Original Article

Changes in Glial Fibrillary Acidic Protein-Immunoreactive Astrocytes in the Prefrontal Cortex of the Male Rat following Chronic Khat Use

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Abstract

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

Introduction

The use of khat (miraa) is prevalent in the Horn of Africa and some Middle Eastern countries. The plant is mainly valued for its central nervous effects, especially euphoriant and stimulating effects.[1] Recently, concerns have arisen due to its long-term effects of khat on cognitive function among users.[2]

While not many studies have been done on the exact mechanism of action of khat on the brain, its effects can be extrapolated from those of amphetamine, which shares similar chemical structure and action at cellular receptors.[3] 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 potentiates the

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release and action of dopamine from neuronal synapses, and recent evidence suggests that it also activates serotonergic synapses.[4]

A great amount of research has been conducted on the behavioral changes associated with acute and subacute administration of khat in experimental animals.[5] Further, some researchers have examined behavioral 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.[6] There is recorded evidence of hallucinations and even overt psychosis among khat users.[7]

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 behaviors.[8] In rodents, the medial prefrontal cortex is involved in attentional processing, modulation of working memory, goal-directed behavior, and behavioral flexibility.[9,10] The ventral medial prefrontal cortex corresponds with the limbic system in humans.[11]

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.[12] They play critical roles in metabolic processes of the brain,[13] formation and maintenance of synapses, transduction of synaptic messages, and processing of data.[14] This implies that any disease processes or substances affecting neuronal function could also affect astrocyte structure and function. Glial fibrillary acidic protein (GFAP) is a prototypical marker of astrocyte reactivity in the central nervous system and, therefore, a critical antibody protein in evaluation of prefrontal astrocytes in disease states.[15]

Despite the importance of Khat in the socio-economic lives of the people of East Africa, it's potential deleterious effect on the brain call for more research to better understand its effects. There is little or no recorded literature elucidating the changes in astrocyte morphology and density on khat users.

Materials and Methods

Experimental animals

Adult male Wistar rats (*Rattus norvegicus*) aged 2–3 months and weighing 200–300 g were purchased from an accredited breeding institution and housed within the Department of Veterinary Anatomy and Physiology of the University of Nairobi. They were housed in cages, with adequate ventilation and provided with a normal light and dark circadian cycle and given adequate and free access to food and water (*ad libitum*), and allowed to acclimatize for 7 days before beginning the experiments.

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

Ethical consideration

This study was carried out within 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), issued on 16th October 2020. Animals were handled in a humane way in accordance with the Declaration of Helsinki. Animals were handled in a humane way and euthanized using intraperitoneal ketamine and xylazine.

Khat extraction

This was performed as described elsewhere, with slight modification.[16] Khat samples were collected from the farm and transported in a cooler box to the laboratory within 4 h of harvesting. After weighing, each bunch was chopped to homogenize the sample and blended with 125 ml of sterile distilled water. The blended mixture was then transferred to 40-ml falcon tubes and centrifuged at 7000 rpm for 6 min. The supernatant was then transferred into 100-ml bottles covered with aluminum foil to minimize exposure to light and stored at refrigerated conditions of 2°C awaiting lyophilization. Supernatant from khat extract was then dispensed in volumes of 3 ml into vials for lyophilization. The vials were first frozen at −80°C for 2 h and then freeze dried under vacuum at 0.103 mbar for 24 h.

Preparation of brain sections

Animals were euthanized by intraperitoneal ketamine. The brains were fixed by cardiac perfusion with 0.9% normal saline followed by 4% paraformaldehyde in 0.1-M phosphate buffer. Brains were quickly removed from the skulls and placed in buffered formaldehyde and embedded in paraffin blocks.

Histological sections 1 mm thick were cut from the medial prefrontal cortex using the atlas of Paxinos and Watson,[17] with the olfactory tract intact. One out of 20 sections was randomly selected for staining from each animal group. Toluidine blue was used to stain brain sections in all the groups to visualize the light microscopic arrangement of layers.

Glial fibrillary acidic protein staining

The paraffin-embedded sections were deparaffinized in xylene for 10 min twice and rehydrated through graded alcohols, from absolute alcohol to 95% alcohol. They were then washed in running tap water. Antigen retrieval was performed using citrate buffer pH 6 overnight in a water bath at 60°C.

On the 2nd day, the sections were allowed to cool to room temperature for 20 min and then rinsed once in PBS buffer. Endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol by immersing the slides in the solution for 20 min. The slides were then washed three times in PBS buffer for 5 min each wash. Slides were then incubated in a moist chamber in 5% normal goat serum for 30 min. Tissues were then incubated with primary antibody overnight at 4°C. GFAP primary antibody dilution of 1:300 was selected. We also ran negative and positive controls for each of the slides from the animal groups.

