THE EFFECT OF *CANNABIS SATIVA* LEAVES AQUEOUS AND ALCOHOLIC EXTRACTS ON CA1 NEURONAL DENSITY IN RATS

Maryam Kehtarpour and Maryam Tehranipour*

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

Summary

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. 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 CA1 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 CA1 were estimated. Statistical analyses showed significant decrease ($p<0/001$) in the CA1 neuronal density in all experimental groups in compare to control groups. *Cannabis sativa* leaves aqueous and alcoholic extract effectively block the release of k^{+} and induced neuronal degeneration in CA1.

Key words: CA1, stereo logy, Cannabis sativa

Running title: The effect of leaves extracts of *Cannabis sativa* on CA1 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

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 (1).

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 (2).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(3).

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"(4).Clinical trials have shown the efficacy of cannabis as a treatment for cancer and AIDS patient (5).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 (5)

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 (6). 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 (7).The hippocampus has been implicated in certain short-term memory. Indeed hippocampus lesions often produce short-term memory deficits (8).The hippocampus is preferentially susceptible to a wide variety of toxic insults and disease processes, including hypoxia-ischemia and hypoglycemia (9).Metabolic diseases such as diabetes and obesity have been associated with increased vulnerability to stress (10) and cognitive dysfunction (11).

Cannabinoids display a variety of central effects such as impairment of hippocampus-dependent learning and memory, modulation of emotional states and analgesia (12). They inhibit stimulus-evoked

synaptic transmission in several brain regions such as the hippocampus (13), nucleus accumbens and prefrontal cortex (14) predominantly via presynaptic mechanisms. Most behavioral effects of cannabinoids are mediated by the cannabinoid receptor type 1 (CB1)(15). Activation of the $G_{i/o}$ protein-coupled CB1 inhibits the adenylyl cyclase-protein kinase A (AC-PKA) pathway and modulates Ca^{2+} and K^{+} conductance (16). CB1 is widely distributed throughout the central nervous system. Among other brain regions, CB1 is highly expressed in the amygdala (17), which is an integral component of the limbic circuitry.

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 hippocampal 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 (18). The aim of this study is the effect of leaves aqueous and alcoholic extracts of *Cannabis sativa* on CA1 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 (19). 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 (20). 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 CA1 was determined, using stereological counting technique; physical dissector (21).

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 (22).

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 CA1 region of hippocampus after one month in rats are shown in (Fig.1, 2,3).

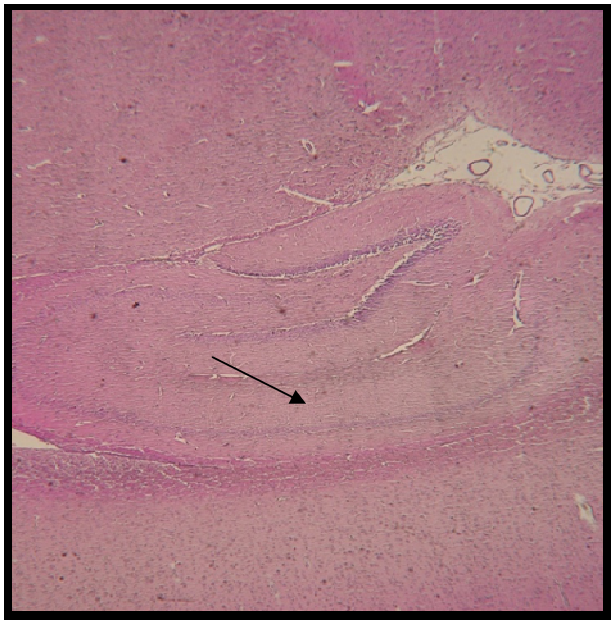

Fig.1.Photomicrograph illustrates neurons of the CA1 region of hippocampus at magnification of (20×). Spikes show the CA1.

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

2-The neuronal cells density in all experimental groups decreased in compare with control group (p<o.o01) (Table.1).

Table.1: CA1 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± 67
Treatment with aqueous extract(25mgkg-1)	10081 ±25
Treatment with aqueous extract(50mgkg-1)	10986 ±51
Treatment with alcoholic extract(25mgkg-1)	20598 ±45
Treatment with alcoholic extract(50mgkg-1)	13379± 60

2- 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.

3- Aqueous extracts have more degeneration effect on CA1 neuron in compare with alcoholic extracts (Fig.2).

4- Alcoholic extract with dose (25mgkg-1) has less degeneration effect on CA1 numerical density (Fig.2).

