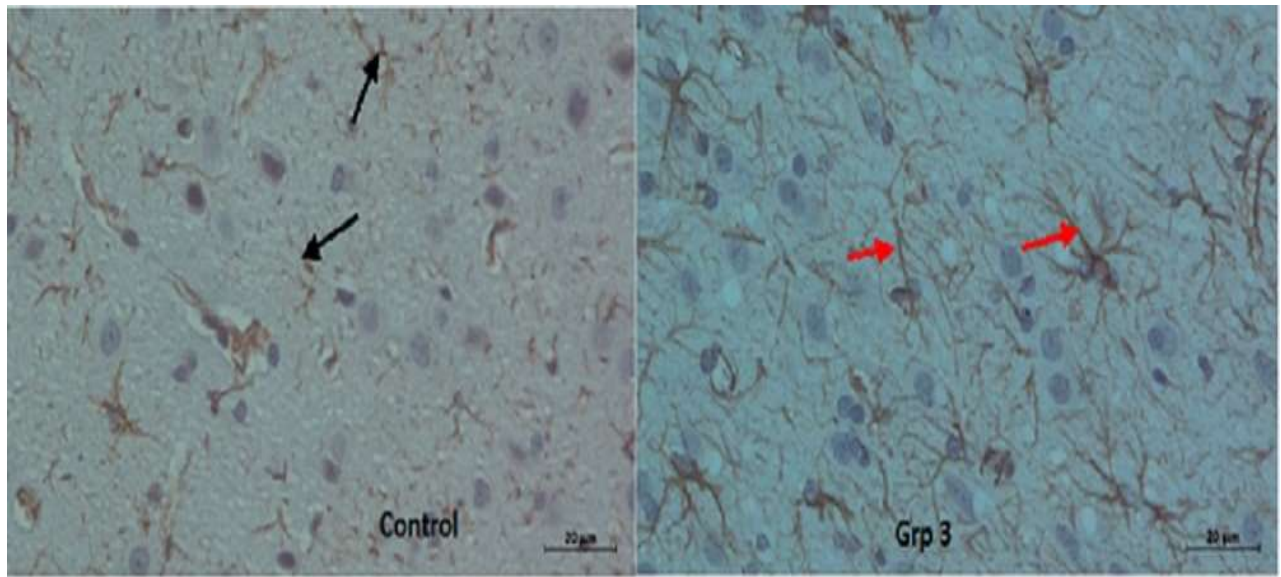
Figure 44: Photomicrographs comparing the astrocytic processes in control group and group 3 (2000mg/kg). Note the increased complexity and thickness of astrocytic processes in the experimental group. (Magnification X400, GFAP).

3.5   CNPase+ Oligodendrocyte Densities

CNPase immunostaining was observed in all rat groups, with varying amounts of myelinating oligodendrocytes demonstrated. The control group shows more abundant myelin fibres compared to the experimental groups.

The immunostaining oligodendrocytes appear smaller than the pyramidal neurons, with the CNPase immunostain appearing more distinct as a rim around the cell.

No change in the CNPase stained oligodendrocyte density was noted on administration of the Khat extracts over the study period. The minor intergroup differences noted were not statistically significant ($p = 0.863$) (*Table 8*). The photomicrographs revealed that the oligodendrocytes were of uniform size and roughly of similar density irrespective of group (*Figure 45*).

Figure 45A: Photomicrograph of the prefrontal cortex of the control group. Note the abundance of Pyramidal neuronal cells (black arrowheads) that do not take up the CNPase stain. Note the CNPase+ oligodendrocytes (red arrowheads) and the abundantly stained myelin (blue arrows) in this group. Magnification = x400.

Figure 45B: Photomicrograph of the prefrontal cortex of the experimental group 1 (500 mg/kg). Note that the density of CNPase+ oligodendrocytes (red arrowheads) is roughly similar to what was seen in the control group. Note that there is a decreased staining of myelin (blue arrows) as compared to the control group. Magnification = x400.

Figure 45C: Photomicrograph of the prefrontal cortex of the experimental group 3 (1000 mg/kg). The CNPase+ oligodendrocyte density (red arrowheads) is similar to what was seen in the control and the experimental group 1. Note that there is also a decreased staining of myelin (blue arrows) as compared to the control group . Magnification = x400.

Figure 45D: Photomicrograph of the prefrontal cortex of the experimental group 3 (2000 mg/kg). Note the CNPase+ oligodendrocytes (red arrowheads) whose density is similar to that of the preceding groups. There is relatively lesser myelin (blue arrows) taking up the CNPase counterstain as compared to the control group (Nissl stain with CNPase counterstain) Magnification = x400.
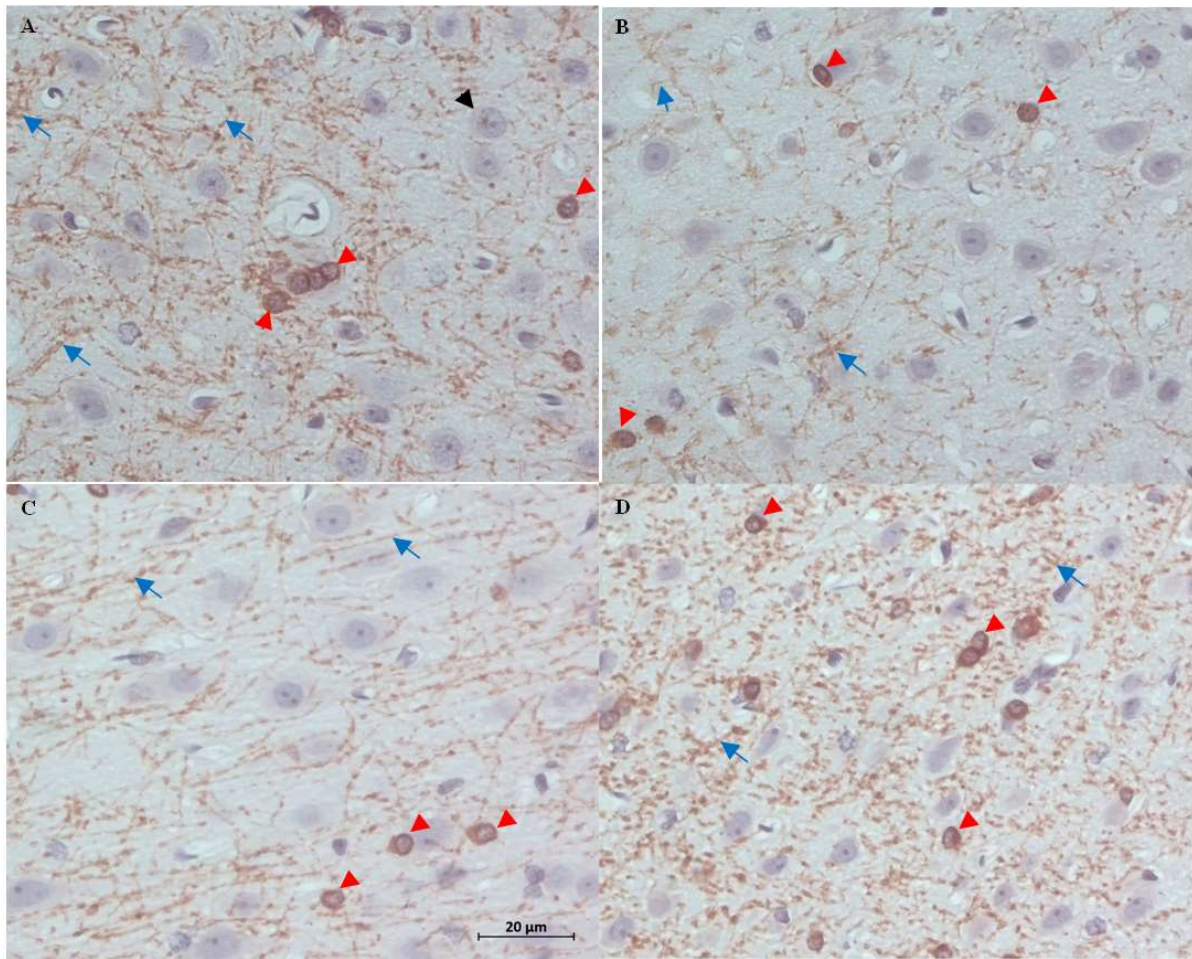
Figure 45A-D: CNPase+ oligodendrocyte changes following Khat extract administration.

Furthermore, it was noted that the amount of stained myelin in the experimental group appeared to be significantly lesser than that in the control group (*Figure 45*). The densities of the CNPase+ oligodendrocytes with an increase in the dosage of Khat per body weight are plotted in the bar graph (*Figure 46*) and box plots below (*Figure 47*).

