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Original Research Article

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

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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.
Results: Double-cortin immunoreactive neurons were observed in all animal groups, and an increase in the number and complexity of the neurons was noted in khat-fed rats compared to controls, with the highest concentration noted in low dose khat (500mg/kg) compared to controls ($p<0.04$). Higher doses of khat were associated with an increase in immunoreactive neurons, but no statistical difference compared to controls.
Conclusion: Khat use may cause an increase in immature neurons possibly due to neuronal damage with attempts at repair.

1. Introduction

Doublecortin (DCX), first described in X-linked lissencephaly, is associated with the arrest of migrating cerebral cortical neurons,[1,2] and is a microtubule-associated protein expressed during development by migrating neuroblasts.[3] It plays a critical role in microtuble stabilization[4] and nuclear translocation during neuronal migration.[5] Initially thought to be only expressed in the areas of active neurogenesis such as the subventricular zone and the hippocampus,[3] it is now known to express in the cerebral cortex indicating that these cells might be developing interneurons.[6]

Cells expressing the marker of immature neurons (DCX) in the neocortex may be critical in glia-to-neuron signalling mediating synaptic and metabolic plasticity.[7] 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.[3,8] Dcx-protein levels reflect high motility and/or structural plasticity of a given cell or a given brain region.

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Khat use is associated with neurocognitive perturbations in chronic use. The role of DCX-immunopositive cells, and their differential expression might be critical in determining the plasticity and high motility of the affected cells. Recent literature favor a strong neuro-regulatory role for DCX in brain besides neurogenesis. Studying the changes in histomorphometry of these neurons in the cortex may therefore shed some light on the role they play and how they signify brain changes associated with chronic khat use.

2. Materials and Methods

2.1. Experimental animals

Young adult male Wistar rats (Rattus Norvigecus) aged 2-3 months and weighing 200-300 grams 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 divided into four experimental clusters of 11 each. The first group served as the control and were fed on a normal diet and 10ml/kg normal saline water as control. The other 3 groups were fed on a once-daily Khat extract at three different doses: 500mg/kg (K500), 1000mg/kg (K1000) and 2000mg/kg (K2000) for a period of 6 weeks, by oral gavage.

The research protocol with animal experimentation was approved by the Biosafety, Animal use and Ethics committee of the University of Nairobi.

2.2. Khat extraction

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

2.3. 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 0.1M Phosphate buffer. Brains were quickly removed from the skulls and placed in buffered formaldehyde and embedded in paraffin blocks.

Histological sections 1mm thick were cut from the medial prefrontal cortex using the atlas of Paxinos and Watson,[9] with the olfactory tract intact. One out of 20 sections were randomly selected for staining from each animal group. Haematoxylin and Eosin was used to stain brain sections in all groups to visualize the light microscopic arrangement of layers.

2.4. Doublecortin staining

On the first day, the sections were deparaffinized in xylene with two washes of 10 minutes each. They were then rehydrated through graded alcohol, washed in running tap water and antigen retrieval performed using citrate buffer pH 6 overnight in a waterbath at 60^0C.

On the second day, sections were cooled, rinsed in Phosphate buffer solution (PBS), and endogenous peroxidase blocked with 1% hydrogen peroxide in methanol. The slides were washed in PBS buffer twice for 5 minutes, then incubated in a moist chamber in 5% normal goat serum for 30 minutes. They were then incubated overnight in DCX primary antibody (dilution of 1:100). They were washed thrice in PBS, incubated in secondary antibody (biotinylated goat antirabbit, Vector labs 1:1000) for 30 minutes, washed again in PBS and incubated for 30 minutes in avidin-biotin-complex, washed an incubated in diaminobenzoate (DAB) working solution for 5 minutes. After counterstaining with hematoxylin, they were hydrated in graded alcohols and mounted ready for viewing.

2.5. Histomorphometric analysis

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. This method is illustrated in Figure 1 and 2 below.

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

2.6. 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

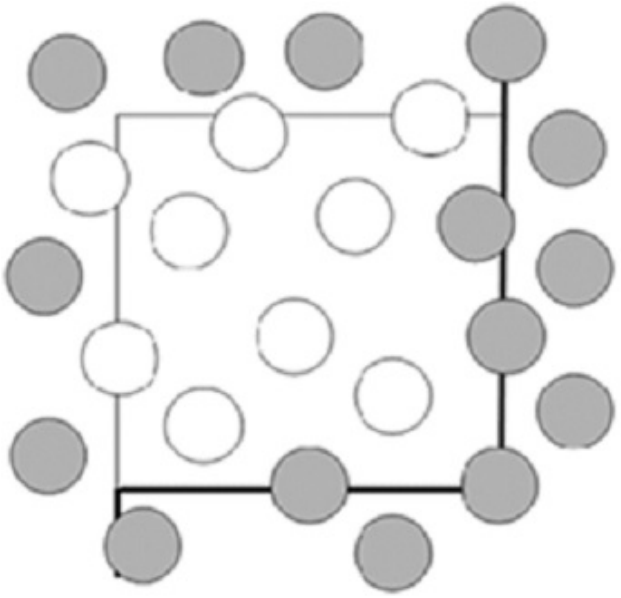


Fig. 1: An illustration of the cell counting method. The bold lines represent the exclusion zone while the lighter lines represent the inclusion zone. All cells within the area bounded and touching the inclusion zone were counted

was the immature DCX positive pyramidal neuronal densities (cells/1,000um^2). Descriptive statistics such as mean, standard deviation, median, interquartile ranges, standard error of the mean were determined for each of the study groups. Due to the inequality in the control and experimental groups, non-parametric tests were run. The Kruskal Wallis H test was used for comparison across the four study groups (control, experimental 1 (500 mg/kg), experimental 2 (1000 mg/kg) and experimental 3 (2000 mg/kg)).

