საქართველოს მეცნიერებათა ეროვნული აკადემიის მოამბე, ტ. 14, №1, 2020
BULLETIN OF THE GEORGIAN NATIONAL ACADEMY OF SCIENCES, vol. 14, no. 1, 2020

Human and Animal Physiology

Antinociceptive Effects of NSAIDs Injected into Central Amygdala are Attenuated by Combined Administration of Opioid and Cannabinoid Receptor Antagonists

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

[*] *Laboratory of Pain and Analgesia, Beritashvili Center for Experimental Biomedicine, Tbilisi, Georgia*
[**] *Department of Physiology, Tbilisi State Medical University, Tbilisi, Georgia*

(Presented by Academy Member Tengiz Zaalishvili)

Pain has a strong emotional component, and negative emotions can exacerbate chronic pain. It is well-documented that the central nucleus of amygdala (CeA) is particularly important for sensory and emotion processing, and is now defined as the "nociceptive amygdala". In this work we investigated the central brain mechanisms of non-opioid antinociception induced by non-steroidal anti-inflammatory drugs (NSAIDs) microinjected into CeA using the formalin test model in male rats. We also studied the role of endogenous opioid and cannabinoid receptors in modulation pain sensation by injection of their antagonists, naloxone and AM-251, respectively into the CeA. Five minutes after intraplantar formalin injection all animals showed a significant reduction in thermal paw withdrawal latency and mechanical withdrawal threshold compared to pre-baseline values. Fifteen minutes after formalin injection, NSAIDs injected into the CeA clearly showed antinociceptive effects of withdrawals. However, post- or pre-treatment with a combined naloxone and AM-251 into the CeA resulted in a significant abolish or reduction of analgesic effects of NSAIDs. The present findings support the concept that NSAIDs-evoked antinociception is mediated via descending endogenous opioid and cannabinoid modulatory systems. *© 2020 Bull. Georg. Natl. Acad. Sci.*

Analgesia, antinociception, formalin test, hyperalgesia, nociception, pain

The most important means of signaling imminent harm is the pain system. Studies of the emotional and motivational basis of pain reveal a diverse and complex set of processes by which the affective experience of pain is realized. On the other hand, pain has a strong emotional component, and negative emotions can exacerbate chronic pain [1].

We have recently shown that microinjections of commonly used non-steroidal anti-inflammatory drugs (NSAIDs) separately, into the anterior cingulate cortex (ACC) or agranular insular cortex (AIC), induce antinociception in rats. Repeated administration of the analgesics in these limbic structures develops tolerance to them [2-4].

The amygdala with its well-documented role in emotion processing and related disorders, such as anxiety, depression, and persistent pain, strongly supports the concept that the central amygdala is a key player in the emotional modulation of chronic pain. The central nucleus of amygdala (CeA) is particularly important for sensory and emotion processing, and is now defined as the "nociceptive amygdala" because of its high content of nociceptive neurons, receiving nociceptive specific information directly from the spino-parabrachio-amygdaloid pain pathway [5,6].

Here we report the role of endogenous opioid and cannabinoid receptors in modulation pain sensation by injection their antagonists, naloxone and AM-251, respectively into the CeA using the formalin test model in male rats.

Materials and Methods

Animals. The research was carried out on adult male Wistar rats weighing 200–250 g, bred at the Beritashvili Exp. BMC. The animals were kept under standard housing conditions (22 ± 2 ºC, 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 Beritashvili Center for Experimental Biomedicine 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 (AP: –1.8; L: +8; H: 3.8) according to the coordinates in the atlas of Paxinos and Watson (1997) [7]. 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 3-4 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. Diclofenac (diclofenac sodium, 75 μg/0.5 μl, Hemofarm, Serbia), ketonal (ketoprofen, 25 μg/0.5 μl, Sandoz, Slovenia), ketorolac (ketorolac thromethamin, 90 μg/0.5 μl, Grindex, Latvia) or xefocam (lornoxicam, 12 μg/0.5 μl, Nycomed, Austria) were injected through the microinjection cannula as we used previously [8]. The guide cannula was then plugged with a stainless steel stylet. Isotonic saline was injected in the same volume (0.5 μl, GalichPharm, Ukraine) and manner in a separate group of rats for controls. Opioid receptor antagonists, non-selective naloxone (0.25 μg/0.5 μl, Sigma-Aldrich, St. Louis, MO, USA) or selective μ-opioid CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2) (100 ng/0.5 μl, Sigma-Aldrich) were injected through the microinjection cannula, and of the cannabinoid 1 (CB1) receptor antagonist AM-251(1 μM/0.5 μl, Sigma-Aldrich, St. Louis, MO, USA) was injected through the microinjection cannula. Solutions were microinjected in about 10-12 seconds.