Table 8: CNPase+ Oligodendrocyte Density in each study group

Group	DCX+ Neuronal Density (cells/1000 μm^2)		p value against:
	Mean ± SD	Median	Control
Control	0.36 ± 0.19	0.25	-
Experimental 1 (500 mg/kg)	0.45 ± 0.32	0.50	0.863
Experimental 2 (1000 mg/kg)	0.43 ± 0.14	0.44	
Experimental 3 (2000 mg/kg)	0.30 ± 0.22	0.28	
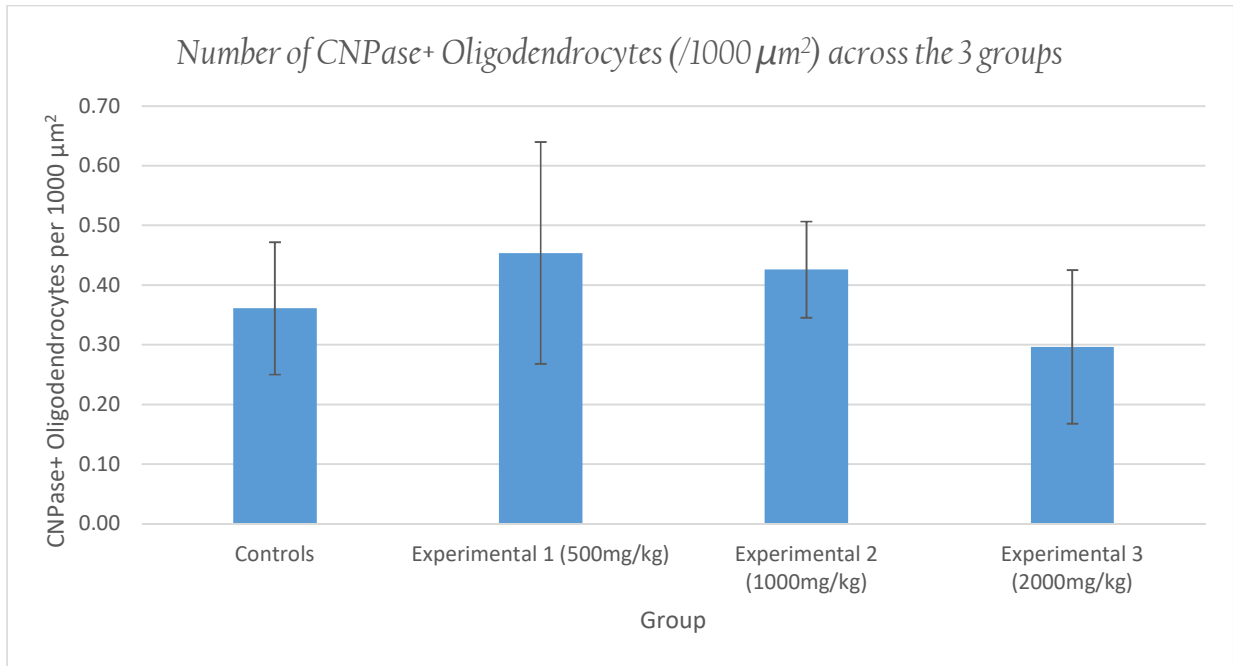
Figure 46: Number of CNPase+ Oligodendrocytes (/1000 μm²) across the 3 groups
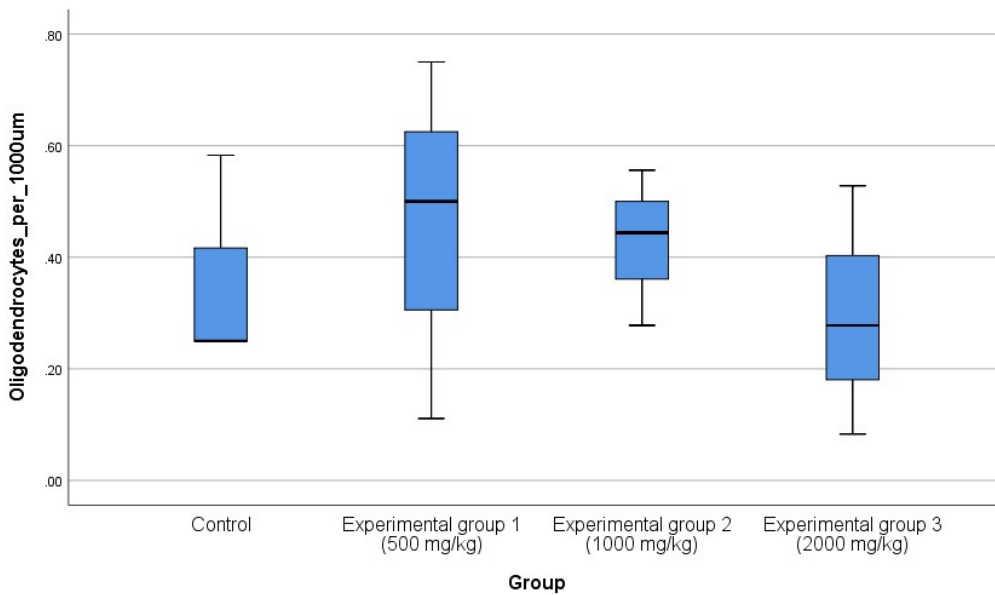
Figure

47: Box plots of the CNPase+ Oligodendrocyte Densities in each study group

CHAPTER 4: DISCUSSION AND CONCLUSIONS

4.1   Overview of gross morphometric indices of the prefrontal cortex of the male rat

Morphometric data is invaluable in providing a glimpse of structure-function relationship in body organs as a result of exposure to drugs and substances(Ishikawa et al., 2003). Further, dose-dependent changes in brain parameters add valuable information on correlation with micro-anatomic as well as imaging studies.

We found a mean brain weight of 2.04g in control group, which is in keeping with what has been reported in a previous study(Muche, 2004). The brain weights of corresponding experimental groups decreased, and the rats fed on highest dose of khat had the lowest brain weight. Muche, (2006)reported a statistically significant decrease in brain weight of 10.04% between khat-fed rats and their corresponding controls. Similar changes in brain weight have been reported in past studies(Hassan et al., 2002).

The present study has found a body/brain weight ratio of 0.005 in control rats, compared to 0.0069 in group 2 (1000mg/kg khat), and the difference was statistically significant. This may be related to changes in both body sizes and neuronal and neuroglial changes with khat consumption. Human studies have reported the ratio of brain to body weight of 0.02 in man(Singh & Dyce, 2018). In rodents, the ratio has been found to be 0.01 for male greater cane rat and 0.006 for females (Byanet et al., 2009). Other animals studied include mouse (0.03), rat (0.007), rabbit (0.004)(Lande, 1979).

According to Russel (Lande, 1979), there exists a relationship between brain size and intelligence. Our study has documented lower absolute brain weights with increasing doses of

khat, with the difference between control rats and highest khat dose being statistically significant. This may be extrapolated to mean that beyond a certain dose of khat, there are deleterious effects of khat at the cellular level which may be reflected at the gross level, and may have an impact on brain function.

The decrease in brain weight may be attributable to amphetamine-like effect of cathinone on the release of norepinephrine in the brain, which activates the satiety centres(Patel, 2009). The result is delayed gastric emptying, suppressed appetite, and hampered neuronal nutrition.

We found a decrease in gross cortical parameters in experimental rat groups compared to controls, with the decrease most significant in higher doses of khat. This decrease observed in the current study could be partially explained by neuronal effects as has been observed before(Carvalho, 2003; Holford, 1987). Neuronal loss in the prefrontal cortex and cerebellum and other brain areas are well reported.

Neuronal death through glutamate-induced excitotoxicity in the cerebral cortex, with release of intracellular calcium and membrane damage is a potential mechanism in khat-induced changes(Holford, 1987).

4.2   Changes in Pyramidal neurons with chronic khat use

We set out to primarily determine and evaluate the changes in normal and apoptotic pyramidal neuronal densities in the PFC of winstar rats with increasing doses of khat.

Pyramidal neurons, the most common neuron in the cerebral cortex, are at the centre of activity of the brain, specifically the prefrontal cortex, and any structural change has a profound effect on brain function. They function as the major source of excitatory cortical synapses, and their dendritic spines are the main postsynaptic target of excitatory synapses. Due to this central structure, they profoundly influence cerebral function at subcellular, cellular and system levels(Passingham et al., 2002; Roth & Dicke, 2005; Treves, 2005).

The number and complexity of pyramidal neurons and their processes determines their biophysical properties, thus influencing their functional capacity and potential for plasticity(Elston et al., 2011; Koch, 1998; London & Häusser, 2005). These specializations may subserve executive functions, which are domiciled in the prefrontal cortex (Courtney et al., 1998; Duncan & Owen, 2000; Funahashi & Takeda, 2002; Fuster, 2001; Treves, 2005; X. J. Wang, 2001).

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. This dose-structure relationship is buttressed by behavioural studies, which 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-Espejo & Rodriguez-Espinosa, 2011).

These changes have been reported elsewhere in the brain. Muche (2006) reported reduction in density and disruption of neuronal density in Purkinje cells of the cerebellum(Muche, 2006).

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).

4.3   Changes in GFAP-immunoreactive astrocytes

Our study has demonstrated an increase in the number, density, branching and gliotic changes in GFAP immunoreactive astrocytes in khat-fed rats. An increase in the dose of khat extracts beyond 1000mg/kg produced greater and more significant changes compared to control rats.

Initially, it was thought that astrocytes played only physical supportive function to the neurons. However, recent research has shown that astrocytes are at the core of neuronal homeostatic, metabolic and protective functions(Ota et al., 2013). They play critical roles in metabolic processes of the brain(Panov et al., 2014), formation and maintenance of synapses, transduction of synaptic messages and processing of data(Newman, 2003a).

Astrocytes have recently been identified as the glial cells which function in close relationship with neurons, and this cross-talk is crucial for normal brain function(Kimelberg, 2007; D. D. Wang & Bordey, 2008). This has been conceptualized as bidirectional communication between neurons and astrocytes, a kind of tripartite synapse (Araque et al., 1999; Newman, 2003b; Perea et al., 2009). It is therefore plausible that any changes in astrocyte number and function would have huge impact on neuron-astrocyte network integrity and activity, and consequently, on behaviour output.

The prefrontal cortex has anatomic and functional linkages with components of the limbic system, justifying its key role in cognitive, mnesic and emotional processing (Heidbreder & Groenewegen, 2003; Hoover & Vertes, 2007).

GFAP is a prototypical marker for immunohistochemical identification of astrocytes. It strongly labels astrocytes that are responding to injuries in the central nervous system(Sofroniew &

Vinters, 2010). GFAP is a cytoskeletal protein, and a malfunction of the proteins leads to accumulation(Rajkowska et al., 2002) and its mRNA expression may be altered in states associated with neuronal pathology(Moehle et al., 2012).

GFAP has been found to increase in many demyelinating and psychotic diseases(Feresten et al., 2013), and may at first be inhibited in alcoholics until late in neuronal disease when extensive neuronal damage occurs(Miguel-Hidalgo, 2009).

Activation of astrocytes, with increased expression of GFAP has been described with administration of cocaine, amphetamines and most psychostimulants(Fattore et al., 2002; Itzhak & Ali, 2006). This upregulation of GFAP may indicate brain injury, neurotoxicity or simply adaptive changes that occur to protect neurons. Treatment with methamphetamine results in loss of dopaminergic terminals without detectable neuronal loss (Hess et al., 1990), and induces astrogliosis with increased GFAP in several areas of the brain such as striatum, hippocampus and frontal cortex (Pubill et al., 2003).

It is now known that expression of GFAP is not essential for the normal appearance and function of most astrocytes in the nervous system, but is an essential component of reactive astrogliosis and glial scar formation(Herrmann et al., 2008; Pekny et al., 1995; Pekny & Pekna, 2004).

GFAP is an intermediate filament protein, and is known to critically affect the processes by which astrocytes control neurogenesis, neural plasticity and regeneration. Many previous researchers have reported an increase in axonal and synaptic regeneration (Bao et al., 2012; Menet et al., 2003; Pekny & Pekna, 2004; Wilhelmsson et al., 2004), and a better environment for transplantation of neural grafts or stem cells with ablation of GFAP in mice (Colucci-Guyon

et al., 1999; Eliasson et al., 1999; Parpura et al., 2012; Pekny et al., 1995; Pekny & Lane, 2007; Pekny & Pekna, 2004).

Whereas in the past it was thought that reactive astrogliosis was a uniformly negative and maladaptive phenomenon that leads to neurotoxicity, current evidence shows that reactive astrocytes are in fact protective to the central nervous system through various mechanisms such as uptake of potentially excitotoxic glutamate(T. G. Bush et al., 1999; Rothstein et al., 1996; Swanson et al., 2004), protection from oxidative stress via glutathione production(Chen et al., 2009; Shih et al., 2003), release of adenosine(Rothstein et al., 1996), degradation of amyloid-beta peptides (M et al., 2004) and repair of the blood-brain barrier(T. G. Bush et al., 1999).

The glial scar formed by astrocytes is composed of chondroitin, keratin and mucopolysaccharides in response to states of brain injury. The chondroitin and keratin inhibit axonal regeneration and prevent nerve processes from entering damaged areas, while mucopolysaccharides cements the damaged area by producing a glial scar (Seifert et al., 2006).

