საქართველოს მეცნიერებათა ეროვნული აკადემიის მოამბე, ტ. 14, №3, 2020
BULLETIN OF THE GEORGIAN NATIONAL ACADEMY OF SCIENCES, vol. 14, no. 3, 2020

Human and Animal Physiology

The Study of Antinociceptive Tolerance to Cannabinol Microinjected into Central Nucleus of Amygdala

Nana Tsiklauri[*], Natia Tsagareli[**], Ivliane Nozadze[§], Gulnaz Gurtskaia[*], Merab G. Tsagareli[*]

[*] *Department of Pain and Analgesia, Iv. Beritashvili Center of Experimental Biomedicine, Tbilisi, Georgia*
[**] *Department of Physiology, Tbilisi State Medical University, Tbilisi, Georgia*
[§] *Ivane Javakhishvili Tbilisi State University, Tbilisi, Georgia*

(Presented by Academy Member Nodar Mitagvaria)

The biological effects of cannabinoids, the major constituents of the ancient medicinal plant *Cannabis sativa* are mediated by two members of the G-protein coupled receptor family, cannabinoid receptors 1, CB1 and CB2. It has been discovered that cannabinoid receptors are expressed in the amygdala, and their activity contributes to the dissociative effect of cannabis on pain perception. There are many studies examining the effects of cannabinoids in relief of acute and chronic inflammatory pain, and many researches try to prove high efficiency and demonstrate new capabilities of these drugs in pain treatment, but much less is known about their side effects like tolerance after repeated administration for long-term treatment. Therefore it was very interesting to figure out if repeated microinjections of cannabinoids into the central nucleus of amygdala would induce tolerance effects to them. Here we found that microinjection of Δ^9-tetrahydrocannabinol (THC) into the central nucleus of amygdala produced antinociception, as detected by increased latency as compared to the baseline and to the control group with saline. On subsequent four days THC microinjections caused progressively less antinociception, so that by day 4 there was no effect. In this paper, thus, we have shown that a long-term use of THC cause development of tolerance toward it, which is one of the side effects of cannabinoids, and drugs used for treatment should be limited. © *2020 Bull. Georg. Natl. Acad. Sci.*

Analgesia, antinociception, cannabinoid, pain, tail-flick, hot-plate

The plant *Cannabis sativa* has long been used for medical purpose throughout human history. The first record can be traced back to ancient China around 5000 years ago, where extracts of the plant were used for relief of cramps and pain. The widely uses of *Cannabis sativa* included antinociception, anti-inflammatory, and anti-convulsant effects [1,2]. Not until a half century ago, the first light was shed on the myth of the versatility of *Cannabis sativa* by the discovery of Δ^9-tetrahydrocannabinol (THC) [3]. There are a number of factors relevant to the chronic diseases that pertain to the cannabinoid system. Endocannabinoid molecules are produced by breakdown of phospholipids, and may constitute the endogenous anti-inflammatory pathway [4]. Furthermore, a wide range of

behavioral studies have shown that cannabinoids produce antinociception in animal models of acute pain, the naturally occurring THC inhibited responses to noxious thermal and mechanical stimuli in the hot-plate (HP), tail-flick (TF) and paw pressure tests [5,6]. It has been shown that one of endocannabinoid ligands anandamide is also an effective against in more persistent nociceptive processes, since its administration results in reducing thermal and mechanical hyperalgesia and mechanical allodynia following peripheral inflammation [7]. Cannabinoid (CB1) receptors are expressed in key nociception areas including the periaqueductal grey, the dorsal horn of the spinal cord and dorsal root ganglion neurons [8]. It has been discovered that cannabinoid receptors are expressed in the amygdala [9,10], and their activity contributes to the dissociative effect of cannabis on pain perception [11]. Pain having a strong affective and emotional dimension and the amygdala, especially its central nucleus (CeA), has also emerged in the last twenty years as key element of the pain matrix. The CeA receives nociceptive information from the brainstem. Hyperactivity in the latero-capsular division of the CeA also termed as the "nociceptive amygdala" [11,12]. In our investigations also have shown the role of CeA in pain perception, microinjections of NSAIDs into the CeA induced antinociception, and repeated administration led to development of tolerance to these non-opioids [13,14,]. In the other study, administration of CB1 receptor antagonist AM-251 into the CeA blocked developing of antinociception to NSAIDs [15].