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On the 3rd day, slides were washed in PBS for three runs of 5 min each. They were then incubated in secondary antibody (biotinylated goat anti-rabbit, Vector Lab, USA 1:1000), for 30 min, and washed off in PBS, three runs of 5 min each. They were incubated for 30 min with avidin–biotin complex and washed thrice in PBS, three runs of 5 min each. Afterward, they were incubated with DAB working solution for 5 min, rinsed in running tap water, and counterstained in hematoxylin for 1 min. After washing off the stain in tap water for 5 min, the slides were run in graded alcohols, cleared in xylene, and mounted with Entellan. Photomicrographs were captured using a digital camera (AxioCam HRc, Zeiss, South Africa) mounted on the light microscope (Axioskop 2 Plus, Zeiss, South Africa) and operating on the ZEN 2010 computer software.

Data analysis

The photomicrographs (×40 magnification) were analyzed using Fiji ImageJ software. Grid lines were centered on the photomicrographs making a total of 15 complete square boxes, each with an area of 1000 um^2 [Figure 1]. Complete astrocytes (with cell body and processes) were counted in all the 15 boxes. The average number of astrocytes per group was entered into Excel for purposes of generating a bar graph. Kruskal–Wallis test and descriptive statistics such as mean, standard deviation, and range were generated using Statistical Package for Social Sciences (SPSS, version 28, IBM, USA) software. Analyzed data from SPSS was then presented in form of box plots.

Results

On toluidine blue staining, the prefrontal cortex appears adjacent to the olfactory bulb, and the associated white matter can be visualized. The layer arrangement of cells is discernible, though the deeper layers III, IV, and V appear to merge, as shown in Figure 2.

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. This arrangement is seen in both the control and experimental groups. Figure 3 shows the layer arrangement of astrocytes.

Astrocyte count increases with increasing doses of khat

There was a general increase in the average number of astrocytes across the groups [Figure 4]. The astrocyte counts in Groups 1, 2, 3, and 4 were 7, 8, 9.7, and 10 per 15,000 um^2, respectively. A similar trend for the astrocyte number is replicated in the respective box plots [Figure 5]. Kruskal–Wallis test reveals a significant difference in astrocyte count in Group 3 (2000 mg/kg) compared to the control rat group ($P < 0.0025$). Figure 6 shows the increase in astrocyte numbers with increasing doses of khat.

Increased glial fibrillary acidic protein 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 7c and d]. Whereas the nucleolus in the control group was almost indistinct [Figure 7a], 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 layers 1 and 2 of the medial prefrontal cortex. It was noted that gliosis was mild in Group 1 (500 mg/kg), moderate in Group 2 (1000 mg/kg), and severe in Group 3 (2000 mg/kg) [Figure 7b-d, respectively]. Little or no features of gliosis were noted in the control group.

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 groups (1, 2, and 3) demonstrated

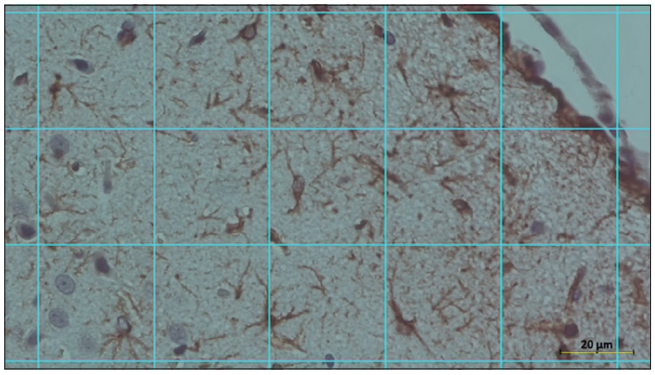

Figure 1: Photomicrograph showing the square grids used in counting of astrocytes using ImageJ Fiji software (GFAP stain, ×400). GFAP: Glial fibrillary acidic protein

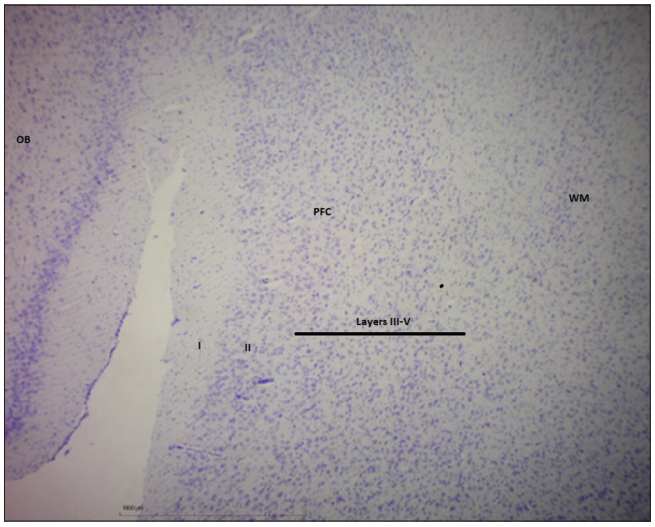

Figure 2: Photomicrograph of a coronal section through the frontal lobe of the right cerebral hemisphere. The PFC and its associated WM are located adjacent to the OC (toluidine blue staining, ×40). PFC: Prefrontal cortex, WM: White matter, OC: Olfactory cortex