The current study is among the first few examining GFAP immunoreactivity in the prefrontal cortex in chronic use of khat. The prefrontal cortex is central in information processing, memory and decision making(Yang & Raine, 2009). Understanding neuroglial morphometric dynamics in this region is therefore a critical step in explaining the complex mechanisms that underly neurocognitive perturbations in khat use.

The finding of dose-dependent changes in astrocyte morphometry and structure further corroborates the findings reported in pyramidal neurons and other prefrontal cortical cells, where

widespread gliosis, neurolysis and other apoptotic changes have been reported with increasing

doses of khat, signifying an intense neuronal injury state(Echoru et al., 2018).

4.4   Doublecortin immunoreactive immature neurons

Doublecortin, first described in X-linked lissencephaly, is associated with the arrest of migrating cerebral cortical neurons(des Portes et al., 1998; Gleeson et al., 1998), and is a microtubule-associated protein expressed during development by migrating neuroblasts(J. P. Brown et al., 2003). It plays a critical role in microtubule stabilization (Gleeson et al., 1999) and nuclear translocation during neuronal migration (Cai et al., 2009). Initially thought to be only expressed in the areas of active neurogenesis such as the subventricular zone and the hippocampus(J. P. Brown et al., 2003), it is now known to express in in the cerebral cortex indicating that these cells might be developing interneurons(Cai et al., 2009).

Cells expressing the marker of immature neurons (DCX) in the neocortex may be critical in glia-to-neuron signalling mediating synaptic and metabolic plasticity(Verwer et al., 2007). During brain injury such as ischemia or seizure induction, the neurogenic response is associated with the transient increased expression of DCX in adult rodent brain (J. P. Brown et al., 2003; Couillard-Despres et al., 2005). Dcx-protein levels reflect high motility and/or structural plasticity of a given cell or a given brain region.

The present study has demonstrated the presence of DCX-positive cells in the prefrontal cortex of the young adult male rat. It was initially thought that DCX, as an immunomarker of migrating immature neurons, was only expressed by cells in neurogenic sites such as the hippocampus and subventricular zone. Further, we demonstrated an increased DCX expression in khat-fed rats compared to controls. Those fed on low doses (500mg/kg) had higher expression of DCX-

immunopositive cells compared to higher doses. An exuberant staining of neuronal processes was also observed.

The current finding of DCX-immunoreactive neurons in a predominantly non-neurogenic site may indicate that in addition to its neurodevelopmental role, DCX plays a role in adult neuronal plasticity and migration. In higher mammalian species, an increase in DCX+ cells is thought to be an evolutionary adaptation to increased brain size in order to retain or increase structural plasticity and interconnectivity(Bloch et al., 2011).

In the Wistar rats we studied, the presence of DCX-immunoreactive neurons may suggest a function in mature nervous system cells. DCX is known to co-precipitate with adapter proteins in protein sorting and vesicular trafficking(Friocourt et al., 2003). It has been speculated that increase in DCX-protein levels reflect high motility and structural plasticity of a given cell or given brain region (Kremer et al., 2013).

A decline in DCX protein levels has been reported in mice with age, possibly reflecting a state of reduced plasticity of interneuronal connections with age (García-Cabezas et al., 2017; Voloboueva & Giffard, 2011). It is not clear to what extent DCX-positive neurons can be dynamically regulated, though our present finding seems to suggest that substances can lead to different levels of expression of DCX in non-neurogenic sites of the brain.

The current findings shed light on DCX dynamics in the frontal cortex in neuronal stress. Previous studies have shown reduction in DCX cells with increasing ethanol consumption(Crews et al., 2006). Since DCX is thought to be expressed by immature migrating and proliferating cells(J. P. Brown et al., 2003; Koizumi et al., 2006), it is possible that chronic khat use induces a

certain amount of neuro-proliferation. This in itself may be a response to brain insult, as previous research has shown that ischemic insults induce a transient increase in DCX expression(Arvidsson et al., 2002).

It is already widely reported that increasing doses of khat induce astrogliosis(Herrmann et al., 2008; Pekny et al., 1995; Pekny & Pekna, 2004). Some of the DCX-positive cells may be destined to become astrocytes and not neurons (Lu et al., 2005). It is therefore possible that increased DCX positivity in the prefrontal cortex may also correlate with attempts at repair and increased astrogenesis.

4.5 CNPase Immunoreactive oligodendrocytes

The current study has found no significant changes in the density of CNPase-positive oligodendrocytes with khat use. However, we noted a reduction in the quantity and complexity of myelinating fibres, although this was not statistically quantified.

Our findings may be interpreted to mean that whereas khat use does not lead to loss or increase in oligodendrocytes, it is possible that loss of myelin is a potential pathway by which brain changes occur in chronic khat use.

Oligodendrocytes, cells responsible for formation and maintenance of myelin, are crucial in ensuring efficient exchange of impulses between brain centres (Nave & Werner, 2014). They not only produce myelin but also signals and growth factors that support axons of neurons and astrocytes in their vicinity (Du & Dreyfus, 2002; Nave & Trapp, 2008; Simons & Nave, 2016).

Whereas substances such as alcohol are known to affect oligodendrocyte function, with myelin damage (Zahr & Pfefferbaum, 2017), there's little literature on the effects of chronic use of amphetamines and amphetamine-like substances like khat on oligodendrocyte structure and function.

Myelin loss orchestrated by alcohol use disorders may cause BBB disruption or nutritional deficits, such as thiamine deficiency, alone or most likely in interaction with direct effects of alcohol on oligodendrocytes considered main culprits for myelin disturbances in chronic alcoholism (Alexander-Kaufman et al., 2007; Baydyuk et al., 2020).

Oligodendrocytes precursors are oligodendrocyte progenitor cells (OPC). These cells have the potential to multiply, migrate and differentiate into mature oligodendrocytes capable of

myelination. New OPCs are usually recruited in case of demyelination to differentiate into myelinating oligodendrocytes to restore myelin and hence ensure proper neuronal function and structure. However, substances of abuse, and their attendant nutritional deficiencies and toxic metabolites hamper this recapitulation process (Franklin & Goldman, 2015).

Precursor cells for oligodendrocytes form about 8% of the cellular population in the central nervous system (Levine et al., 2001) and are found in high numbers and density in areas such as the optic nerve(Shi et al., 1998), motor cortex, corpus callosum(Clarke et al., 2012), and cerebellum(Zezula et al., 2001). Here, they ensure a steady cource of new oligodendrocytes, sustaining remyelination as and when needed. Adult OPCs have limited potential for myelin formation (Bradl & Lassmann, 2010), but can be quite efficient during development or injury(Shi et al., 1998).

Oligodendrocytes are produced at a steady rate in the healthy adult brain. In human brain, although the oligodendrocyte turnover is very stable, with an annual change of 0.3% (Yeung et al., 2014), new studies reveal that myelination and remodelling of myelin is a life-long process, not just during childhood and adolescence. Actually, it was shown that inhibiting the formation of new oligodendrocytes during adulthood, without compromising preexisting oligodendrocytes and myelin, prevented mice from learning new motor skills(McKenzie et al., 2014), suggesting that the formation of new myelinating oligodendrocytes during adult life is an important mechanism for neuroplasticity.

The potential alterations in neuroplasticity orchestrated by drugs and substances therefore may have their genesis from loss of oligodendrocytes, with attendant loss of myelin and their other support functions on neurons and astrocytes.

CONCLUSIONS

The present study on neuronal, astrocytic and oligodendroglial changes in the prefrontal cortex of the male rat with chronic khat use has made the following conclusions:

1. Chronic use of khat results in a dose-dependent decrease in gross cortical morphometric indices such as brain weight, cortical length and width, especially with doses beyond 1000mg/kg. This implies that neurocognitive changes associated with high dose chronic khat use have gross anatomic correlates.

2. Chronic khat use demonstrated a decrease in pyramidal neuronal density, as well as an increase in density of apoptotic pyramidal neurons with increasing doses. Being central to brain signalling, decrease in pyramidal neurons signifies perturbed brain function.

3. There was increased GFAP immunoreactivity, associated astrogliosis and increased astroglial complexity with increasing doses of khat. This may mean an increase in brain injury with attempts at repair, or a state of disturbed brain homeostasis with formation of scar tissue.

4. A strong DCX immunopositivity in the prefrontal cortex, with increase in DCX immunoreactive cells with khat use. This provides insight into potential adult neurogenesis in the prefrontal cortex in which may be a repair process after an initial loss of neurons associated with khat use.

5. There is apparent myelin loss with increasing doses of khat, signifying reduced oligodendrocyte function, with attendant slowed conduction of brain impulses.

LIMITATIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

The current study has the following limitations

1. The time-effect relationship was not exhaustively captured: changes at time zero, and along the feeding process would have given more insights on when changes start and how they progress.

2. Markers of neuronal apoptosis such as ki-67 should have been included to identify and quantify the apoptotic process

3. The exclusion of female rats in the study limited our chance to further elaborate the neuronal and neuroglial changes in the female gender.

Recommendations

We recommend the following for follow-up or future studies on this subject

- A longitudinal study should be designed to identify histological and immunohistochemical changes along the feeding process

- Ki-67 and caspase staining to elucidate the process of neuronal loss

- A comparative study between male and female rats may further unravel the mechanisms of cellular injury and the innate protective mechanisms that exist.

