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Evidence for Tolerance Effects Induced by Non-Opioid Analgesics Microinjected into the Central Nucleus of Amygdala in the Rat Hot Plate Test

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(Presented by Academy Member T. Ioseliani)

ABSTRACT. Recent investigations have shown that rats repeatedly injected with metamizol (dipyrone) and lysine-acetylsalicylate in the midbrain periaqueductal gray matter (PAG) developed tolerance to these drugs and cross-tolerance to morphine. Our previous findings also have shown the same effects of tolerance in intraperitoneal injections of non-steroidal anti-inflammatory drugs (NSAIDs) analgine (metamizol), ketorolac, and xefocam. Moreover, we recently found that microinjections of these NSAIDs into the central nucleus of amygdala (Ce) both unilaterally and bilaterally produced tolerance to these analgesics and cross-tolerance to morphine in the rat tail-flick test. The purpose of this work was to determine the same effects of tolerance to other NSAIDs clodifen and voltaren injected into Ce both unilaterally and bilaterally by another hot plate test. Our investigation revealed that microinjection of clodifen and voltaren into Ce both unilaterally (the left side) and bilaterally produced antinociception as indicated by a latency increase in paw withdrawal in the hot plate test to compare with control rats with saline.

However, when these drugs microinjection subsequent testing also took place on the following days the antinociceptive effects progressively diminished so that on the fifth experimental day the hot plate latency was similar to that in the rats that received repeated injections of only saline. Furthermore, the latencies of the non-opioid tolerant rats were not altered by morphine microinjections, i.e. they showed cross-tolerance to morphine. These facts indicate the development of tolerance to these non-opioid analgesics. This is similar to opioid tolerance. Taken together, our present and previous data support the notion that endogenous opioids are involved and play a key role in the development of non-opioid tolerance to NSAIDs. *© 2009 Bull. Georg. Natl. Acad. Sci.*

***Key words**: anti-nociception, non-opioid tolerance, morphine cross-tolerance.*

Introduction

It has recently been established that pain modulation system includes the midbrain periaqueductal gray matter (PAG) and rostral ventro-medial medulla (RVM). The RVM involves the midline nucleus raphe magnus and adjacent reticular formation. The PAG is part of CNS circuit that controls nociceptive transmission at the level of spinal cord mainly through the RVM. The PAG-RVM system is central substrate for the actions of opioid analgesic drugs. Endogenous opioid peptides are

present in neural somata and/or terminal fields in several components of this network. In animals, PAG electrical stimulation inhibits simple noxious-stimulus reflexes, such as the tail-flick (TF) or paw withdrawal reflexes. Furthermore, this circuit contributes to opiate analgesia and opioid dependence [1].

Recent investigations have shown that in some brain areas, particularly, in PAG and RVM, the microinjection of non-opioid analgesics, metamizol, and lysine-acetylsalicylate (LASA) causes antinociception with some effects of tolerance [2-5]. Our previous findings also have shown the same effects of tolerance in intraperitoneal (i.p.) injections of analgine (metamizol), ketorolac, and xefocam [6-8]. Taken together these studies support the notion that contribution of the downstream pain-control system to the tolerance effects of the above-mentioned non-steroidal anti-inflammatory drugs (NSAIDs) involve endogenous opioidergic mechanisms.

The amygdala, which receives massive input from the hippocampus and the neocortex, is a major source of afferents to PAG [9]. Analgesia resulting from microinjection of opioid agonists into the basolateral amygdala is blocked by lidocaine inactivation of, or opioid antagonist injection into, the PAG [10,11]. Cortical afferents to the amygdala largely target its basolateral component. The basolateral amygdala then projects to the central nucleus of amygdala (Ce), which in turn projects densely to the PAG [12]. The Ce also receives nociceptive input, both directly from the spinal cord, and indirectly via a large projection from the dorsal horn to the parabrachial nucleus [13,14]. Other authors have provided evidence that Ce is an integral component of the endogenous pain-modulatory circuit. This nucleus is critical for systemic morphine-induced suppression of TF nociceptive reflex [15].