There is now considerable evidence supporting a role for cannabinoids in antinociception, however, much less is known about cannabinoids side effects like tolerance, hence, studying the mechanisms of cannabinoid actions is very actual and important for pharmacological and clinical practice. Here we report antinociceptive tolerance effects induced by THC injected into CeA for four consecutive days in rats.

Materials and Methods

Animals. The research was carried out on adult male Wistar rats weighing 200-250 g, bred at the Beritashvili Center for Experimental Biomedicine (BMC). The animals were kept under standard housing conditions (22 ± 2ºC, 65% humidity, and light from 7:00 a.m. to 8:00 p.m.) and kept on a standard dry diet with water freely available. Every effort was made to minimize both the number of animals used and their suffering. Six rats were used for each experimental and control groups. The local Bioethic Committee of the BMC approved the experimental protocols, adhering to the Guidelines of the International Association for the Study of Pain regarding investigations of experimental pain in conscious animals.

Surgical procedures. Under anesthesia with intramuscular administration of ketamine (100 mg/kg, "KharkovPharm", Ukraine), a 12-mm-long stainless steel guide cannula (Small Parts, Inc., USA) was stereotaxically implanted bilaterally into the CeA according to the coordinates in the atlas of Paxinos and Watson [16]. The guides were anchored to the cranium by dental cement. The guide cannula was plugged with a stainless steel stylet. Thereafter, the animals were handled every day for 2-3 days for 15-20 min to get familiar with the testing protocol and experimental environment. During this time, the stylet was removed and 14 mm-long stainless steel microinjection cannula was inserted into the guide cannula to reach the CeA, but no drug was injected. Five days after surgery the microinjection cannula, attached to Hamilton syringe (Hamilton, Inc., USA), was joined to the guide cannula, and the drug was introduced through it while the rat was gently restrained.

Drugs. TCH 0.05 μg in 0.5 μl solution (100 μg dissolved in 1 ml heptanes, Sigma Aldrich.USA) was injected through the microinjected cannula as we used previously [13-15]. The guide cannula was then plugged with a stainless steel styled. Isotonic saline was injected in the same volume 0.5 μl,

(GalichPharm, Ukraine) and manner in a separate group of rats for controls. Solutions were microinjected in about 10-15 seconds.

Behavioral testing. Twenty minutes post microinjection of TCH or saline into the CeA 10-min before the peak of the drugs' effect is normally reached, rats were tested for antinociception. For the TF test the distal part of the tail was stimulated with a light beam induce TF reflex (33 Tail Flick Analgesia Meter, IITC, USA), and the latency measured until the tail was reflexively flicked away from the beam. The holding temperature was 45°C and the cut-off time was 20 s. Only a single stimulus to the tail was applied daily for each animal. For the HP test, the rat was placed on a 55°C hot plate (39 Hot Plate Analgesia Meter, IITC, USA) and the latency of paw lick or jump was measured. The cut-off time was 20 s. The Paw Pressure (PP) test nociceptive withdrawal threshold was estimated by using the Randall-Selitto electronic algesimeter (digital analgesia-paw pressure meter, #37215, Ugo Basile. Italy). Before testing, each animal received 5 min of handling to get used to manipulation; then it was carefully immobilized by hand used to hold the tested paw. The test consisted of the application of an increasing mechanical force, in which the tip of the device was applied onto the medial portion of the dorsal surfaces hind paws until a withdrawal response resulted. Each rat was tested for all latencies and threshold in the same session.

Histology. At the end of each set of experiments, the microinjection sites were marked with 2 μl of saturated solution of Pontamine Sky Blue (Sigma-Aldrich, USA) and the animal was euthanized with pentobarbital. After fixation by immersion in 10% formalin, the brain was sectioned and counter-stained with Cresyl Violet. The microinjection sites were histologically verified and plotted according to Paxinos and Watson (1997) stereotaxic atlas coordinates [16].