CHAPTER 5: REFERENCES AND APPENDICES

REFERENCES

Abdulwaheb, M., Makonnen, E., Debella, A., & Abebe, D. (2007). Effect of Catha edulis foresk (khat) extracts on male rat sexual behavior. *Journal of Ethnopharmacology*, *110*(2), 250–256. https://doi.org/10.1016/j.jep.2006.09.019

Abebe, M., Kindie, S., & Adane, K. (2015). Adverse Health Effects of Khat: A Review. *Family Medicine & Medical Science Research*, *04*. https://doi.org/10.4172/2327-4972.1000154

Ahmed, A., Ruiz, M. J., Cohen Kadosh, K., Patton, R., & Resurrección, D. M. (2021). Khat and neurobehavioral functions: A systematic review. *PLOS ONE*, *16*(6), e0252900. https://doi.org/10.1371/journal.pone.0252900

Alele, P. E., Ajayi, A. M., & Imanirampa, L. (2013). Chronic khat (Catha edulis) and alcohol marginally alter complete blood counts, clinical chemistry, and testosterone in male rats. *Journal of Experimental Pharmacology*, *5*, 33–44. https://doi.org/10.2147/JEP.S46635

Alexander-Kaufman, K., Dedova, I., Harper, C., & Matsumoto, I. (2007). Proteome analysis of the dorsolateral prefrontal region from healthy individuals. *Neurochemistry International*, *51*(6), 433–439. https://doi.org/10.1016/j.neuint.2007.04.016

Al-Habori, M. (2005). The potential adverse effects of habitual use of Catha edulis (khat). *Expert Opinion on Drug Safety*, *4*(6), 1145–1154. https://doi.org/10.1517/14740338.4.6.1145

Al-Motarreb, A., Baker, K., & Broadley, K. J. (2002). Khat: Pharmacological and medical aspects and its social use in Yemen. *Phytotherapy Research: PTR*, *16*(5), 403–413. https://doi.org/10.1002/ptr.1106

Araque, A., Parpura, V., Sanzgiri, R. P., & Haydon, P. G. (1999). Tripartite synapses: Glia, the unacknowledged partner. *Trends in Neurosciences*, *22*(5), 208–215. https://doi.org/10.1016/s0166-2236(98)01349-6

Arnsten, A. F. T., Raskind, M. A., Taylor, F. B., & Connor, D. F. (2014). The effects of stress exposure on prefrontal cortex: Translating basic research into successful treatments for post-traumatic stress disorder. *Neurobiology of Stress*, *1*, 89–99. https://doi.org/10.1016/j.ynstr.2014.10.002

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., & Lindvall, O. (2002). Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nature Medicine*, *8*(9), 963–970. https://doi.org/10.1038/nm747

Balint, E. E., Falkay, G., & Balint, G. A. (2009). Khat – a controversial plant. *Wiener Klinische Wochenschrift*, *121*(19–20), 604–614. https://doi.org/10.1007/s00508-009-1259-7

Bao, Y., Qin, L., Kim, E., Bhosle, S., Guo, H., Febbraio, M., Haskew-Layton, R. E., Ratan, R., & Cho, S. (2012). CD36 is Involved in Astrocyte Activation and Astroglial Scar Formation. *Journal of Cerebral Blood Flow & Metabolism*, *32*(8), 1567–1577. https://doi.org/10.1038/jcbfm.2012.52

Barres, B. A. (2008). The Mystery and Magic of Glia: A Perspective on Their Roles in Health and Disease. *Neuron*, *60*(3), 430–440. https://doi.org/10.1016/j.neuron.2008.10.013

Baumann, N., & Pham-Dinh, D. (2001). Biology of Oligodendrocyte and Myelin in the Mammalian Central Nervous System. *Physiological Reviews*, *81*(2), 871–927. https://doi.org/10.1152/physrev.2001.81.2.871

Baydyuk, M., Morrison, V. E., Gross, P. S., & Huang, J. K. (2020). Extrinsic Factors Driving Oligodendrocyte Lineage Cell Progression in CNS Development and Injury. *Neurochemical Research*, *45*(3), 630–642. https://doi.org/10.1007/s11064-020-02967-7

Blaesing, B., Nossoll, M., Teuchert-Noodt, G., & Dawirs, R. R. (2001). Postnatal maturation of prefrontal pyramidal neurones is sensitive to a single early dose of methamphetamine in

gerbils (Meriones unguiculatus). *Journal of Neural Transmission*, *108*(1), 101–113.

https://doi.org/10.1007/s007020170101

Bloch, J., Kaeser, M., Sadeghi, Y., Rouiller, E. M., Redmond, D. E., & Brunet, J.-F. (2011).

Doublecortin-positive cells in the adult primate cerebral cortex and possible role in brain

plasticity and development. *The Journal of Comparative Neurology*, *519*(4), 775–789.

https://doi.org/10.1002/cne.22547

Bock, J., Gruss, M., Becker, S., & Braun, K. (2005). Experience-induced changes of dendritic

spine densities in the prefrontal and sensory cortex: Correlation with developmental time

windows. *Cerebral Cortex (New York, N.Y.: 1991)*, *15*(6), 802–808.

https://doi.org/10.1093/cercor/bhh181

Bradl, M., & Lassmann, H. (2010). Oligodendrocytes: Biology and pathology. *Acta*

Neuropathologica, *119*(1), 37–53. https://doi.org/10.1007/s00401-009-0601-5

Brenneisen, R., Fisch, H. U., Koelbing, U., Geisshüsler, S., & Kalix, P. (1990). Amphetamine-

like effects in humans of the khat alkaloid cathinone. *British Journal of Clinical*

Pharmacology, *30*(6), 825–828.

Brown, J. P., Couillard-Després, S., Cooper-Kuhn, C. M., Winkler, J., Aigner, L., & Kuhn, H. G.

(2003). Transient expression of doublecortin during adult neurogenesis. *The Journal of*

Comparative Neurology, *467*(1), 1–10. https://doi.org/10.1002/cne.10874

Brown, S. M., Henning, S., & Wellman, C. L. (2005). Mild, Short-term Stress Alters Dendritic

Morphology in Rat Medial Prefrontal Cortex. *Cerebral Cortex*, *15*(11), 1714–1722.

https://doi.org/10.1093/cercor/bhi048

Karau, PhD Thesis, Uon, 2023

Bush, null, Luu, null, & Posner, null. (2000). Cognitive and emotional influences in anterior
cingulate cortex. *Trends in Cognitive Sciences*, *4*(6), 215–222.
https://doi.org/10.1016/s1364-6613(00)01483-2

Bush, T. G., Puvanachandra, N., Horner, C. H., Polito, A., Ostenfeld, T., Svendsen, C. N.,
Mucke, L., Johnson, M. H., & Sofroniew, M. V. (1999). Leukocyte infiltration, neuronal
degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in
adult transgenic mice. *Neuron*, *23*(2), 297–308. https://doi.org/10.1016/s0896-
6273(00)80781-3

Butt, A. M., & Dinsdale, J. (2005). Fibroblast growth factor 2 induces loss of adult
oligodendrocytes and myelin in vivo. *Experimental Neurology*, *192*(1), 125–133.
https://doi.org/10.1016/j.expneurol.2004.11.007

Byanet, O., Onyeanusi, B. I., & Ibrahim, N. D. G. (2009). Dimorfismo Sexual en Relación con la
Investigación Macro-Morfométrica del Cerebro y Cerebelo del Grasscutter (Thryonomys
swinderianus). *International Journal of Morphology*, *27*(2), 361–365.
https://doi.org/10.4067/S0717-95022009000200010

Cai, Y., Xiong, K., Chu, Y., Luo, D.-W., Luo, X.-G., Yuan, X.-Y., Struble, R. G., Clough, R. W.,
Spencer, D. D., Williamson, A., Kordower, J. H., Patrylo, P. R., & Yan, X.-X. (2009).
Doublecortin expression in adult cat and primate cerebral cortex relates to immature
neurons that develop into GABAergic subgroups. *Experimental Neurology*, *216*(2), 342–
356. https://doi.org/10.1016/j.expneurol.2008.12.008

Carrier, N., & Klantschnig, G. (2018). Quasilegality: Khat, cannabis and Africa's drug laws.
Third World Quarterly, *39*(2), 350–365. https://doi.org/10.1080/01436597.2017.1368383

Carvalho, F. (2003). The toxicological potential of khat. *Journal of Ethnopharmacology*, *87*(1), 1–2. https://doi.org/10.1016/s0378-8741(03)00100-4

Chen, P.-C., Vargas, M. R., Pani, A. K., Smeyne, R. J., Johnson, D. A., Kan, Y. W., & Johnson, J. A. (2009). Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: Critical role for the astrocyte. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(8), 2933–2938. https://doi.org/10.1073/pnas.0813361106

Chudasama, Y., Baunez, C., & Robbins, T. W. (2003). Functional Disconnection of the Medial Prefrontal Cortex and Subthalamic Nucleus in Attentional Performance: Evidence for Corticosubthalamic Interaction. *Journal of Neuroscience*, *23*(13), 5477–5485. https://doi.org/10.1523/JNEUROSCI.23-13-05477.2003

Clarke, L. E., Young, K. M., Hamilton, N. B., Li, H., Richardson, W. D., & Attwell, D. (2012). Properties and Fate of Oligodendrocyte Progenitor Cells in the Corpus Callosum, Motor Cortex, and Piriform Cortex of the Mouse. *Journal of Neuroscience*, *32*(24), 8173–8185. https://doi.org/10.1523/JNEUROSCI.0928-12.2012

Colucci-Guyon, E., Giménez Y Ribotta, M., Maurice, T., Babinet, C., & Privat, A. (1999). Cerebellar defect and impaired motor coordination in mice lacking vimentin. *Glia*, *25*(1), 33–43. https://doi.org/10.1002/(SICI)1098-1136(19990101)25:1<33::AID-GLIA4>3.0.CO;2-J

Condé, F., Maire-Lepoivre, E., Audinat, E., & Crépel, F. (1995). Afferent connections of the medial frontal cortex of the rat. II. Cortical and subcortical afferents. *The Journal of Comparative Neurology*, *352*(4), 567–593. https://doi.org/10.1002/cne.903520407

Karau, PhD Thesis, Uon, 2023

Corkery, J. M., Schifano, F., Oyefeso, A., Ghodse, A. H., Tonia, T., Naidoo, V., & Button, J. (2011). Overview of literature and information on 'khat-related' mortality: A call for recognition of the issue and further research. *Annali Dell'Istituto Superiore Di Sanita*, *47*(4), 445–464. https://doi.org/10.4415/ANN_11_04_17

Couillard-Despres, S., Winner, B., Schaubeck, S., Aigner, R., Vroemen, M., Weidner, N., Bogdahn, U., Winkler, J., Kuhn, H.-G., & Aigner, L. (2005). Doublecortin expression levels in adult brain reflect neurogenesis. *The European Journal of Neuroscience*, *21*(1), 1–14. https://doi.org/10.1111/j.1460-9568.2004.03813.x

Courtney, S. M., Petit, L., Haxby, J. V., & Ungerleider, L. G. (1998). The role of prefrontal cortex in working memory: Examining the contents of consciousness. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *353*(1377), 1819–1828. https://doi.org/10.1098/rstb.1998.0334

Coutureau, E., & Killcross, S. (2003). Inactivation of the infralimbic prefrontal cortex reinstates goal-directed responding in overtrained rats. *Behavioural Brain Research*, *146*(1–2), 167–174. https://doi.org/10.1016/j.bbr.2003.09.025

Cox, G., & Rampes, H. (2003). Adverse effects of khat: A review. *Advances in Psychiatric Treatment*, *9*(6), 456–463. https://doi.org/10.1192/apt.9.6.456

Crews, F. T., Mdzinarishvili, A., Kim, D., He, J., & Nixon, K. (2006). Neurogenesis in adolescent brain is potently inhibited by ethanol. *Neuroscience*, *137*(2), 437–445. https://doi.org/10.1016/j.neuroscience.2005.08.090

Dalley, J. W., Cardinal, R. N., & Robbins, T. W. (2004). Prefrontal executive and cognitive functions in rodents: Neural and neurochemical substrates. *Neuroscience and Biobehavioral Reviews*, *28*(7), 771–784. https://doi.org/10.1016/j.neubiorev.2004.09.006

Karau, PhD Thesis, Uon, 2023

Damasio, A. R., Grabowski, T. J., Bechara, A., Damasio, H., Ponto, L. L., Parvizi, J., & Hichwa, R. D. (2000). Subcortical and cortical brain activity during the feeling of self-generated emotions. *Nature Neuroscience*, *3*(10), 1049–1056. https://doi.org/10.1038/79871