The present study was designed to examine whether microinjection of clodifen and voltaren into the Ce leads to the development of tolerance in rats, and to ascertain whether Ce is the pain-modulating pathway through PAG. Previously for this purpose we have used the tail flick test in microinjections of metamizol, ketorolac and xefocam into Ce [16]. Here we report that hot plate (HP) withdrawal latency test indicates the development of tolerance to two other NSAIDs as voltaren and clodifen.

Methods

The experiments were carried out on male white rats, 200-250g in body weight, bred at the Beritashvili Institute of Physiology. Guidelines of the International Association for the Study of Pain regarding animal experimentation were followed throughout. Under anesthesia with thiopental (55 mg/kg, i.p. "Kievmed" Ukraine) 12- mm-long stainless steel guide cannula (Plastic One, Inc., USA) was stereotaxically implanted unilaterally on the left side or bilaterally into the Ce amygdala by the atlas of Paxinos & Watson (1998) and anchored to the cranium by dental cement. The guide cannula was plugged with a stainless steel stylet. Thereafter, the rats were handled every day for 15 min to get familiar with the testing protocol and experimental environment during three days. During this time, the stylet was removed and the injection cannula was inserted into the guide cannula, but no drug was injected. This helped to habituate the rats to the injection procedure and to reduce artifacts arising from mechanical manipulation during the test days. Five days after surgery 10 mm length tubing was attached to a 50 µl Hamilton syringe (Hamilton, Inc., USA) and was then joined to the guide cannula, and the drug was introduced through it while the rat was gently restrained. Voltaren (diclofenac sodium, 75µg/3µl, "Novartis Pharma" AG, Switzerland), and Clodifen (diclofenac sodium, 75µg/3µl, "E.I.P.I." Comp., Egypt), or saline (3µl, "Galichpharm" Ltd. Ukraine) were then injected through the microinjection cannula; then the guide cannula was plugged with stainless steel stylet. Twenty minutes post microinjection, i.e. 10- min before the peak of the drugs' effect is normally reached, hind paw withdrawal latency (to lick paw or jump when standing on 52ºC plate) was measured by Hot Plate Analgesia Meter (39 HP, IITC, Life science, Inc., USA). A similar procedure was followed for the repeated microinjection of analgine, ketorolac, xefocam or saline for five consecutive days. On fifth experimental day, all animals received a Ce microinjection of morphine hydrochloride (3µg/2µl, "Laboratoires Stella", France) and HP latencies were measured 20 min thereafter. At the end of each experiment, after fifth day the microinjection site was marked with, 2 µl, of a saturated solution of Pontamine Sky Blue (Sigma Chemical Co., USA), and the animal was killed with ester. After fixation by immersion in 10% formalin the brain was sectioned and the microinjection site was identified with the aid of Paxinos & Watson' stereotaxic atlas (1998). All data are presented as Mean±S.E.M. Analysis of variance (ANOVA) subsequent to Tukey-Kramer multiple comparison test was used for statistical evaluations. The statistical software utilized was InStat 3.05 (GraphPad Software, Inc, USA). Data are comparisons between treated and saline groups. Statistical significance was acknowledged if $P<0.05$.

Results

Only rats with microinjections into Ce were included in data analysis (Fig. 1). These data consisted of 7 rats microinjected with voltaren, 6 with clodifen, and 8 with control saline, respectively. Injection sites outside the boundaries of the Ce (the shaded region in the fig. 1) were not included in data analysis.

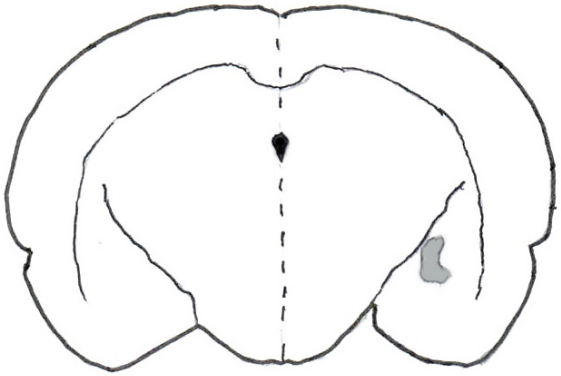

Fig. 1. Location of microinjection sites in the Ce. Only NSAIDs and saline injections within the shaded region were included in data analysis. Coronal sections are taken from the atlas of Paxinos and Watson (1998). The Distance from interaural line 5.7 mm and from the bregma -3.3 mm respectively. All injections fell within ±0.5 mm of this coronal plane.