Behavioral testing. Twenty minutes post microinjection of NSAIDs or saline into the CeA, i.e. 10 min before the peak of the drugs' effect is normally reached, rats were tested for antinociception using the thermal paw withdrawal (Hargreaves) test (IITC #390, IITC Life science, Inc., Woodland Hills, CA, USA) and mechanical paw withdrawal threshold (von Frey) test (IITC Life science, Inc., USA). For Hargreaves' test Rats were first habituated over three successive daily sessions

to stand on a glass surface heated to 30 ± 1 °C within a ventilated Plexiglas enclosure. Before formal testing, baseline latencies for paw withdrawals evoked by radiant thermal stimulation were measured five times per paw, with at least 5 min intervals between tests of a given paw. A light beam (Plantar Test 390, IITC) was focused onto the plantar surface of the hindpaw through a glass plate from below, and the latency from onset of the light to brisk withdrawal of the stimulated paw was measured. To prevent potential tissue damage, a cutoff time of 20 s was used if no paw movement occurred. For von Frey test baseline mechanical withdrawal thresholds were assessed using an electronic von Frey filament with 90 g range (1601C, IITC) pressed against the plantar surface of one hindpaw. This device registered the force (g) at the moment that the hindpaw was withdrawn from the filament.

In the second set of experiments, pretreatment of rats with AM-251in the CeA was followed by thermal and mechanical tests. 10 min after they were treated with NSAIDs in the same dose as in the first set of experiments and were then retested again. Different animal groups were used for the first and second sets of experiments. The number of rats in each group was six.

Formalin-induced nociception test. Rats were placed in plastic cylinders on a room temperature glass surface and allowed to acclimate for approximately one hour before injection. The formalin solution was prepared at 10% in saline from a formalin stock (Sigma-Aldrich, USA) and a unilateral intraplantar injection (right hindpaw) was made in a volume of 50 ml. The formalin stock corresponded to a 37% formaldehyde solution. In rodents, intraplantar injections of formalin produce a biphasic behavioral reaction consisting of an initial phase of paw-flinching occurring about 3–5 min after the injection, followed by a quiescent period, then the second phase of flinching beginning after 20–30 min. The intensities of these behaviors are dependent on the concentration of formalin that is administered. We presently collected data at 5 minutes post-formalin injections representing the first phase, and at 15 and 60 minutes post-formalin injections representing the second phase.

Histology. At the end of each set of experiments, the microinjection site was 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 counterstained with Cresyl Violet. The microinjection sites were histologically verified and plotted according to Paxinos and Watson (1997) stereotaxic atlas coordinates [7].

Statistical analysis. 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, and treated and naloxone+AM-251 groups, respectively. 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 NSAIDs, and with combined naloxone and AM-251 were acknowledged as statistically significant if $P < 0.05$.

Results and Discussion

Five minutes following intraplantar formalin injection all animals showed a strong hyperalgesia expressing a significant reduction in thermal paw withdrawal latency and mechanical withdrawal threshold compared to pre-baseline values. Fifteen minutes after formalin injection, NSAIDs injected into the CeA clearly showed antinociceptive effects of withdrawals. However, when post-treated with a combined naloxone and AM-251 we found a significant abolish of analgesic effects of NSAIDs (Fig. 1). In the second session of this study, after formalin testing, when pre-treated with a combination of these antagonists we revealed a completely reduction of NSAIDs-induced antinociception (Fig. 2).