Davidson, R. J., Putnam, K. M., & Larson, C. L. (2000). Dysfunction in the Neural Circuitry of Emotion Regulation—A Possible Prelude to Violence. *Science*, *289*(5479), 591–594. https://doi.org/10.1126/science.289.5479.591

des Portes, V., Pinard, J. M., Billuart, P., Vinet, M. C., Koulakoff, A., Carrié, A., Gelot, A., Dupuis, E., Motte, J., Berwald-Netter, Y., Catala, M., Kahn, A., Beldjord, C., & Chelly, J. (1998). A novel CNS gene required for neuronal migration and involved in X-linked subcortical laminar heterotopia and lissencephaly syndrome. *Cell*, *92*(1), 51–61. https://doi.org/10.1016/s0092-8674(00)80898-3

Du, Y., & Dreyfus, C. F. (2002). Oligodendrocytes as providers of growth factors. *Journal of Neuroscience Research*, *68*(6), 647–654. https://doi.org/10.1002/jnr.10245

Duncan, J., & Owen, A. M. (2000). Common regions of the human frontal lobe recruited by diverse cognitive demands. *Trends in Neurosciences*, *23*(10), 475–483. https://doi.org/10.1016/s0166-2236(00)01633-7

Echoru, I., Bukenya, E. E. M., Masilili, G., Owembabazi, E., Lemuel, A. M., & Ahimbisibwe, J. (2018). Khat distorts the prefrontal cortex histology and function of adult Wistar rats. *Anatomy Journal of Africa*, *7*(1), 1121–1131. https://doi.org/10.4314/aja.v7i1.169485

Eliasson, C., Sahlgren, C., Berthold, C.-H., Stakeberg, J., Celis, J. E., Betsholtz, C., Eriksson, J. E., & Pekny, M. (1999). Intermediate Filament Protein Partnership in Astrocytes *. *Journal of Biological Chemistry*, *274*(34), 23996–24006. https://doi.org/10.1074/jbc.274.34.23996

Elston, G., Benavides-Piccione, R., Elston, A., Manger, P., & Defelipe, J. (2011). Pyramidal

Cells in Prefrontal Cortex of Primates: Marked Differences in Neuronal Structure Among

Species. *Frontiers in Neuroanatomy*, *5*.

https://www.frontiersin.org/article/10.3389/fnana.2011.00002

Euston, D. R., Gruber, A. J., & McNaughton, B. L. (2012). The Role of Medial Prefrontal Cortex

in Memory and Decision Making. *Neuron*, *76*(6), 1057–1070.

https://doi.org/10.1016/j.neuron.2012.12.002

Fattore, L., Puddu, M. C., Picciau, S., Cappai, A., Fratta, W., Serra, G. P., & Spiga, S. (2002).

Astroglial in vivo response to cocaine in mouse dentate gyrus: A quantitative and

qualitative analysis by confocal microscopy. *Neuroscience*, *110*(1), 1–6.

https://doi.org/10.1016/s0306-4522(01)00598-x

Feresten, A. H., Barakauskas, V., Ypsilanti, A., Barr, A. M., & Beasley, C. L. (2013). Increased

expression of glial fibrillary acidic protein in prefrontal cortex in psychotic illness.

Schizophrenia Research, *150*(1), 252–257. https://doi.org/10.1016/j.schres.2013.07.024

Fernandez-Espejo, E., & Rodriguez-Espinosa, N. (2011). Psychostimulant Drugs and

Neuroplasticity. *Pharmaceuticals*, *4*(7), 976–991. https://doi.org/10.3390/ph4070976

Feyissa, A. M., & Kelly, J. P. (2008). A review of the neuropharmacological properties of khat.

Progress in Neuro-Psychopharmacology and Biological Psychiatry, *32*(5), 1147–1166.

https://doi.org/10.1016/j.pnpbp.2007.12.033

Franklin, R. J. M., & Goldman, S. A. (2015). Glia Disease and Repair-Remyelination. *Cold

Spring Harbor Perspectives in Biology*, *7*(7), a020594.

https://doi.org/10.1101/cshperspect.a020594

Friocourt, G., Koulakoff, A., Chafey, P., Boucher, D., Fauchereau, F., Chelly, J., & Francis, F. (2003). Doublecortin Functions at the Extremities of Growing Neuronal Processes. *Cerebral Cortex*, *13*(6), 620–626. https://doi.org/10.1093/cercor/13.6.620

Fujisawa, S., Amarasingham, A., Harrison, M. T., & Buzsáki, G. (2008). Behavior-dependent short-term assembly dynamics in the medial prefrontal cortex. *Nature Neuroscience*, *11*(7), 823–833. https://doi.org/10.1038/nn.2134

Funahashi, S., & Takeda, K. (2002). Information processes in the primate prefrontal cortex in relation to working memory processes. *Reviews in the Neurosciences*, *13*(4), 313–345. https://doi.org/10.1515/revneuro.2002.13.4.313

Fuster, J. M. (2001). The prefrontal cortex--an update: Time is of the essence. *Neuron*, *30*(2), 319–333. https://doi.org/10.1016/s0896-6273(01)00285-9

García-Cabezas, M. Á., Joyce, M. K. P., John, Y. J., Zikopoulos, B., & Barbas, H. (2017). Mirror trends of plasticity and stability indicators in primate prefrontal cortex. *European Journal of Neuroscience*, *46*(8), 2392–2405. https://doi.org/10.1111/ejn.13706

Gleeson, J. G., Allen, K. M., Fox, J. W., Lamperti, E. D., Berkovic, S., Scheffer, I., Cooper, E. C., Dobyns, W. B., Minnerath, S. R., Ross, M. E., & Walsh, C. A. (1998). Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. *Cell*, *92*(1), 63–72. https://doi.org/10.1016/s0092-8674(00)80899-5

Gleeson, J. G., Lin, P. T., Flanagan, L. A., & Walsh, C. A. (1999). Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron*, *23*(2), 257–271. https://doi.org/10.1016/s0896-6273(00)80778-3

Goldman-Rakic, P. S. (1987). Circuitry of Primate Prefrontal Cortex and Regulation of Behavior by Representational Memory. In R. Terjung (Ed.), *Comprehensive Physiology* (1st ed., pp. 373–417). Wiley. https://doi.org/10.1002/cphy.cp010509

Graziani, M., Milella, M. S., & Nencini, P. (2008). Khat Chewing from the Pharmacological Point of View: An Update. *Substance Use & Misuse*, *43*(6), 762–783. https://doi.org/10.1080/10826080701738992

Gregoriou, G. G., Rossi, A. F., Ungerleider, L. G., & Desimone, R. (2014). Lesions of prefrontal cortex reduce attentional modulation of neuronal responses and synchrony in V4. *Nature Neuroscience*, *17*(7), 1003–1011. https://doi.org/10.1038/nn.3742

Guérit, S., Fidan, E., Macas, J., Czupalla, C. J., Figueiredo, R., Vijikumar, A., Yalcin, B. H., Thom, S., Winter, P., Gerhardt, H., Devraj, K., & Liebner, S. (2021). Astrocyte-derived Wnt growth factors are required for endothelial blood-brain barrier maintenance. *Progress in Neurobiology*, *199*, 101937. https://doi.org/10.1016/j.pneurobio.2020.101937

Hadland, K. A., Rushworth, M. F. S., Gaffan, D., & Passingham, R. E. (2003). The effect of cingulate lesions on social behaviour and emotion. *Neuropsychologia*, *41*(8), 919–931. https://doi.org/10.1016/s0028-3932(02)00325-1

Hassan, N. A., Gunaid, A. A., El-Khally, F. M., & Murray-Lyon, I. M. (2002). The effect of chewing Khat leaves on human mood. *Saudi Medical Journal*, *23*(7), 850–853.

Heidbreder, C. A., & Groenewegen, H. J. (2003). The medial prefrontal cortex in the rat: Evidence for a dorso-ventral distinction based upon functional and anatomical characteristics. *Neuroscience & Biobehavioral Reviews*, *27*(6), 555–579. https://doi.org/10.1016/j.neubiorev.2003.09.003

Herrmann, J. E., Imura, T., Song, B., Qi, J., Ao, Y., Nguyen, T. K., Korsak, R. A., Takeda, K., Akira, S., & Sofroniew, M. V. (2008). STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *28*(28), 7231–7243. https://doi.org/10.1523/JNEUROSCI.1709-08.2008

Hertz, L., & Zielke, H. R. (2004). Astrocytic control of glutamatergic activity: Astrocytes as stars of the show. *Trends in Neurosciences*, *27*(12), 735–743. https://doi.org/10.1016/j.tins.2004.10.008

Hess, A., Desiderio, C., & McAuliffe, W. G. (1990). Acute neuropathological changes in the caudate nucleus caused by MPTP and methamphetamine: Immunohistochemical studies. *Journal of Neurocytology*, *19*(3), 338–342. https://doi.org/10.1007/BF01188403

Holford, N. H. G. (1987). Clinical Pharmacokinetics of Ethanol: *Clinical Pharmacokinetics*, *13*(5), 273–292. https://doi.org/10.2165/00003088-198713050-00001

Hoover, W. B., & Vertes, R. P. (2007). Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. *Brain Structure & Function*, *212*(2), 149–179. https://doi.org/10.1007/s00429-007-0150-4

Hutsler, J., & Galuske, R. A. W. (2003). Hemispheric asymmetries in cerebral cortical networks. *Trends in Neurosciences*, *26*(8), 429–435. https://doi.org/10.1016/S0166-2236(03)00198-X

Ishikawa, M., Matsumoto, M., Fujimura, Y., Chiba, K., & Toyama, Y. (2003). Changes of cervical spinal cord and cervical spinal canal with age in asymptomatic subjects. *Spinal Cord*, *41*(3), 159–163. https://doi.org/10.1038/sj.sc.3101375

Itzhak, Y., & Ali, S. F. (2006). Role of nitrergic system in behavioral and neurotoxic effects of amphetamine analogs. *Pharmacology & Therapeutics*, *109*(1), 246–262. https://doi.org/10.1016/j.pharmthera.2005.08.004

Kalix, P. (1981). Cathinone, an alkaloid from khat leaves with an amphetamine-like releasing effect. *Psychopharmacology*, *74*(3), 269–270. https://doi.org/10.1007/BF00427108

Kalix, P. (1996). Catha edulis, a plant that has amphetamine effects. *Pharmacy World and Science*, *18*(2), 69–73. https://doi.org/10.1007/BF00579708

Kimelberg, H. K. (2007). Supportive or information-processing functions of the mature protoplasmic astrocyte in the mammalian CNS? A critical appraisal. *Neuron Glia Biology*, *3*(3), 181–189. https://doi.org/10.1017/S1740925X08000094

Kinouchi, R., Takeda, M., Yang, L., Wilhelmsson, U., Lundkvist, A., Pekny, M., & Chen, D. F. (2003). Robust neural integration from retinal transplants in mice deficient in GFAP and vimentin. *Nature Neuroscience*, *6*(8), 863–868. https://doi.org/10.1038/nn1088

Klüver, H., & Bucy, P. C. (1937). 'Psychic blindness' and other symptoms following bilateral temporal lobectomy in Rhesus monkeys. *American Journal of Physiology*, *119*, 352–353.

