EVALUATION OF CA2 NEURONAL DENSITY IN RATS WITH EXOGENUS *CANNABIS SATIVA* LEAVES AQUEOUS AND ALCOHOLIC EXTRACTS

Maryam Tehranipour* and Maryam Kehtarpour

Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran.

Summary

The endogenous cannabinoid system has been shown recently to play a crucial role in the extinction of aversive memories. In *Cannabis sativa* was found more than sixty one substance that cannabinoid was named. The aim of this study is the effect of leaves aqueous and alcoholic extracts of *Cannabis sativa* on CA2 neuronal density of hippocampus in male rats. Forty male Wistar Rats weight (300-350 g) were completely divided into four experimental groups and one control group. *Cannabis sativa* was extracted with Soxhlet apparatus. aqueous and alcoholic extract was injected peritonealy (I.P.) in experimental groups with dosage (50 mg kg^{-1}, 25 mg kg^{-1}) for three week. After one month, Animal was decapitated and their brain dissected, fixed in 10% formalin, sectioned in 7µm thickness and stained by H.E. By applying stereological techniques and systematic random sampling scheme the neuronal density of CA2 were estimated. Statistical analyses showed significant decrease ($p<0/001$) in the CA2 neuronal density in all experimental groups except alcoholic extract dose (25mgkg-1) in compare to control groups.

Key words: CA2, Hippocampus, Cannabis sativa

Running title: the effect of leaves extracts of *Cannabis sativa* on CA2 neuronal density

Corresponding Author: Tehranipour Maryam, Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran. Tel: +98511835050 Fax: +985118424020E mail: maryam_tehranipour@mshdiau.ac.ir

Introduction

Cannabinoids, the principal psychoactive constituents of marijuana, have a wide range of effects on the CNS, including loss of concentration, impairment of memory, enhancement of sensory perception, and mild euphoria(1). Brain regions that participate in the regulation and coordination of motor activity are densely populated with CB_1 receptors (2). The basal ganglia, cerebellum and related structures are enriched with CB_1 receptors and could therefore mediate the powerful motor effects of cannabinoids (3). The hippocampus is an important structure for memory processing. It is a particularly vulnerable and sensitive region of the brain that is also very important for declarative and spatial learning and memory (4). Hippocampus neurons are vulnerable to seizures, strokes, and head trauma, as well as responding to stressful experiences. At the same time they show remarkable plasticity, involving long-term synaptic potentiation and depression, dendrite remodeling (synaptic turnover, and neurogenesis in the case of the dentate gyrus (5).The hippocampus has been implicated in certain short-term memory.

Indeed hippocampus lesions often produce short-term memory deficits (6).The hippocampus is preferentially susceptible to a wide variety of toxic insults and disease processes, including hypoxia-ischemia and hypoglycemia (7).Metabolic diseases such as diabetes and obesity have been associated with increased vulnerability to stress (8) and cognitive dysfunction (9).

Cannabis is an annual, dioeciously, flowering herb. The leaves are palmate compound or digit ate, with serrate leaflets. *Cannabis* normally has imperfect flowers, with staminate "male" and pistil late "female" flowers occurring on separate plants (10).*Cannabis* plants produce a unique family of terpeno-phenolic compounds called cannabinoids, which produce the "high" one experiences from smoking marijuana. The two cannabinoids usually produced in greatest abundance are cannabidiol (CBD) and/or Δ^9-tetrahydrocannabinol (THC), but only THC is psychoactive (11).A synthetic form of the main psychoactive cannabinoid in *Cannabis*, Δ^9-tetrahydrocannabinol (THC), is used as a treatment for a wide range of medical conditions (12).