Statistics. All mean control and experimental groups' values are presented as mean ± S.E.M. One-way analysis of variance (ANOVA) with post-hoc Tukey-Kramer or Dunnett's multiple comparison tests were used for statistical evaluation of comparisons between treated and saline groups. The Kolmogorov-Smirnov test was applied to verify normality. The statistical software utilized was InStat 3.05 (GraphPad Software, San Diego, CA, USA). Differences between means of saline control and treated groups with TCH, were acknowledged as statistically significant if $P < 0.05$.

Results and Discussion

Microinjection of THC into the CeA produced antinociception, as detected by increased in the latency as compared to the baseline and to the control group with saline microinjections in CeA. ANOVA revealed statistical significant values for the THC in the TF [ANOVA, $F(4,25) = 35.309$, $P < 0.0001$, $n = 6$], the HP [ANOVA, $F(4,25) = 22.237$, $P < 0.0001$, $n = 6$], and mechanical (PP) (Randall-Selitto) [ANOVA, $F(4,55) = 84.603$, $P < 0.0001$, $n = 12$] tests, respectively, but not for the saline-control group in the TF [ANOVA, $F(4,25) = 0.1453$, $P = 0.9634$, not significant, $n = 6$], in the HP [ANOVA, $F(4,25) = 1.607$, $P = 0.2036$, not significant, $n = 6$], and in the PP [ANOVA, $F(4,55) = 1.171$, $P = 0.3338$, not significant, $n = 12$] tests, respectively.

Dunnett's multiple comparison test showed significant increase latencies compare to baseline values for TF reflex in the first ($t = 9.533$, $P < 0.01$), and the second experimental days ($t = 6.089$, $P < 0.01$) (Fig. A); for HP test in the first ($t = 7.786$, $P < 0.01$), and the second experimental days ($t = 3.923$, $P < 0.01$) (Fig. 1B); and for PP test in the first ($t = 14.780$, $P < 0.01$), the second ($t = 7.937$, $P < 0.01$), and the third days ($t = 2.596$, $P < 0.05$) (Fig. 1C). On subsequent days THC microinjections into CeA caused progressively less antinociception, so that by day 4 there were not analgesic effects of THC, i.e. developed tolerance in all three tests (Fig. 1).

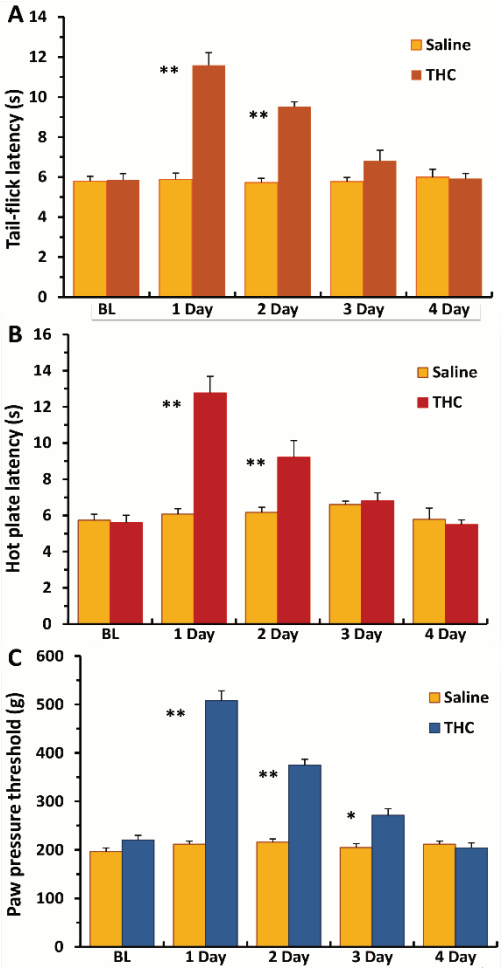

Fig. Antinociceptive tolerance to THC injected into CeA for the tail-flick (A), hot plate (B), and PP (Randall Selitto) (C) tests. Note the progressively decrease in latency and threshold for the consecutively four days develops tolerance to NSAIDs.