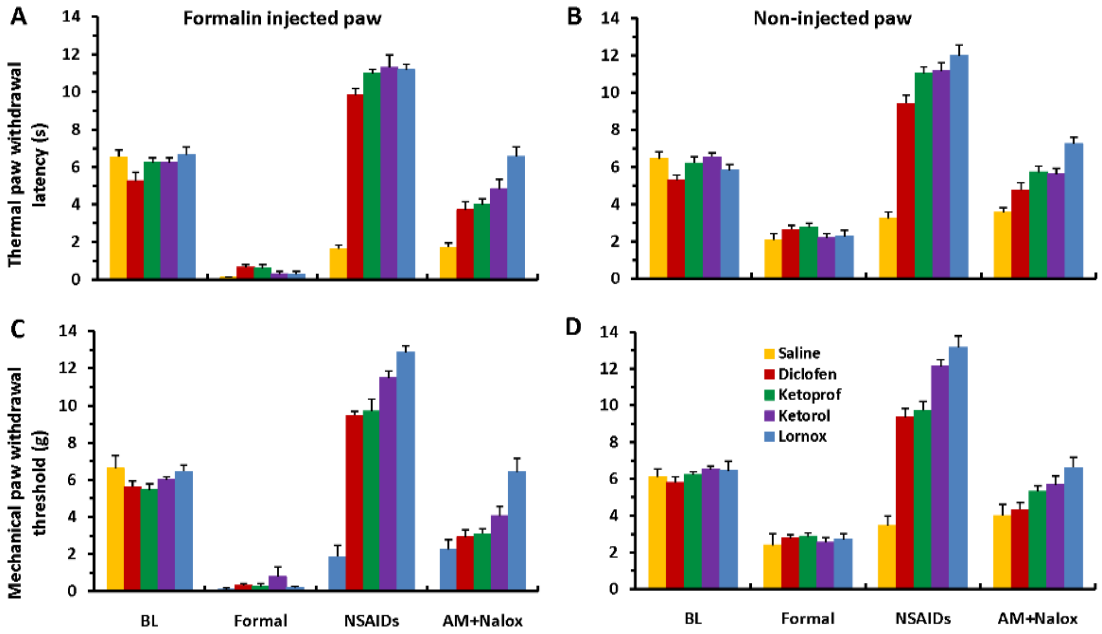

Fig. 1. Post-treatment with combined CB1 receptor antagonist AM-251 and naloxone significantly reduces analgesic effects of NSAIDs in ipsilateral (formalin injected) paw (A, C) and contralateral (non-injected) paw (B, D) in latencies of the thermal paw withdrawal reflex (s) (A, B) and thresholds of the mechanical paw withdrawal reflex (g) (C, D) for post-formalin phase II (30 min), respectively.

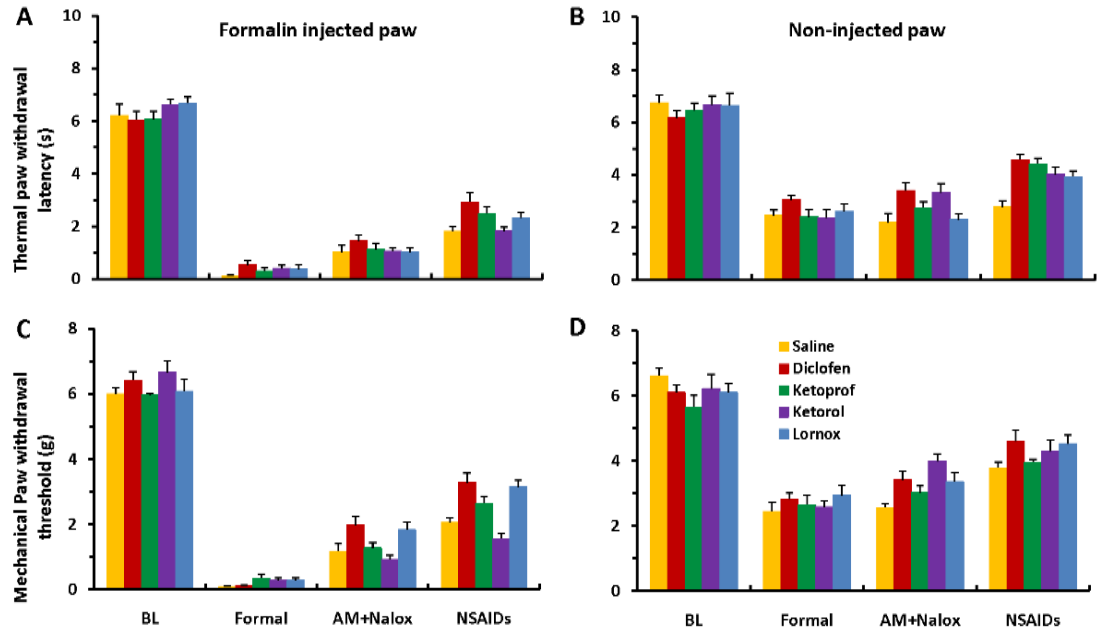

Fig. 2. Pre-treatment with combined AM-251 and naloxone in CeA prevents analgesic effects of NSAIDs in ipsilateral (formalin injected) paw (A, C) and contralateral (non-injected) paw (B, D) in latencies of the thermal paw withdrawal reflex (s) (A, B) and thresholds of the mechanical paw withdrawal reflex (g) (C, D) for post-formalin phase II (30 min), respectively.