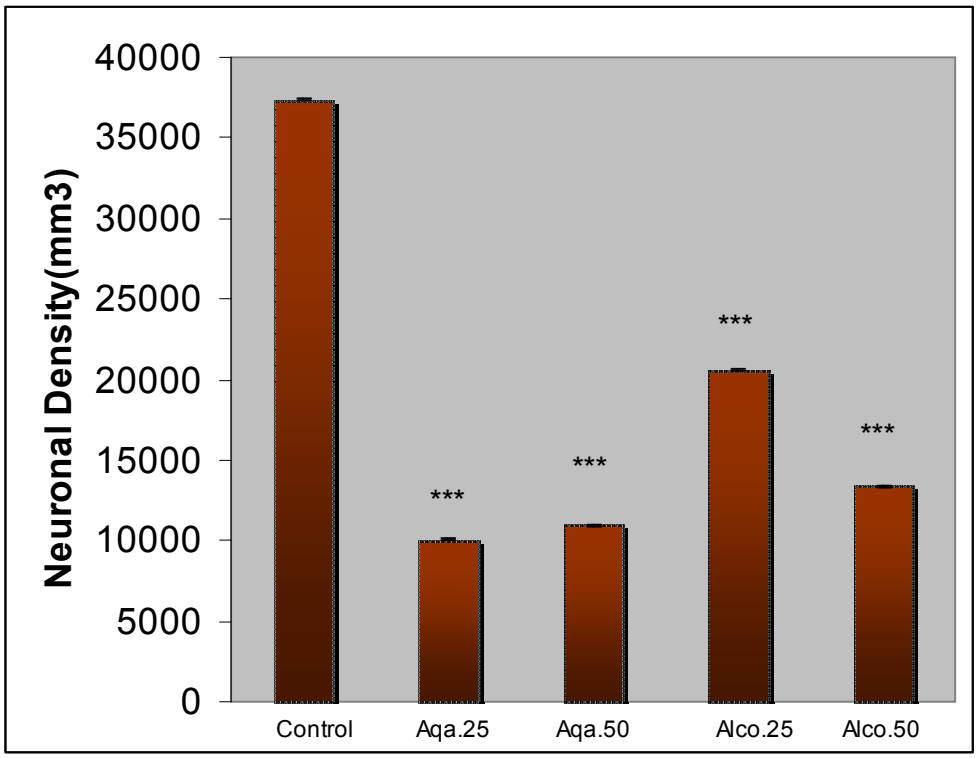

Fig. 2: Effects of Cannabis sativa leaves extracts on the intact neurons numerical density of hippocampus CA1 region in rats. Results are expressed as Mean± SD of 8 rats and data were analyzed by one-way ANOVA followed by Tukey-kramer multiple comparisons test.

Discussion

The endogenous cannabinoid system has been shown recently to play a crucial role in the extinction of aversive memories (23).The hippocampus is especially important because it has been shown to be involved in certain aspects of learning and memory (24) the degeneration of which are primary effects of Alzheimer's. The reasons for this degeneration are many-fold.

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

neuron 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 less decreased in compare with other groups (Fig.2)

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 (25). 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 (26).

Further experiments need to identify the molecular mechanisms underlying this cannabinoid-receptor-mediated decrease in neurotransmitter release. Cannabinoid receptor activation had no effect on the size of the presynaptic pool of readily releasable neurotransmitter-filled vesicles, eliminating reduction in pool size as a mechanism for cannabinoid-receptor-mediated effects. After blockade of Q- and N-type calcium channels with omega-agatoxin TK and omega-conotoxin GVIA; however, activation of cannabinoid receptors reduced EPSC size by only 14%. These results indicate that cannabinoid receptor activation reduces the probability that neurotransmitter will be released in response to an action potential via an inhibition of presynaptic Q- and N-type calcium channels. This molecular mechanism most likely contributes to the impairment of learning and memory produced by cannabinoids and may participate in the analgesic, antiemetic, and anticonvulsive effects of these drugs as well (27).

Generally, Cannabis consumption results in impaired learning. The results of present study indicated that ***cannabis sativa*** alcoholic and aqueous extracts have degeneration effects on central nervous system neurons especially CA1 regain of hippocampus.

Acknowledgments

Authors would like to thanks the Islamic Azad university Mashhad branch for financial Supports.

References

1. Lebel-Hardenack S, Grant S R. Genetics of sex determination in flowering plants. Trends in Plant Science (1997); 2(4): 130–136.

2. Small E, Beckstead HD. Common cannabinoid phenotypes in 350 stocks of Cannabis. Lloydia (1973); 36: 144–165.

3. Costa B. "On the pharmacological properties of Delta9-tetrahydrocannabinol (THC)". Chemistry & Biodiversity (2007); 4 (8): 1664–77.

4. King SA . Cannabinoids and Pain. Psychiatric Times (2008); 25 (2): 56-59.

5. Alsasua del val A. Implication of cannabinoids in neurological disease .Cellular and molecular neurobiology (2006); 26:571-591.