These findings are similar to our recent data showing that antinociceptive tolerance effects of NSAIDs injected into CeA are attenuated by combined injection of opioid receptor antagonist naloxone and cannabinoid CB1 receptor antagonist AM-251 [14], thereby confirm that spinal nociceptive reflexes in rats are mediated via descending opioid and cannabinoid modulatory system with key functions of periaqueductal grey matter and rostral ventro-medial medulla [13,17]. Recently we also have found that CB1 receptor antagonist AM-251 [15] and opioid receptor antagonists, naloxone and CTOP [18] attenuated NSAIDs-induced antinociceptive tolerance in the anterior cingulate cortex of rats, the second important pain matrix structure in the brain.

In conclusion, the presented work has shown that four days using of THC causes development of tolerance to this drug, therefore this undesirable side effect of THC chronic pain should be taken into account in clinical practice.

This research was supported by the grant from Shota Rustaveli National Science Foundation of Georgia to N. Tsiklauri (# YS17-53).

ადამიანისა და ცხოველთა ფიზიოლოგია

ამიგდალას ცენტრალურ ბირთვში კანაბინოლის მიკროინექციით გამოწვეული ანტინოციცეპციური ტოლერანტობის შესწავლა

ნ. წიკლაური*, ნ. ცაგარელი**, ი. ნოზაძე§, გ. ღურწკაია*, მ. ცაგარელი*

* *ივ. ბერიტაშვილის ექსპერიმენტული ბიომედიცინის ცენტრი, ტკივილისა და ანალგეზიის ლაბორატორია, თბილისი, საქართველო*
** *თბილისის სახელმწიფო სამედიცინო უნივერსიტეტი, ფიზიოლოგიის დეპარტამენტი, თბილისი, საქართველო*
§ *ივანე ჯავახიშვილის სახ. თბილისის სახელმწიფო უნივერსიტეტი, თბილისი, საქართველო*

(წარმოდგენილია აკადემიის წევრის ნ. მითაგვარიას მიერ)

სამკურნალო მცენარის, კანაფის (*Cannabis sativa*) მთავარი შემადგენელი კომპონენტების, კანაბინოიდების ბიოლოგიური ეფექტები განპირობებულია G-ცილასთან შეუღლებული CB1 და CB2 კანაბინოიდური რეცეპტორების გააქტივებით. ნაჩვენებია, რომ ამიგდალას ცენტრალურ ბირთვში კანაბინოიდური რეცეპტორების გააქტივება ცვლის ტკივილის შეგრძნებას. ასევე ცნობილია მწვავე და ქრონიკული ანთებითი ტკივილის მართვაში კანაბინოიდების მაღალი ეფექტურობა, მაგრამ მცირეა ცნობები მათი ისეთი გვერდითი ეფექტის შესახებ, როგორიცაა ტოლერანტობა. აქედან გამომდინარე, ძალიან საინტერესო იყო იმის გარკვევა, იწვევს თუ არა ამიგდალას ცენტრალურ ბირთვში კანაბინოლის განმეორებითი მიკროინექციები ტოლერანტობის განვითარებას. ჩვენ აღმოვაჩინეთ, რომ Δ^9-ტეტრაჰიდროკანაბინოლის (THC) მიკროინექცია ამიგდალას ცენტრალურ ბირთვში იწვევდა ანტინოციცეპციას, რაც გამოიხატებოდა ტკივილის შეგრძნების ლატენტური პერიოდისა და მექანიკური დაწოლის ზღურბლის გაზრდით, საცდელი ვირთაგვების ბაზისურ მონაცემებსა და საკონტროლო ცხოველების ჯგუფთან შედარებით. მომდევნო ოთხი დღის განმავლობაში, THC მიკროინექციით გამოწვეული ანტინოციცეპცია პროგრესულად მცირდებოდა ისე, რომ ექსპერიმენტის მეოთხე დღეს ლატენტური პერიოდის ხანგრძლივობა და მექანიკური გაღიზიანების ზღურბლი უტოლდებოდა ბაზისურ და საკონტროლო მაჩვენებლებს. წარმოდგენილი კვლევით დასტურდება, რომ ქრონიკული ტკივილის მკურნალობაში THC -ის გამოყენება საჭიროებს მისი გვერდითი ეფექტის, კერძოდ, ტოლერანტობის ფენომენის გათვალისწინებას.