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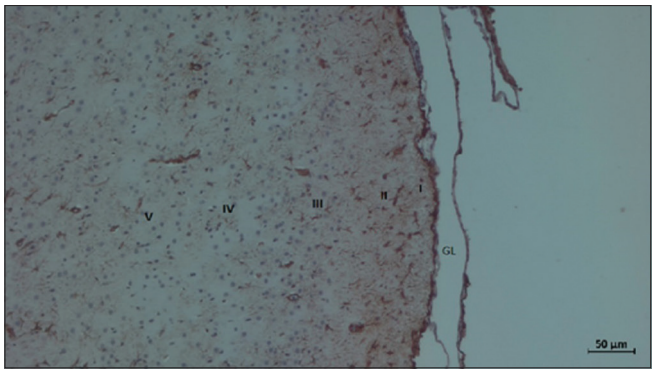

Figure 3: Photomicrograph showing arrangements of astrocytes in the layers of the prefrontal cortex, with predominance of astrocytes in layers II and III (magnification ×100, GFAP Stain). GFAP: Glial fibrillary acidic protein

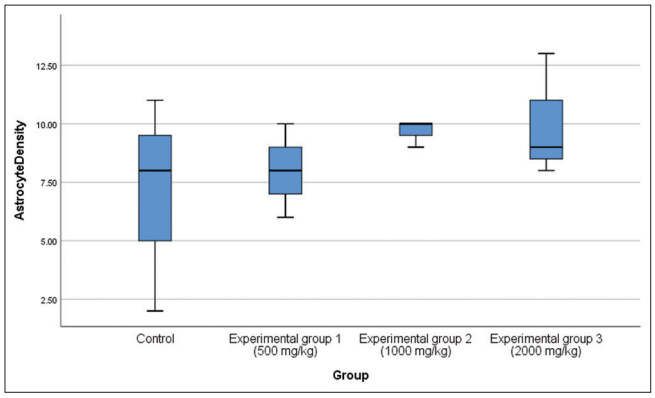

Figure 5: Box plots of the astrocyte densities in each study group

numerous, stout, and ramifying processes, with the most complex being in the third experimental group. This is depicted in Figure 8.

Discussion

Our study has found 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 1000 mg/kg produced greater and more significant changes compared to control rats.

GFAP is a prototypical marker for immunohistochemical identification of astrocytes. It strongly labels astrocytes that are responding to injuries in the central nervous system.[18] GFAP is a cytoskeletal protein, and a malfunction of the proteins leads to accumulation,[19] and its mRNA expression may be altered in states associated with neuronal pathology.[20]

Our study demonstrated an increase in number and density of astrocytes with increasing doses of khat compared to controls. Whereas there are few studies in the literature documenting alterations in astrocyte number and density with chronic use of khat, self-administration of heroin has been documented to result in increased density of astrocytes, a process thought to be due to glial injury and attempts at brain repair.[21]

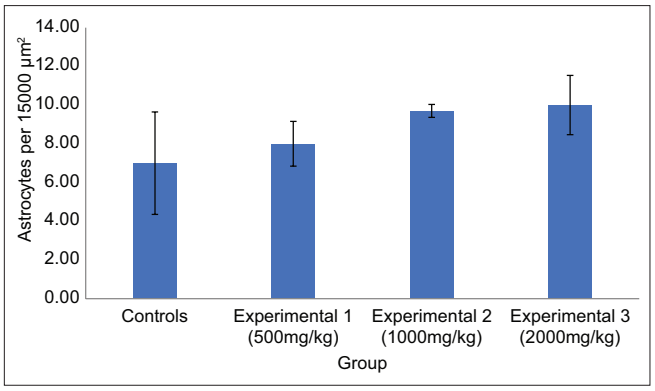

Figure 4: Graph depicting the general trend of astrocyte densities in each study group