3. Results

Across the four experimental groups, DCX-immunopositive 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.

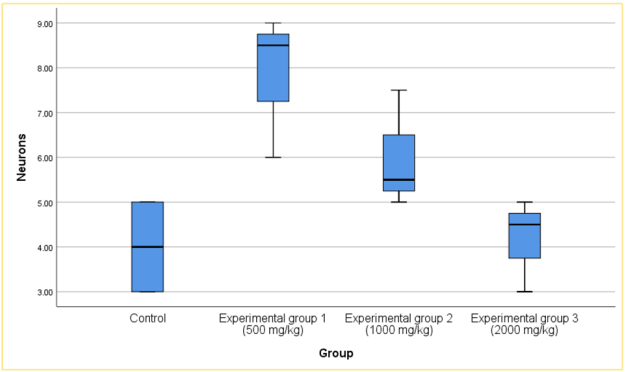
Some cells had sturdy neuronal processes, with occasionally double processes from opposite ends. In some cases, the processes appeared to establish a relationship with distant cells.

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 1).

Table 1: Showing the densities of DCX-positive immature neuronal density in controls and experimental groups

Group	DCX+ Neuronal Density (cells/1000 μm2)		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	

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 3). 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 and box plots below (Graph 1).



Graph 1: Box plots of the DCX-positive pyramidal neuronal densities in each study group

4. Discussion

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

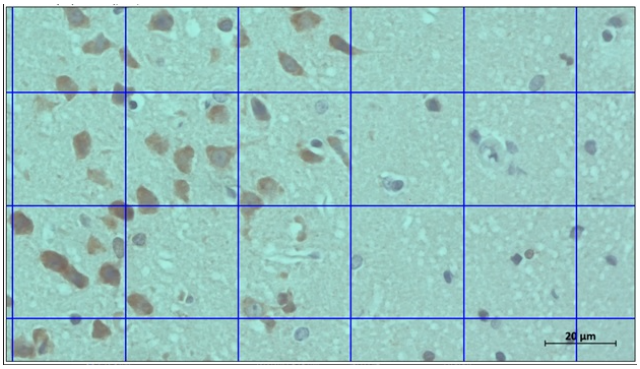


Fig. 2: Figure displaying the grids used to determine the immature neuronal densities

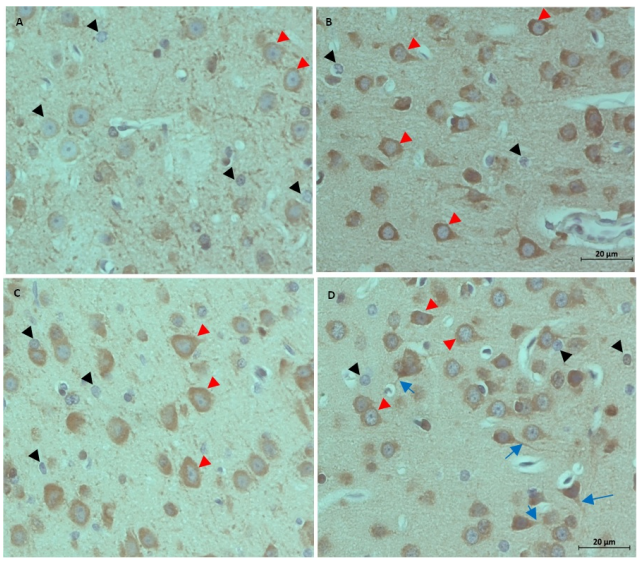


Fig. 3: Prefrontal cortex of the control group showing abundance of pyramidal neurons (black arrow heads) that do not take up the DCX stain (arrowheads), **B:** Shows group 1 (500 mg/kg) with increased DCX+ Pyramidal cells with brown staining of the cytoplasm within the cell body(red arrowheads) and less DCX-negative neurons (black arrowheads). **C:** Demonstrates prefrontal cortex of the experimental group 3 (1000mg/kg) showing a reduction in number of the DCX- Pyramidal cells (blackarrow heads) in comparison to the control group, and lower density of DCX+pyramidal neuronal cells (red arrowheads) when compared to experimental group 1. In the 3^{rd} group (**D**), note the reduced density of DCX+ cells (redarrow heads) 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

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

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.[10] Since DCX is thought to be expressed by immature migrating and proliferating cells,[3,5] 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.[3,11]

It is already widely reported that increasing doses of khat induce astrogliosis.[12–14] Some of the DCX-positive cells may be destined to become astrocytes and not neurons.[15] It is therefore possible that increased DCX positivity in the prefrontal cortex may also correlate with attempts at repair and increased astrogenesis.

5. Conclusion

The present study demonstrates strong DCX positivity in the prefrontal cortex of the male rat, with increase in DCX-immunoreactive staining cells with khat use. This forms a basis for understanding dynamics of adult neurogenesis and the role of immature neurons in drug use states.

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7. Conflict of Interest

The authors declare no conflict of interest.

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