REFERENCES

1. Iversen L. (2000) The Science of Marijuana. Oxford: Oxford University Press.
2. Tsagareli M. (2018) Pain Concept and Treatment: from Alkmaeon to Patrick Wall. LAMBERT Acad. Publ.
3. Pacher P., Batkai S., Kunos G. (2006) The endocannabinoid system as an emerging target of pharmacotherapy. Pharmacol. *Rev.*, **58:** 389-462 (doi: 10.1124/pr.58.3.2).
4. Fitzcharles M.A., Shir Y. (2008) New concepts in rheumatic pain. *Rheum Dis. Clin. North Am.,* **34:** 267-283.
5. Guindon J., Hohmann A.G. (2008) Cannabinoid CB_2 receptors as therapeutic target for the treatment of inflammatory and neuropathic pain. *Brit. J. Pharmacol.,* **153**(2): 319-334.
6. Martin B.R, Lichtman A.H. (1998) Cannabinoid transmission and pain perception. *Neurobiology of Desease,* **5:** 447-461 (doi.org/10.1006/nbdi. 0218).
7. Clapper G., Moreno-Sanz R., Russo A., Guijarro F.,Vacondio A., Duranti A., Tontini A. (2010) Anandamide suppresses pain initiation through a peripheral endocannabinoid mechanism. *Nature Neurosci.,* **13**(10): 265-1270.
8. Katona I., Rancz E.A., Acsady L., Ledent C., Mackie K., Hajos N., Freund T.F. (2001) Distribution of CB1 cannabinoid receptors in the amygdala and their role in the control of GABA-ergic transmission. *J. Neurosci.,* **21** (23): 9506–9518.
9. Pietrzak R.H., Huang Y., Corsi-Travali S., Zheng M.Q., Lin S.F., Henry S., Potenza M.N., Piomelli D., Carson R.E., Neumeister A. (2014) Cannabinoid type 1 receptor availability in the amygdala mediates threat processing in trauma survivors. *Neuropsychopharmacol.,* **39**(11): 2519-2528 (doi: 10.1038/npp).
10. Lee M.C., Ploner M., Wiech K., Brooks J., Menon D.K. (2013) Amygdala activity contributes to the dissociative effect of cannabis on pain perception. *Pain,* **154**(1): 124–134.
11. Neugebauer V. (2015) Amygdala pain mechanisms. *Handb. Exp. Pharmacol.,* **227:** 261–284. (doi: 10.1007/978-3-662-46450-2_13).
12. Veinante P., Yalcin I., Barrot M. (2013) The amygdala between sensation and affect: a role in pain. *J. Mol. Psychiatry.* **1**(1): 9 (doi: 10.1186/2049-9256-1-9).
13. Tsagareli M.G., Tsiklauri N., Nozadze I., Gurtskaia G. (2012) Tolerance effects of non-steroidal anti-inflammatory drugs microinjected into central amygdala, periaqueductal grey, and nucleus raphe. *Neural Regen. Res.,* **7**(13): 1029 –1039 (doi: 10.3969/j.issn.1673-5374.2012.13.010).
14. Tsagareli N., Tsiklauri N., Nozadze I., Gurtskaia G., Kvachadze I., Tsagareli M.G. (2020) Antinociceptive effects of NSAIDs injected into central amygdala are attenuated by combined administration of opioid and cannabinoid receptor antagonists. *Bull. Georg. Natl. Acad. Sci.,* **14**(1): 120-126.
15. Tsiklauri N., Tsagareli N., Nozadze I., Gurtskaia G., Tsagareli M.G. (2020) Endogenous opioid and cannabinoid receptors are involved in antinociceptive effects NSAIDs injected into anterior cingulate cortex of rats. *Int. J. Adv. Sci. Tech.,* **8**(1): 61-65.
16. Paxinos G., Watson C. (1997) The rat brain in stereotaxic coordinates. San Diego: Academic Press.
17. Gurtskaia G., Tsiklauri N., Nozadze I., Tsagareli M.G. (2014) An overview of antinociceptive tolerance to non-steroidal anti-inflammatory drugs. *Annu. Res. Review Biol.,* **4**(12): 1887-1901.
18. Tsagareli N., Tsiklauri N., Tsagareli M., Kvachadze I. (2019) Naloxone and CTOP block NSAIDs-induced antinociception in anterior cingulate cortex of rats. *Georgian Med. News,* **26**(1) (286): 116-122.

Received July, 2020