Klüver, H., & Bucy, P. C. (1938). An Analysis of Certain Effects of Bilateral Temporal Lobectomy in the Rhesus Monkey, with Special Reference to "Psychic Blindness". *The Journal of Psychology*, *5*(1), 33–54. https://doi.org/10.1080/00223980.1938.9917551

Klüver, H., & Bucy, P. C. (1939). Preliminary analysis of functions of the temporal lobes in monkeys. *Archives of Neurology & Psychiatry*, *42*, 979–1000. https://doi.org/10.1001/archneurpsyc.1939.02270240017001

Koch, C. (1998). *Biophysics of Computation: Information Processing in Single Neurons*. Oxford University Press. https://doi.org/10.1093/oso/9780195104912.001.0001

Koizumi, H., Higginbotham, H., Poon, T., Tanaka, T., Brinkman, B. C., & Gleeson, J. G. (2006). Doublecortin maintains bipolar shape and nuclear translocation during migration in the adult forebrain. *Nature Neuroscience*, *9*(6), 779–786. https://doi.org/10.1038/nn1704

Kremer, T., Jagasia, R., Herrmann, A., Matile, H., Borroni, E., Francis, F., Kuhn, H. G., & Czech, C. (2013). Analysis of adult neurogenesis: Evidence for a prominent 'non-neurogenic' DCX-protein pool in rodent brain. *PloS One*, *8*(5), e59269. https://doi.org/10.1371/journal.pone.0059269

Krettek, J. E., & Price, J. L. (1977). Projections from the amygdaloid complex to the cerebral cortex and thalamus in the rat and cat. *The Journal of Comparative Neurology*, *172*(4), 687–722. https://doi.org/10.1002/cne.901720408

Lande, R. (1979). Quantitative genetic analysis of multivariate evolution, applied to brain:body size allometry. *Evolution*, *33*(1Part2), 402–416. https://doi.org/10.1111/j.1558-5646.1979.tb04694.x

Liu, C.-Y., Yang, Y., Ju, W.-N., Wang, X., & Zhang, H.-L. (2018). Emerging Roles of Astrocytes in Neuro-Vascular Unit and the Tripartite Synapse With Emphasis on Reactive Gliosis in the Context of Alzheimer's Disease. *Frontiers in Cellular Neuroscience*, *12*, 193. https://doi.org/10.3389/fncel.2018.00193

London, M., & Häusser, M. (2005). Dendritic computation. *Annual Review of Neuroscience*, *28*, 503–532. https://doi.org/10.1146/annurev.neuro.28.061604.135703

Lovatt, D., Sonnewald, U., Waagepetersen, H. S., Schousboe, A., He, W., Lin, J. H.-C., Han, X., Takano, T., Wang, S., Sim, F. J., Goldman, S. A., & Nedergaard, M. (2007). The transcriptome and metabolic gene signature of protoplasmic astrocytes in the adult

murine cortex. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *27*(45), 12255–12266. https://doi.org/10.1523/JNEUROSCI.3404-07.2007

Lu, G., Wai, S. M., Poon, W. S., & Yew, D. T. (2005). Ki67 and doublecortin positive cells in the human prefrontal cortices of normal aging and vascular dementia. *Microscopy Research and Technique*, *68*(5), 255–257. https://doi.org/10.1002/jemt.20242

M, K., S, L., X, W., M, E., D, K., J, H., R, H., F, L., S, M., Kr, B., & Sm, P. (2004). Apolipoprotein E promotes astrocyte colocalization and degradation of deposited amyloid-beta peptides. *Nature Medicine*, *10*(7). https://doi.org/10.1038/nm1058

McEwen, B. S., & Milner, T. A. (2017). Understanding the broad influence of sex hormones and sex differences in the brain: Sex Hormones Affect the Whole Brain. *Journal of Neuroscience Research*, *95*(1–2), 24–39. https://doi.org/10.1002/jnr.23809

McKenzie, I. A., Ohayon, D., Li, H., de Faria, J. P., Emery, B., Tohyama, K., & Richardson, W. D. (2014). Motor skill learning requires active central myelination. *Science (New York, N.Y.)*, *346*(6207), 318–322. https://doi.org/10.1126/science.1254960

Menet, V., Prieto, M., Privat, A., & Ribotta, M. G. y. (2003). Axonal plasticity and functional recovery after spinal cord injury in mice deficient in both glial fibrillary acidic protein and vimentin genes. *Proceedings of the National Academy of Sciences*, *100*(15), 8999–9004. https://doi.org/10.1073/pnas.1533187100

Miguel-Hidalgo, J. J. (2009). The Role of Glial Cells in Drug Abuse. *Current Drug Abuse Reviews*, *2*(1), 76–82. https://doi.org/10.2174/1874473710902010076

Miller, E. K., & Wallis, J. D. (2009). Executive Function and Higher-Order Cognition: Definition and Neural Substrates. In *Encyclopedia of Neuroscience* (pp. 99–104). Elsevier. https://doi.org/10.1016/B978-008045046-9.00418-6

Karau, PhD Thesis, Uon, 2023

Minzenberg, M. J., Fan, J., New, A. S., Tang, C. Y., & Siever, L. J. (2008). Frontolimbic structural changes in borderline personality disorder. *Journal of Psychiatric Research*, *42*(9), 727–733. https://doi.org/10.1016/j.jpsychires.2007.07.015

Moehle, M. S., Webber, P. J., Tse, T., Sukar, N., Standaert, D. G., DeSilva, T. M., Cowell, R. M., & West, A. B. (2012). LRRK2 inhibition attenuates microglial inflammatory responses. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *32*(5), 1602–1611. https://doi.org/10.1523/JNEUROSCI.5601-11.2012

Morris, R., Pandya, D. N., & Petrides, M. (1999). Fiber system linking the mid-dorsolateral frontal cortex with the retrosplenial/presubicular region in the rhesus monkey. *Journal of Comparative Neurology*, *407*(2), 183–192. https://doi.org/10.1002/(SICI)1096-9861(19990503)407:2<183::AID-CNE3>3.0.CO;2-N

Muche, A. (2004). *Effect of Ethanol and Khat (Catha Edulis Forsk)on Cerebellar Cortex of the Rat* [Thesis, Addis Abeba University]. http://etd.aau.edu.et/handle/123456789/252

Muche, A. (2006). The effect of ethanol and khat (catha edulis forsk) on the cerebellar cortex of early postnatal rats. Pharmacologyonline 3: 862-876

Murmu, M. S., Salomon, S., Biala, Y., Weinstock, M., Braun, K., & Bock, J. (2006). Changes of spine density and dendritic complexity in the prefrontal cortex in offspring of mothers exposed to stress during pregnancy. *The European Journal of Neuroscience*, *24*(5), 1477–1487. https://doi.org/10.1111/j.1460-9568.2006.05024.x

Mwenda, J. M., Owuor, R. A., Kyama, C. M., Wango, E. O., M'Arimi, M., & Langat, D. K. (2006). Khat (Catha edulis) up-regulates testosterone and decreases prolactin and cortisol levels in the baboon. *Journal of Ethnopharmacology*, *103*(3), 379–384. https://doi.org/10.1016/j.jep.2005.08.016

Karau, PhD Thesis, Uon, 2023

Nägler, K., Mauch, D. H., & Pfrieger, F. W. (2001). Glia-derived signals induce synapse

formation in neurones of the rat central nervous system. *The Journal of Physiology*,

533(Pt 3), 665–679. https://doi.org/10.1111/j.1469-7793.2001.00665.x

Nave, K.-A., & Trapp, B. D. (2008). Axon-glial signaling and the glial support of axon function.

Annual Review of Neuroscience, *31*, 535–561.

https://doi.org/10.1146/annurev.neuro.30.051606.094309

Nave, K.-A., & Werner, H. B. (2014). Myelination of the nervous system: Mechanisms and

functions. *Annual Review of Cell and Developmental Biology*, *30*, 503–533.

https://doi.org/10.1146/annurev-cellbio-100913-013101

Nelson, A. J. D., Perry, J. C., & Vann, S. D. (2018). The Papez Circuit and Recognition

Memory. In *Handbook of Behavioral Neuroscience* (Vol. 27, pp. 217–226). Elsevier.

https://doi.org/10.1016/B978-0-12-812012-5.00015-X

Newman, E. A. (2003a). Glial cell inhibition of neurons by release of ATP. *The Journal of*

Neuroscience: The Official Journal of the Society for Neuroscience, *23*(5), 1659–1666.

Newman, E. A. (2003b). New roles for astrocytes: Regulation of synaptic transmission. *Trends*

in Neurosciences, *26*(10), 536–542. https://doi.org/10.1016/S0166-2236(03)00237-6

Oberheim, N. A., Wang, X., Goldman, S., & Nedergaard, M. (2006). Astrocytic complexity

distinguishes the human brain. *Trends in Neurosciences*, *29*(10), 547–553.

https://doi.org/10.1016/j.tins.2006.08.004

Omar, Y. S., Jenkins, A., Altena, M. van R., Tuck, H., Hynan, C., Tohow, A., Chopra, P., &

Castle, D. (2015). Khat Use: What Is the Problem and What Can Be Done? *BioMed*

Research International, *2015*, 1–7. https://doi.org/10.1155/2015/472302

Ota, Y., Zanetti, A. T., & Hallock, R. M. (2013). The Role of Astrocytes in the Regulation of
Synaptic Plasticity and Memory Formation. *Neural Plasticity*, *2013*, 1–11.
https://doi.org/10.1155/2013/185463

Panov, A., Orynbayeva, Z., Vavilin, V., & Lyakhovich, V. (2014). Fatty acids in energy
metabolism of the central nervous system. *BioMed Research International*, *2014*,
472459. https://doi.org/10.1155/2014/472459

PAPEZ, J. W. (1937). A proposed mechanism of emotion. *Archives of Neurology & Psychiatry*,
38(4), 725–743. https://doi.org/10.1001/archneurpsyc.1937.02260220069003

Parpura, V., Basarsky, T. A., Liu, F., Jeftinija, K., Jeftinija, S., & Haydon, P. G. (1994).
Glutamate-mediated astrocyte-neuron signalling. *Nature*, *369*(6483), 744–747.
https://doi.org/10.1038/369744a0

Parpura, V., Heneka, M. T., Montana, V., Oliet, S. H. R., Schousboe, A., Haydon, P. G., Stout Jr,
R. F., Spray, D. C., Reichenbach, A., Pannicke, T., Pekny, M., Pekna, M., Zorec, R., &
Verkhratsky, A. (2012). Glial cells in (patho)physiology. *Journal of Neurochemistry*,
121(1), 4–27. https://doi.org/10.1111/j.1471-4159.2012.07664.x

Parpura, V., & Zorec, R. (2010). Gliotransmission: Exocytotic release from astrocytes. *Brain
Research Reviews*, *63*(1–2), 83–92. https://doi.org/10.1016/j.brainresrev.2009.11.008

Passingham, R. E., Stephan, K. E., & Kötter, R. (2002). The anatomical basis of functional
localization in the cortex. *Nature Reviews Neuroscience*, *3*(8), 606–616.
https://doi.org/10.1038/nrn893

Patel, N. (2009). Mechanism of action of cathinone: The active ingredient of Khat (*Catha
Edulis*. *East African Medical Journal*, *77*(6). https://doi.org/10.4314/eamj.v77i6.46651

Karau, PhD Thesis, UoN, 2023

Pekny, M., & Lane, E. B. (2007). Intermediate filaments and stress. *Experimental Cell Research*, *313*(10), 2244–2254. https://doi.org/10.1016/j.yexcr.2007.04.023

Pekny, M., Levéen, P., Pekna, M., Eliasson, C., Berthold, C. H., Westermark, B., & Betsholtz, C. (1995). Mice lacking glial fibrillary acidic protein display astrocytes devoid of intermediate filaments but develop and reproduce normally. *The EMBO Journal*, *14*(8), 1590–1598.