In the United States there has been considerable interest in its use for the treatment of a number of conditions, including glaucoma, AIDS wasting, neuropathic pain, treatment of spasticity associated with multiple sclerosis, and chemotherapy-induced nausea," the agency has not approved "medical marijuana"(13).Clinical trials have shown the efficacy of cannabis as a treatment for cancer and AIDS patient (14).Potential therapeutic uses of cannabinoid receptor agonists include the management of spasticity and tremor in multiple sclerosis/spinal cord injury, pain, inflammatory disorders, glaucoma, bronchial asthma, cancer, and vasodilatation that accompanies advanced cirrhosis. CB (1) receptor antagonists have therapeutic potential in Parkinson's disease (14).Cannabinoids, such as marijuana, are known to impair learning and memory perhaps through their actions in the hippocampus where cannabinoid receptors are expressed at high density. Although cannabinoid receptor activation decreases glutamatergic synaptic transmission in cultured hippocampus neurons, the mechanisms of this action are not known. Cannabinoid receptor activation also inhibits calcium channels that support neurotransmitter release in these cells, making modulation of these channels a candidate for cannabinoid-receptor-mediated effects on synaptic transmission (15). The aim of this study is the effect of leaves aqueous and alcoholic extracts of *Cannabis sativa* on CA2 neuronal density of hippocampus in male rats.

Materials and methods

All experiment was conducted in faculty of science, Islamic Azad University of Mashhad, Iran (2010-2011).

Animal subjects:

Forty male, Wistar rats weighting between 300-350 g served as subjects for these experiments. All animals were housed individually and maintained on a 12/12 light/dark cycle, with lights on at 6.00h. Ambient temperature in the animal facility was kept at 22±2C$^{\circ}$ .Food and water was given ad libitum.

Extraction

Cannabis sativa was collected from a reign around Mashhad and was coded with Islamic Azad University of Mashhad, Iran herbarium (herbarium code 2521). For extraction 50g powder leaves with 300 cc methanol were mixed and extraction performs with Soxhlet apparatus (16). For aqueous extract 300 cc pure water as dissolver was used. After obtaining extract, it was situated in oven with temperature (45°±2°) for 48 hours to remove solvent.

Groups:

1. Controls (N=8)

2. Treatment with aqueous extract of Cannabis sativa leaves (25mgkg $^{-1}$, i.p., 3 time every week) (N=8)

3. Treatment with aqueous extract of Cannabis sativa leaves (50mgkg $^{-1}$, i.p. ,3 time every week) (N=8)

4. Treatment with alcoholic extract of Cannabis sativa leaves (25mgkg $^{-1}$, i.p.,3 time every week) (N=8)

5. Treatment with alcoholic extract of Cannabis sativa leaves (50mgkg $^{-1}$, i.p.,3 time every week) (N=8)

Surgery:

After one month animals were anesthetized under interaperitoneal injection of an initial dose of 60mg kg^{-1}ketamine and 6 mg kg^{-1} xylazine (ip) and decapitated. The whole brain was removed and fixed in 10% paraformaldehyde. NaCl was added to the fixative to make the tissue float in order to overcome deformities during the fixation period (17). Paraffin embedded tissue blocks were sectioned at 7mμ thickness coronally and stained. A uniform random sampling scheme was employed so that about 10 sections from each block were sampled. With each section thus selected its immediately preceding neighbor was also collected. Sections were stained with toluidine blue staining method with special buffer of acetic acid, sodium acetate and distilled water (pH 4.65). Neuronal density in CA2 was determined, using stereological counting technique; physical dissector (18).

The dissector principle was used to determine the numbers of neurons in each section. Form each section and it's adjacent neighbor two photos were taken, one from each section with a final magnification of 100. A two-dimensional unbiased counting frame was overlaid in a uniform, random manner on to regions of any two photos taken of both sections. Those cell nuclei selected by the frame on the reference plane but not appearing on the adjacent look-up frame section were deemed to have their tops in the volume described by the product of the area of the counting frame and the distance between sections. These nuclei were counted (Q) to provide the numerical density of cells (NV) in the ventral horns of 100-spinal cord according to the equation:

$$NV = \frac{\sum a}{\sum frame \times V_{di\,\mathrm{sec}\,tor}}$$

Where $\sum a$ is the sum of counted neurons, h is the depth of the dissector equal to the section thickness (7 micron) and a (frame) is the scaled area of the dissector frame (19).

Statistical analyze

The ratio of numerical density of neurons in samples of brain was then used as an index of neuronal death. All quantitative data were analyzed using ANOVA and t-test. Student's t test was used for comparison when only 2 groups were analyzed. Statistical significance was chosen as $p<0.05$.All results are reported as mean ± SEM.

Results

The effects of Cannabis sativa extracts on the numbers of intact neurons in the CA2 region of hippocampus after one month in rats are shown in (Fig.1, 2, 3).