6. Artola A. Diabetes, stress and ageing-related changes in synaptic plasticity in hippocampus and neocortex the same metaplastic process? Eur J Pharmacol.(2008); 6; 585(1):153-62.

7. McEwen BS. Stress and the aging hippocampus. Front Neuroendocrinol. (1999); 20(1):49-70.

8. Mumby DG, Gaskin S, Glenn MJ, Schramek TE, Lehmann H. Hippocampal damage and exploratory Preferences in rats: memory for objects, places, and contexts. Learn Mem.(2002); 9(2):49-57.

9. Piotrowski P, Wierzbicka K, Smiałek M. Neuronal death in the rat hippocampus in experimental diabetes and cerebral ischaemia treated withantioxidants. Folia Neuropathol.(2001); 39(3):147-54.

10. Damasceno DC, Volpato GT, de Mattos Paranhos Calderon I, Cunha Rudge MV. Oxidative stress and diabetes in pregnant rats. Anim Reprod Sci.(2002); 15;72(3-4):235-44.

11.Nazer J, Ramírez R.Congenital malformations in the offspring of diabetic mothers. Rev Med Chil.(2000); 128(9):1045-52.

12. Hajos N, Ledent C, Freund TF. Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. Neuroscience. (2001); 106:1–4.

13. Misner DL, Sullivan JM. Mechanism of cannabinoid effects on long-term potentiation and depression in hippocampal CA1 neurons. J Neurosci. (1999); 19:6795–6805

14. Auclair N, Otani S, Soubrie P, Crepel F. Cannabinoids modulate synaptic strength and plasticity at glutamatergic synapses of rat prefrontal cortex pyramidal neurons. J Neurophysiol.(2000);83 :3287–3293.

15. Ledent C, Valverde O, Cossu G, Petitet F, Aubert JF, Beslot F, Bohme GA, Imperato A, Pedrazzini T, Roques BP, et al. Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. Science. (1999); 283:401–404

16. Deadwyler SA, Hampson RE, Mu J, Whyte A, Childers S. Cannabinoids modulate voltage sensitive potassium A-current in hippocampal neurons via a cAMP-dependent process. J Pharmacol Exp Ther. (1995); 273:734–743

17. Marsicano G, Lutz B. Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. Eur J Neurosci. (1999); 11:4213–4225

18. Sullivan JM. Mechanisms of cannabinoid-receptor-mediated inhibition of synaptic transmission in cultured hippocampal pyramidal neurons. J Neurophysiol. (1999); 82(3):1286-94

19. Cicchetti E, Chaintreau A. Comparison of extraction techniques and modeling of accelerated solvent extraction for the authentication of natural vanilla flavors.J Sep Sci (2009);32(11):1957-64

20- Behnam-Rasouli M, Nikravesh M, Mahdavi N and Tehranipour M. Post-treatment time after sciatic nerve crush on the number of alpha motoneurons, using a stereological counting method (disector). Iranian Biomedical Journal (2000); 4(1): 45–49.

21- Tehranipour M, Khakzad M.Effect of mathernal diabetes on hippocampus Neuronal density in neonatal rats.J. of biological Sciences.(2008);8(6).1027-1032.

22- Gundersen HJG, Bendtsen TF and et al. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS (1988); 96(5):379-394.

23. Manzoni OJ, Bockaert J. Cannabinoids inhibit GABAergic synaptic transmission in mice nucleus accumbens. Eur J Pharmacol. (2001); 412:R3–R5

24. Barnes CA, Rao G, Foster TC, McNaugton BL. Region-specific age effects on AMPA sensitivity: electrophysiological evidence for loss of synaptic contacts in hippocampal field CA1. Hippocampus (1992); 2:457–468.

25. Shahnaz Christina Azad,[1,2,4] Matthias Eder,[1] Giovanni Marsicano,[3] Beat Lutz,[3]Walter Zieglgänsberger,[1] and Gerhard Rammes[1] Activation of the Cannabinoid Receptor Type 1 Decreases Glutamatergic and GABAergic Synaptic Transmission in the Lateral Amygdala of the Mouse. Learn Mem. (2003); 10(2): 116–128.

26. Maier N, Morris G, Schuchmann S, Korotkova T, Ponomarenko A, Böhm C, Wozny C, Schmitz D. Cannabinoids disrupt hippocampal sharp wave-ripples via inhibition of glutamate release. Hippocampus. (2011)18. doi: 10.1002/hipo.20971. [Epub ahead of prin

27. Sullivan JM. Mechanisms of cannabinoid-receptor-mediated inhibition of synaptic transmission in cultured hippocampal pyramidal neurons. J Neurophysiol. (1999);82(3):1286-94