The present data demonstrated that microinjections of commonly used NSAIDs, diclofenac, ketoprofen, ketorolac, and lornoxicam (xefocam) into the CeA result in antinociception in the formalin test model of rats. These data are similar to our previous findings in an acute pain model with tail-flick and hot plate tests, in which metamizol (analgin), diclofenac, ketorolac or lornoxicam were given systemically, or microinjected into the periaqueductal grey matter, the CeA, the nucleus raphe magnus or the dorsal hippocampus [4,8-10].

Just recently we have revealed that pre- or post-treatment with naloxone and CTOP injected into the other limbic brain area such as the ACC significantly prevented or diminished NSAIDs-induced antinociception [11] as well as CB1 receptor antagonist AM-251 similarly attenuated NSAIDs-induced analgesia by diclofenac, ketoprofen, ketorolac and lornoxicam [12,13]. Regard as our presented data, we cannot claim that the results are a synergistic or cumulative effect, but anyway, the attenuation of NSAID antinociception is significant (Figs. 1, 2).

We have shown thus that microinjection of commonly used NSAIDs in the CeA induces antinociception in the formalin test. Furthermore, we have revealed that pre- or post-treatment with a combined naloxone and AM-251 injected into the CeA significantly prevented or diminished NSAIDs-induced antinociception. Overall, the present findings support the concept of involvement of endogenous opioidergic descending pain control circuits. The latter consists of the brainstem pain modulatory periaqueductal grey – rostral ventro-medial medulla axis underscoring the strong convergence of antinociceptive mechanisms via descending endogenous opioid and cannabinoid modulatory systems.

Shota Rustaveli National Science Foundation of Georgia (grant # 217148).

ადამიანისა და ცხოველთა ფიზიოლოგია

ცენტრალურ ამიგდალაში ინიექცირებული არასტეროიდული ანთების საწინააღმდეგო პრეპარატებით გამოწვეული ანტინოციცეპტური ეფექტები სუსტდება ოპიოიდური და კანაბინოიდური რეცეპტორების ანტაგონისტების კომბინირებული შეყვანით

ნ. ცაგარელი[*,**], ნ. წიკლაური[*], ი. ნოზაძე[*], გ. ღურწკაია[*], ი. კვაჭაძე[**], მ. ცაგარელი[*,**]

** ივ. ბერიტაშვილის ექსპერიმენტული ბიომედიცინის ცენტრი, ტკივილისა და ანალგეზიის ლაბორატორია, თბილისი, საქართველო*

*** თბილისის სახელმწიფო სამედიცინო უნივერსიტეტი, ფიზიოლოგიის დეპარტამენტი, თბილისი, საქართველო*

(წარმოდგენილია აკადემიის წევრის თ. ზაალიშვილის მიერ)