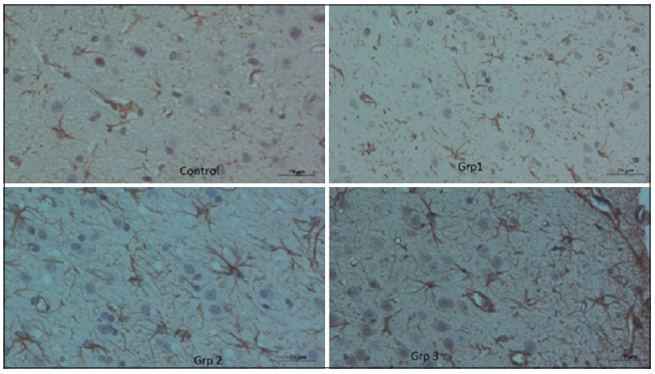

Figure 6: Representative photomicrograph of the prefrontal cortex showing an increase in density GFAP-immunoreactive astrocytes with increasing doses of khat with relatively more cells and more exuberant branching in Group 2 (1000 mg/kg) and Group 3 (2000 mg/kg) (GFAP stain, ×400). GFAP: Glial fibrillary acidic protein

Administration of opioids such as morphine and amphetamines (which have structure and function almost similar to cathine and cathinone in khat) has been associated with an increase in astrocytes compared to controls. One study in 2002 on the astrocytes of rats' dentate gyrus under the influence of cocaine showed that GFAP expression in the affected cocaine group has increased compared to controls. Furthermore, in morphological and morphometric analysis, they were observed significant changes in size and number of astrocytes.[22] Hippocampal and dentate gyrus astrocytes significantly increased in number and complexity with subacute administration of morphine in Wistar rats, and this correlated with neurocognitive behavioral changes.[23]

GFAP has also been found to increase in many demyelinating and psychotic diseases,[24] and may at first be inhibited in alcoholics until late in neuronal disease when extensive neuronal damage occurs.[25]

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.[26-28] Whereas in the past it was thought that reactive astrogliosis was a uniformly negative

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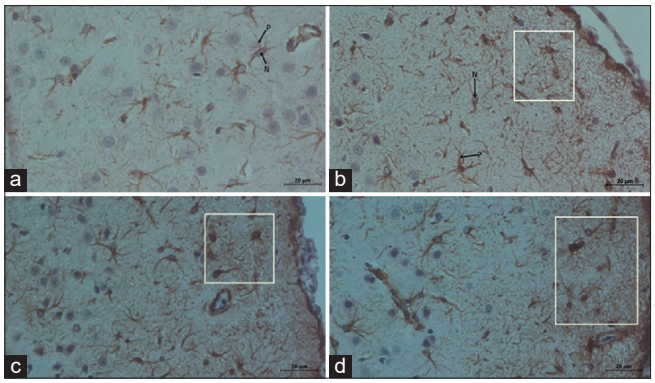

Figure 7: Photo micrograph of the prefrontal cortex of the control group (a). Note the appearance of a normal astrocyte having a relatively smaller nucleus (N) with indistinct nucleolus and fewer and shorter processes (P). In group 1 (b) (500mg/kg), the numerous astrocytes with stout ramifying processes (P). Nucleolus (N) become noticeable and evidence of mild astrogliosis is seen in the area delimited by a rectangle. While in group 2 (c) (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 (d) (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)

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,[29-31] protection from oxidative stress via glutathione production,[32,33] release of adenosine,[34] degradation of amyloid-beta peptides,[35] and repair of the blood–brain barrier.[36]

We noted an increase in astrogliosis, especially in rats fed on high doses of khat at 2000 mg/kg. 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 cement the damaged area by producing a glial scar.[36]

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.[37] Understanding neuroglial morphometric dynamics in this region is, therefore, a critical step in explaining the complex mechanisms that underly neurocognitive functional changes 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.[38]

Conclusion

Chronic use of khat at high doses causes an increase in GFAP immunoreactivity in astrocytes, associated gliosis, and increased complexity of astrocytes, implying a central role of astrocytes in cognitive changes associated with khat use.

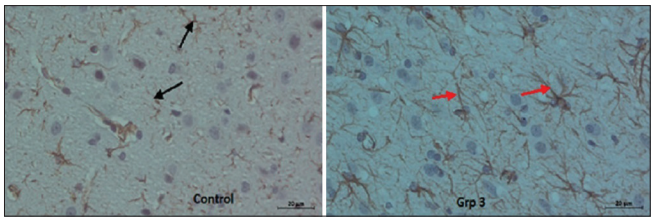

Figure 8: Photomicrographs comparing the astrocytic processes in control group and group 3 (2000 mg/kg). Note the increased complexity and thickness of astrocytic processes in the experimental group. The black arrows show astrocytic processes in the control group while the red arrows demonstrate increased complexity and thickness of astrocytic processes in group 3 rats (Magnification ×400, GFAP)

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Conflicts of interest

There are no conflicts of interest.

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