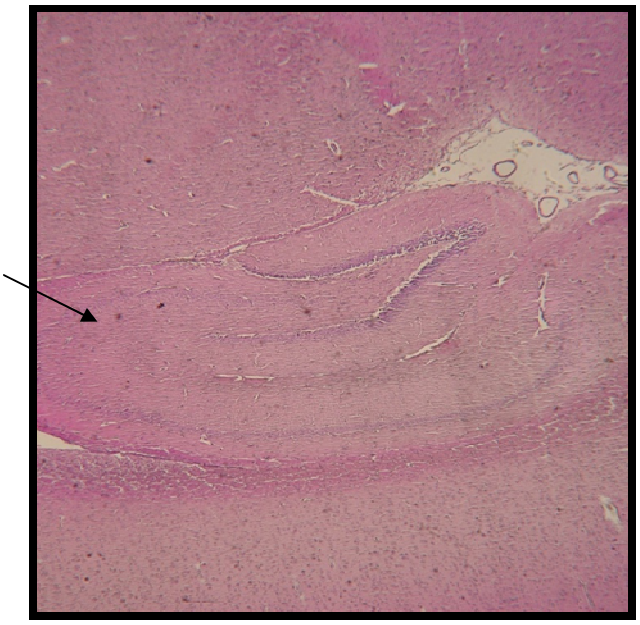

Fig.1.Photomicrograph illustrates neurons of the CA2 region of hippocampus at magnification of (20×). Spikes show the CA2.

Table.1: CA2 Neuronal cells density in different groups.

Data are presented as mean± S.E. (n=8 in each group).

Groups	NV (Mean ±S.E.)
Control	37396± 449
Treatment with aqueous extract(25mgkg-1)	14648 ± 284
Treatment with aqueous extract(50mgkg-1)	17147 ± 378
Treatment with alcoholic extract(25mgkg-1)	41446 ± 345
Treatment with alcoholic extract(50mgkg-1)	21339± 387

1-The control group revealed healthy neuronal cells amounted by (37394 ± 67) intact neurons.

2-The neuronal cells density in all experimental groups except alcoholic extract (25mgkg-1)dose decreased in compare with control group ($p<0.001$)(Table.1).