Pekny, M., & Pekna, M. (2004). Astrocyte intermediate filaments in CNS pathologies and regeneration. *The Journal of Pathology*, *204*(4), 428–437. https://doi.org/10.1002/path.1645

Perea, G., Navarrete, M., & Araque, A. (2009). Tripartite synapses: Astrocytes process and control synaptic information. *Trends in Neurosciences*, *32*(8), 421–431. https://doi.org/10.1016/j.tins.2009.05.001

Phan, K. L., Wager, T., Taylor, S. F., & Liberzon, I. (2002). Functional Neuroanatomy of Emotion: A Meta-Analysis of Emotion Activation Studies in PET and fMRI. *NeuroImage*, *16*(2), 331–348. https://doi.org/10.1006/nimg.2002.1087

Pubill, D., Canudas, A. M., Pallàs, M., Camins, A., Camarasa, J., & Escubedo, E. (2003). Different glial response to methamphetamine- and methylenedioxymethamphetamine-induced neurotoxicity. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *367*(5), 490–499. https://doi.org/10.1007/s00210-003-0747-y

Quinn, R. (2005). Comparing rat's to human's age: How old is my rat in people years? *Nutrition (Burbank, Los Angeles County, Calif.)*, *21*(6), 775–777. https://doi.org/10.1016/j.nut.2005.04.002

Radley, J. J., Sisti, H. M., Hao, J., Rocher, A. B., McCall, T., Hof, P. R., McEwen, B. S., & Morrison, J. H. (2004). Chronic behavioral stress induces apical dendritic reorganization in pyramidal neurons of the medial prefrontal cortex. *Neuroscience*, *125*(1), 1–6. https://doi.org/10.1016/j.neuroscience.2004.01.006

Ragozzino, M. E., Detrick, S., & Kesner, R. P. (1999). Involvement of the prelimbic-infralimbic areas of the rodent prefrontal cortex in behavioral flexibility for place and response learning. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *19*(11), 4585–4594.

Rajkowska, G., Miguel-Hidalgo, J. J., Makkos, Z., Meltzer, H., Overholser, J., & Stockmeier, C. (2002). Layer-specific reductions in GFAP-reactive astroglia in the dorsolateral prefrontal cortex in schizophrenia. *Schizophrenia Research*, *57*(2–3), 127–138. https://doi.org/10.1016/s0920-9964(02)00339-0

Rana, A., Massa, P. T., & Chen, X. J. (2022). A Gravity Fed Transcardial Perfusion Method for Histologic Analysis of the Mouse Central Nervous System. *Journal of Visualized Experiments : JoVE*, *179*, 10.3791/63386. https://doi.org/10.3791/63386

Reagan-Shaw, S., Nihal, M., & Ahmad, N. (2008). Dose translation from animal to human studies revisited. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *22*(3), 659–661. https://doi.org/10.1096/fj.07-9574LSF

Robinson, R. G., Kubos, K. L., Starr, L. B., Rao, K., & Price, T. R. (1984). MOOD disorders in stroke patients: importance of location of lesion. *Brain*, *107*(1), 81–93. https://doi.org/10.1093/brain/107.1.81

Karau, PhD Thesis, UoN, 2023

Rolls, E. T., O'Doherty, J., Kringelbach, M. L., Francis, S., Bowtell, R., & McGlone, F. (2003). Representations of pleasant and painful touch in the human orbitofrontal and cingulate cortices. *Cerebral Cortex (New York, N.Y.: 1991)*, *13*(3), 308–317. https://doi.org/10.1093/cercor/13.3.308

Roth, G., & Dicke, U. (2005). Evolution of the brain and intelligence. *Trends in Cognitive Sciences*, *9*(5), 250–257. https://doi.org/10.1016/j.tics.2005.03.005

Rothstein, J. D., Dykes-Hoberg, M., Pardo, C. A., Bristol, L. A., Jin, L., Kuncl, R. W., Kanai, Y., Hediger, M. A., Wang, Y., Schielke, J. P., & Welty, D. F. (1996). Knockout of Glutamate Transporters Reveals a Major Role for Astroglial Transport in Excitotoxicity and Clearance of Glutamate. *Neuron*, *16*(3), 675–686. https://doi.org/10.1016/S0896-6273(00)80086-0

Samue Kindie, M. A. (2015). Adverse Health Effects of Khat: A Review. *Family Medicine & Medical Science Research*, *04*(01). https://doi.org/10.4172/2327-4972.1000154

Seifert, G., Schilling, K., & Steinhäuser, C. (2006). Astrocyte dysfunction in neurological disorders: A molecular perspective. *Nature Reviews. Neuroscience*, *7*(3), 194–206. https://doi.org/10.1038/nrn1870

Sengupta, P. (2013). The Laboratory Rat: Relating Its Age With Human's. *International Journal of Preventive Medicine*, *4*(6), 624–630.

Shi, J., Marinovich, A., & Barres, B. A. (1998). Purification and Characterization of Adult Oligodendrocyte Precursor Cells from the Rat Optic Nerve. *Journal of Neuroscience*, *18*(12), 4627–4636. https://doi.org/10.1523/JNEUROSCI.18-12-04627.1998

Shih, A. Y., Johnson, D. A., Wong, G., Kraft, A. D., Jiang, L., Erb, H., Johnson, J. A., & Murphy, T. H. (2003). Coordinate regulation of glutathione biosynthesis and release by

Karau, PhD Thesis, Uon, 2023

Nrf2-expressing glia potently protects neurons from oxidative stress. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *23*(8), 3394–3406.

Simons, M., & Nave, K.-A. (2016). Oligodendrocytes: Myelination and Axonal Support. *Cold Spring Harbor Perspectives in Biology*, *8*(1), a020479. https://doi.org/10.1101/cshperspect.a020479

Singh, B., & Dyce, K. M. (Eds.). (2018). *Dyce, Sack, and Wensing's textbook of veterinary anatomy* (Fifth edition). Saunders.

Sofroniew, M. V., & Vinters, H. V. (2010). Astrocytes: Biology and pathology. *Acta Neuropathologica*, *119*(1), 7–35. https://doi.org/10.1007/s00401-009-0619-8

Swanson, R. A., Ying, W., & Kauppinen, T. M. (2004). Astrocyte influences on ischemic neuronal death. *Current Molecular Medicine*, *4*(2), 193–205. https://doi.org/10.2174/1566524043479185

Thomas, S., & Williams, T. (2013). Khat (Catha edulis): A systematic review of evidence and literature pertaining to its harms to UK users and society. *Drug Science, Policy and Law*, *1*, 2050324513498332. https://doi.org/10.1177/2050324513498332

Tong, M., Yu, R., Silbermann, E., Zabala, V., Deochand, C., & de la Monte, S. M. (2015). Differential Contributions of Alcohol and Nicotine-Derived Nitrosamine Ketone (NNK) to White Matter Pathology in the Adolescent Rat Brain. *Alcohol and Alcoholism (Oxford, Oxfordshire)*, *50*(6), 680–689. https://doi.org/10.1093/alcalc/agv102

Totah, N. K. B., Jackson, M. E., & Moghaddam, B. (2013). Preparatory attention relies on dynamic interactions between prelimbic cortex and anterior cingulate cortex. *Cerebral Cortex (New York, N.Y.: 1991)*, *23*(3), 729–738. https://doi.org/10.1093/cercor/bhs057

Treves, A. (2005). Frontal latching networks: A possible neural basis for infinite recursion.

Cognitive Neuropsychology, *22*(3), 276–291.

https://doi.org/10.1080/02643290442000329

Uher, R., Murphy, T., Brammer, M. J., Dalgleish, T., Phillips, M. L., Ng, V. W., Andrew, C. M.,

Williams, S. C. R., Campbell, I. C., & Treasure, J. (2004). Medial prefrontal cortex

activity associated with symptom provocation in eating disorders. *The American Journal*

of Psychiatry, *161*(7), 1238–1246. https://doi.org/10.1176/appi.ajp.161.7.1238

Uylings, H. B. M., Groenewegen, H. J., & Kolb, B. (2003). Do rats have a prefrontal cortex?

Behavioural Brain Research, *146*(1–2), 3–17. https://doi.org/10.1016/j.bbr.2003.09.028

Van Eden, C. G., & Uylings, H. B. (1985). Postnatal volumetric development of the prefrontal

cortex in the rat. *The Journal of Comparative Neurology*, *241*(3), 268–274.

https://doi.org/10.1002/cne.902410303

Verwer, R. W. H., Sluiter, A. A., Balesar, R. A., Baayen, J. C., Noske, D. P., Dirven, C. M. F.,

Wouda, J., van Dam, A. M., Lucassen, P. J., & Swaab, D. F. (2007). Mature astrocytes in

the adult human neocortex express the early neuronal marker doublecortin. *Brain: A*

Journal of Neurology, *130*(Pt 12), 3321–3335. https://doi.org/10.1093/brain/awm264

Voloboueva, L. A., & Giffard, R. G. (2011). Inflammation, mitochondria, and the inhibition of

adult neurogenesis. *Journal of Neuroscience Research*, *89*(12), 1989–1996.

https://doi.org/10.1002/jnr.22768

Wabe, N. T. (2011). Chemistry, Pharmacology, and Toxicology of Khat (Catha Edulis Forsk): A

Review. *Addiction & Health*, *3*(3–4), 137–149.