ტკივილს ძლიერი ემოციური კომპონენტი ახლავს და, თავის მხრივ, უარყოფითი ემოციები ამძაფრებს ქრონიკულ ტკივილს. დადასტურებულია, რომ ამიგდალას ცენტრალური ბირთვი (CeA) განსაკუთრებით მნიშვნელოვანია სენსორული და ემოციური პროცესებისთვის და მას უწოდებენ „ნოციცეპტურ ამიგდალას“. წარმოდგენილ შრომაში გამოკვლეულია არასტეროიდული ანთების საწინააღმდეგო მედიკამენტების (NSAIDs) ამიგდალას ცენტრალურ ბირთვში მიკროინიექციების შედეგად განვითარებული ანალგეზიის ცენტრალური მექანიზმები, ფორმალინის ტესტის მოდელის გამოყენებით მამრ ვირთაგვებში. ასევე შესწავლილია ენდოგენური ოპიოიდური და კანაბინოიდური რეცეპტორების როლი მათი ანტაგონისტების, ნალოქსონის და AM-251 ინიექციის გზით, CeA-ში. ფორმალინის ტერფქვეშა (ინტრაპლანტარული) ინიექციიდან ხუთი წუთის შემდეგ ყველა ცხოველს აღენიშნებოდა თერმული და მექანიკური ფარული პერიოდის მნიშვნელოვანი შემცირება. ფორმალინის ინიექციიდან თხუთმეტი წუთის შემდეგ, CeA-ში შეყვანილმა არასტეროიდულმა პრეპარატებმა გამოიწვია სარწმუნო ანალგეზია. ამასთან, კომბინირებული ნალოქსონისა და AM-251-ის პრე- და პოსტინიექციებმა CeA-ში გამოიწვია NSAID-ების ანტინოციცეპტური ეფექტების მნიშვნელოვანი შემცირება. მიღებული შედეგები მხარს უჭერს კონცეფციას, რომ არასტეროიდული ანთების საწინააღმდეგო პრეპარატებით გამოწვეული ანტინოციცეპცია განპირობებულია ენდოგენური ოპიოიდური და კანაბინოიდური მოდულატორული დაღმავალი სისტემების შუამავლობით.

REFERENCES

1. Seymour B., Dolan R.J. (2013) Emotion, motivation and pain. In: wall and Melzacks' textbook of pain, 248-255. Elsevier.
2. Pirkulashvili N., Tsiklauri N., Tsagareli M.G. (2017) Antinociceptive tolerance to NSAIDs in the agranular insular cortex is mediated by opioid mechanism. *J. Pain Res.,* **10**:1561–1568 (doi: 10.2147/JPR. S138360).
3. Tsiklauri N., Nozadze I., Gurtskaia G., Tsagareli M.G. (2018) Antinociceptive tolerance to NSAIDs in the anterior cingulate cortex is mediated by an opioid mechanism. *BMC Pharmacol. Toxicol.,* **19**(1), art. 2 (doi: 10.1186/ s40360-017-0193-y).
4. Tsiklauri N., Nozadze I., Tsagareli N., Gurtskaia G., Abzianidze E., Tsagareli M.G. (2018) Brain limbic areas involved in the tolerance of NSAIDs via opioid mechanism. In: *Systemic, Cellular and Molecular Mechanisms of Physiological Functions and Their Disorders.* **2**, chapter 23: 281-299. New York, Nova Biomedical.
5. Neugebauer V. (2015) Amygdala pain mechanisms. In: H.-G. Schaible (ed.) Pain Control, Handb. *Exp. Pharmacol.*, **227**: 261-284 (doi: 10.1007/978-3-662-46450-2_13).
6. Thompson J.M., Neugebauer V. (2017) Amygdala plasticity and pain. Pain Res. Manag., 2017, art. 8296501 (doi: 10.1155/2017/8296501).
7. Paxinos G., Watson C. (1997) The rat brain in stereotaxic coordinates. San Diego, Academic Press.
8. Tsagareli M.G., Tsiklauri N., Gurtskaia G., Nozadze I., Abzianidze E. (2010) The central nucleus of amygdala is involved in tolerance to the antinociceptive effect of NSAIDs. *Health,* **2**: 64–68.
9. Tsagareli M.G., Tsiklauri N. (2012) Behavior study of 'non-opioid tolerance. New York, Nova Biomedical.
10. Tsiklauri N., Nozadze I., Gurtskaia G., Tsagareli M.G. (2016) Cellular mechanisms of antinociceptive tolerance to non-steroidal anti-inflammatory drugs. In: *Systemic,Cellular and Molecular Mechanisms of Physiological Functions and Their Disorders.* **1**, chapter 26: 339-362. New York, Nova Biomedical.
11. 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): 116-122.
12. Tsiklauri N., Tsagareli N., Nozadze I., Gurtskaia G., Kvachadze I., Tsagareli M.G. (2018) Influence of the endogenous cannabinoid system on antinociceptive effects of NSAIDs microinjected into anterior cingulate cortex of rats. *Bull. Georg. Natl. Acad. Sci*., **12**(2): 121-127.
13. Tsagareli N., Tsiklauri N., Kvachadze I., Tsagareli M. (2018) Antinociceptive tolerance to NSAIDs partially mediated via in anterior cingulate cortex of rats. *Georgian Med. News.,* **25**(7-8):120-125.

Received December, 2019