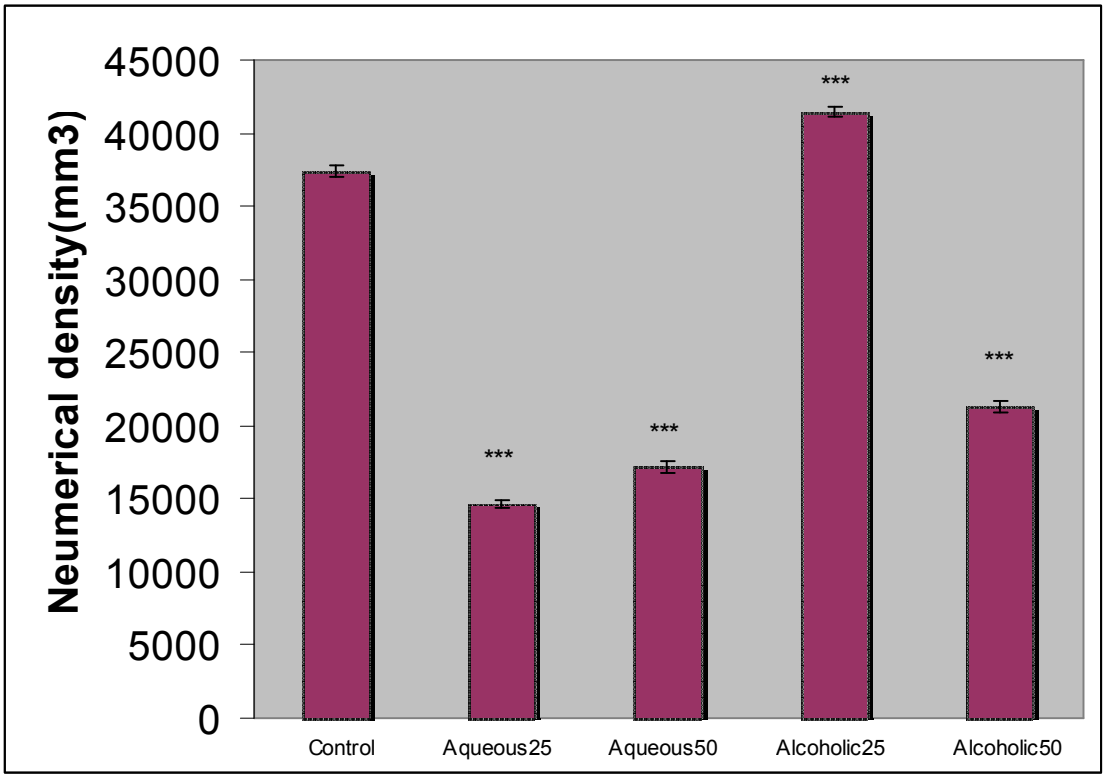

Fig.2: Effects of Cannabis sativa leaves extracts on the intact neurons numerical density of hippocampus CA2 region in rats. Results are expressed as Mean± SD of 8 rats and data were analyzed by one-way ANOVA followed by Tukey-kramer multiple comparisons test.

3- As has shown in fig.2, the effect of Cannabis sativa extracts resulted in massive neuronal damage manifested as a significant ($P<0.001$)50% decrease in the number of normal appearing neurons after one month.

4- Aqueous extracts have more degeneration effect on CA2 neuron in compare with alcoholic extracts (Fig.2).

5- Alcoholic extract with dose (25mgkg-1) has an increase effect on CA2 numerical density (Fig.2).

Discussion

Our findings demonstrate that exogenous cannabinoids play an important role in the degeneration and survival the cell. The data show that there is a remarkable change in the number of neurons in different groups. In control animals resulted in a significant ($P<0.001$) increase in the number of intact neurons, respectively as compared to treatments groups (Fig. 2). It means that in control group endogenous factor acts as a survival factor and suppresses the degeneration phenomena. So it is possible that ***cannabis sativa*** alcoholic and aqueous extracts have degeneration effect on central nervous system neurons and the results of this research show this hypothesis. It was shown that the neuronal density in all treatment groups decreased in compare with control group (Table.1).But in treatment group with alcoholic extract (25mgkg-1) the neuronal density was increased in compare with control group (Fig.2). May be low doses of alcoholic extract (25mgkg-1) has neurogenic effect and make change the media for regenerating neurons or survival the cell. It is not clear that how this extract induces nerogenesis but data show that the numerical density of CA2 in this group was increased.

Cannabinoids are known to inhibit stimulus-evoked synaptic transmission in several brain regions such as hippocampus (20). Cannabinoids inhibit GABAergic synaptic transmission in different brain regions (21). These results indicate that, in the brain, cannabinoids modulate both excitatory and inhibitory synaptic transmission via CB1. WIN-2-induced changes of paired-pulse ratio and of spontaneous and miniature postsynaptic currents suggest a presynaptic site of action. Inhibition of G(i/o) proteins and blockade of voltage-dependent and G protein-gated inwardly rectifying K(+) channels inhibited WIN-2 action on basal synaptic transmission(22). In contrast, modulation of the adenylyl cyclase-protein kinase A pathway, and blockade of presynaptic N- and P/Q- or of postsynaptic L- and R/T-type voltage-gated Ca(2+) channels did not affect WIN-2 effects. Our results indicate that the mechanisms underlying cannabinoid action in the LA partly resemble those observed in the nucleus accumbens and differ from those described for the hippocampus (22).

Also, it is possible that cannabinoids depress the corticostriatal glutamatergic synaptic transmission through the activation of presynaptic CB1 receptors to inhibit N-type Ca2+ channel activity, which in turn reduces glutamate release. The presynaptic action of cannabinoids is mediated by a PTX-sensitive Gi/o protein-coupled signaling pathway (23). It is clear that, Cannabis consumption results in impaired learning. The proper synchronization of neuronal activity in the mammalian hippocampus gives rise to network rhythms that are implicated in memory formation (24).

Generally, the results of present study indicated that ***cannabis sativa*** alcoholic and aqueous extracts have degeneration effects on central nervous system neurons especially CA2 regain of hippocampus. But just low dose of alcoholic extract (25mgkg-1) has neurogenic effect and could protect neurons from death.

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