Wang, D. D., & Bordey, A. (2008). The astrocyte odyssey. *Progress in Neurobiology*, *86*(4),

342–367. https://doi.org/10.1016/j.pneurobio.2008.09.015

Karau, PhD Thesis, UoN, 2023

Wang, X. J. (2001). Synaptic reverberation underlying mnemonic persistent activity. *Trends in Neurosciences*, *24*(8), 455–463. https://doi.org/10.1016/s0166-2236(00)01868-3

Weissman, D. H., Giesbrecht, B., Song, A. W., Mangun, G. R., & Woldorff, M. G. (2003). Conflict monitoring in the human anterior cingulate cortex during selective attention to global and local object features. *NeuroImage*, *19*(4), 1361–1368. https://doi.org/10.1016/S1053-8119(03)00167-8

Wellman, C. L. (2001). Dendritic reorganization in pyramidal neurons in medial prefrontal cortex after chronic corticosterone administration. *Journal of Neurobiology*, *49*(3), 245–253. https://doi.org/10.1002/neu.1079

Widestrand, A., Faijerson, J., Wilhelmsson, U., Smith, P. L. P., Li, L., Sihlbom, C., Eriksson, P. S., & Pekny, M. (2007). Increased neurogenesis and astrogenesis from neural progenitor cells grafted in the hippocampus of GFAP-/- Vim-/- mice. *Stem Cells (Dayton, Ohio)*, *25*(10), 2619–2627. https://doi.org/10.1634/stemcells.2007-0122

Wilhelmsson, U., Li, L., Pekna, M., Berthold, C.-H., Blom, S., Eliasson, C., Renner, O., Bushong, E., Ellisman, M., Morgan, T. E., & Pekny, M. (2004). Absence of glial fibrillary acidic protein and vimentin prevents hypertrophy of astrocytic processes and improves post-traumatic regeneration. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *24*(21), 5016–5021. https://doi.org/10.1523/JNEUROSCI.0820-04.2004

Winstanley, C. A., Theobald, D. E. H., Dalley, J. W., Cardinal, R. N., & Robbins, T. W. (2006). Double dissociation between serotonergic and dopaminergic modulation of medial prefrontal and orbitofrontal cortex during a test of impulsive choice. *Cerebral Cortex (New York, N.Y.: 1991)*, *16*(1), 106–114. https://doi.org/10.1093/cercor/bhi088

Karau, PhD Thesis, Uon, 2023

Yang, Y., & Raine, A. (2009). Prefrontal Structural and Functional Brain Imaging findings in

Antisocial, Violent, and Psychopathic Individuals: A Meta-Analysis. *Psychiatry

Research*, *174*(2), 81–88. https://doi.org/10.1016/j.pscychresns.2009.03.012

Yeung, M. S. Y., Zdunek, S., Bergmann, O., Bernard, S., Salehpour, M., Alkass, K., Perl, S.,

Tisdale, J., Possnert, G., Brundin, L., Druid, H., & Frisén, J. (2014). Dynamics of

oligodendrocyte generation and myelination in the human brain. *Cell, 159*(4), 766–774.

https://doi.org/10.1016/j.cell.2014.10.011

Zahr, N. M., & Pfefferbaum, A. (2017). Alcohol's Effects on the Brain: Neuroimaging Results in

Humans and Animal Models. *Alcohol Research: Current Reviews*, *38*(2), 183–206.

Zezula, J., Casaccia-Bonnefil, P., Ezhevsky, S. A., Osterhout, D. J., Levine, J. M., Dowdy, S. F.,

Chao, M. V., & Koff, A. (2001). P21 *cip1* is required for the differentiation of

oligodendrocytes independently of cell cycle withdrawal. *EMBO Reports*, *2*(1), 27–34.

https://doi.org/10.1093/embo-reports/kve008

APPENDICES

APPENDIX 1: Data Collection Form

1. Date: _____
2. Average weights

Week/ Rat Group	Controls	Group 1	Group 2	Group 3
Week 1				
Week 2				
Week 3				
Week 4				
Week 5				
Week 6				
At sacrifice				

3. Gross brain indices

Gross brain measurements	Controls	Group 1	Group 2	Group 3
Body weight (g)				
Absolute brain weight (g)				
Relative brain weight (Ratio)				
Maximum cerebral cortical width (mm)				
Maximum cortex length (mm)				
Supero-inferior brain lenghth				

4. Normal and apoptotic pyramidal neuronal density

	Controls	Group 1	Group 2	Group 3
Normal PND				
Apoptotic pyramidal neurons				

5.

	Controls	Group 1	Group 2	Group 3
GFAP + Astrocytes				

6.

	Controls	Group 1	Group 2	Group 3
DCX+ Neurons				

APPENDIX 2: ETHICAL APPROVAL

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

P.O. Box 30197,

00100 Nairobi,

Tel: 4449004/4442014/ 6
Ext. 2300
Direct Line. 4448648

REF: FVM

BAUEC/2020/276

Dr. Paul Bundi Karau,
University of Nairobi,
Dept. of Human Anatomy
16th October 2020

Dear Dr. Karau,

RE: Approval of proposal by Faculty Biosafety, Animal use and Ethics committee

Neuronal and Astrocytic structural changes in the prefrontal of the rat following chronic khat use.

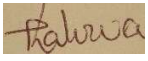**Dr Paul Bundi   H80/56794/2020**

We refer to your PhD proposal submitted to our committee for review and your application letter dated 22nd September 2020. We have reviewed your application for ethical clearance for the study.

Animal handling, euthanasia and brain harvesting protocols meet required standards. The histological, cytoarchitecture and Golgi staining protocol to elucidate neuronal extensions also meets minimum standards of the Faculty of Veterinary medicine ethical regulation guidelines.
We have also noted that Prof Andrew Makanya (KVB number 525) a registered veterinary surgeon will supervise the laboratory protocols.

We hereby give approval for you to proceed with the project as outlined in the submitted proposal.

Karau, PhD Thesis, Uon, 2023

Yours sincerely,

Dr. Catherine Kaluwa, Ph.D

Chairperson, Biosafety, Animal Use and Ethics Committee,

Faculty of Veterinary Medicine,

University of Nairobi

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

Paul Bundi Karau[1,2], Paul Odula[2], Moses Obimbo[2]

[1] Department of Human Anatomy, School of Medicine, Kenya Methodist University

[2] Department of Human Anatomy, Faculty of Health Sciences, The University of Nairobi, Kenya

Correspondence to Dr Paul Bundi Karau, P.O. Box 2375-60200, Meru Kenya, Email pbkarau@gmail.com, +254721484624

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.

Karau, PhD Thesis, Uon, 2023

Original Research Article

Changes in double-cortin immunoreactive neurons in the prefrontal cortex of male rat following chronic khat use

Paul Bundi Karau[1,*], Paul Odula[1], Moses Obimbo[1], Amadi Ogonda-Ihunwo[2], Nkomozepi Pilani[3], Muriira Karau[4]

[1]*Dept. of Human Anatomy & Physiology, The University of Nairobi, Nairobi, Kenya*

[2]*The University of the Witwatersrand, School of Anatomical Sciences, Johannesburg, South Africa*
[3]*Dept. of Anatomy, University of Johannesburg, Johannesburg, South Africa*
[4]*Kenya Bureau of Standards, Directorate of Quality Assurance, Nairobi, Kenya*

ARTICLE INFO

ABSTRACT

Objectives: Chronic use of khat has been associated with neurocognitive changes. Among the cells affected and responsible for the changes in cognition are immature neurons. This study aims to outline the changes in histomorphometry of immature neurons on chronic use of khat.

Materials and Methods: Young adult wistar rats were randomized into controls, and three experimental groups to receive 500mg/kg, 1000mg/kg and 2000mg/kg crude khat extracts respectively. After 6 weeks the animals were sacrificed and their brains removed. We performed immunohistochemical visualization of immature neurons using double-cortin staining. Photomicrographs of the stained sections were transferred to Image J-Fiji software to study the staining neurons. We used Kruskal-Wallis test to correlate the four animal groups in terms of astrocyte densities.

---

[1] . Introduction   zone and the hippocampus,[3] it is now known to express in the cerebral cortex indicating that these cells might be

Changes in Glial Fibrillary Acidic Protein‐Immunoreactive Astrocytes in the Prefrontal Cortex of the Male Rat following

Chronic Khat Use

Paul Bundi Karau[1,2], Paul Odula[2], Moses Obimbo[2], Amadi Ogonda Ihunwo[3], Pilani Nkomozepi[4], Muriira Karau[5]

[1]Department of Human Anatomy, School of Medicine and Health Sciences, Kenya Methodist University, Meru, [2]Department of Human Anatomy and Physiology,
Faculty of Health Sciences, The University of Nairobi, [5]Department of Quality Assurance, Kenya Bureau of Standards, Nairobi, Kenya, [3]Department of Morphological Sciences, School of Anatomical Sciences, Faculty of Health Sciences, University of the Witwatersrand, [4]Department of Anatomy, Johannesburg University,
Johannesburg, South Africa

Background: Long-term khat consumption is associated with significant neurocognitive changes, which have been elucidated in behavioral studies. With current research showing the centrality of astrocytes and other glial cells in neuronal signaling, there is possibility that these cells are also affected by chronic khat use. There is little literature on the structural changes in the prefrontal cortex neuronal and astrocytic cytoarchitecture and morphometry in chronic khat users. **Objective:** The objective of this study was to describe the changes in astrocyte morphometry and structure in rats after long-term use of khat (miraa). **Materials and Methods:** Adult male Wistar rats, aged 2–3 months, weighing 200–300 g were randomized into four groups of 10 each (control, Group 1, Group 2, and Group 3) to correspond with those used as controls and those that received 500 mg/kg, 1000 mg/kg, and 2000 mg/kg body weight khat extracts, respectively. Fresh khat leaves were purchased from Maua market in Meru, and crude extract was prepared using lyophilization. The control rats were fed on normal diet, while the experimental groups were fed on normal diet and khat extracts using oral gavage for 6 weeks. The animals were sacrificed and their brains were removed. We performed immunohistochemical visualization of astrocytes using glial fibrillary acidic protein. Photomicrographs of the stained sections were transferred to ImageJ Fiji software to study the astrocyte density and astrocytic processes. We used Kruskal–Wallis test to correlate the four animal groups in terms of astrocyte densities. **Results:** We observed an increase in the average number of astrocytes with increasing doses of khat compared to controls, with those in Group 3 (2000 mg/kg) having an exuberant reactive astrocytosis. Further, escalating khat doses resulted in increased glial fibrillary acidic protein immunoreactivity in the nuclei and astrocytic processes, gliotic changes, and increased complexity of astrocytic processes. **Conclusion:** Chronic khat use, especially at high doses, results in reactive astrocytosis and astrogliosis, which may be part of the mechanisms involved in the cognitive changes associated with its use.

Keywords: Chronic consumption, glial fibrillary acidic protein, khat, reactive astrocytes

How to cite this article: Karau PB, Odula P, Obimbo M, Ihunwo AO, Nkomozepi P, Karau M. Changes in glial fibrillary acidic protein-immunoreactive astrocytes in the prefrontal cortex of the male rat following chronic khat use. J Microsc Ultrastruct 0;0:0.