Our investigation showed that microinjection of NSAIDs into the Ce unilaterally (the left side) produced antinociception as revealed by a latency increase in HP compared to controls with saline microinjected into the same nucleus, on the first experimental day for clodifen ($p<0.001$) and voltaren ($p<0.05$) (Fig. 2) respectively (ANOVA: $F(2,19)=11.95$, $P=0.0003$). However, when these drugs microinjection subsequent testing also took place in the following days the antinociceptive effects progressively diminished, so that on the fourth experimental day the TF latency was similar to that in the rats that received repeated injections of only saline. This was akin to the development of tolerance to morphine administration to PAG in similar preparations [17, 18], and we will therefore refer to it as "non-opioid tolerance". On day 5 both experimental and control groups of rats received a morphine hydrochloride microinjection into the same Ce sites, and only the saline-treated animals responded with antinociception ($P<0.001$). The latencies of the non-opioid tolerant rats were not altered by the morphine microinjections, i.e. they showed cross-tolerance to morphine (Fig. 2).

Bilateral microinjections into the Ce also increased the latency of HP compared to control rats on the first day for both clodifen and voltaren ($P<0.001$) (Fig. 3) (ANOVA: $F(2,19)=25$, $P<0.0001$). On the second day after repeated microinjections, the HP latency decreased (ANOVA: $F(2,19)=11.67$, $P=0.0004$) for clodifen ($P<0.001$) and voltaren ($P<0.01$) respectively (Fig. 3), so on the fourth and fifth days of experiments, the HP latency was similar to that in the rats receiving bilateral injection of only saline. Between unilateral and bilat-

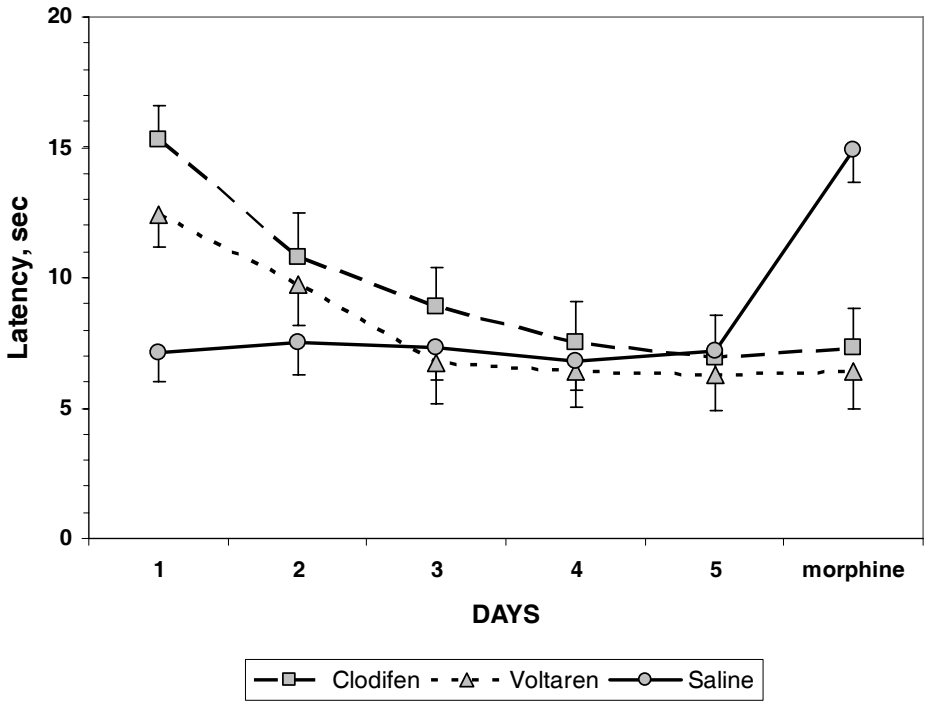

Fig. 2. Response latency in HP for five consecutive experimental days to unilateral microinjections into Ce of clodifen and voltaren following morphine.

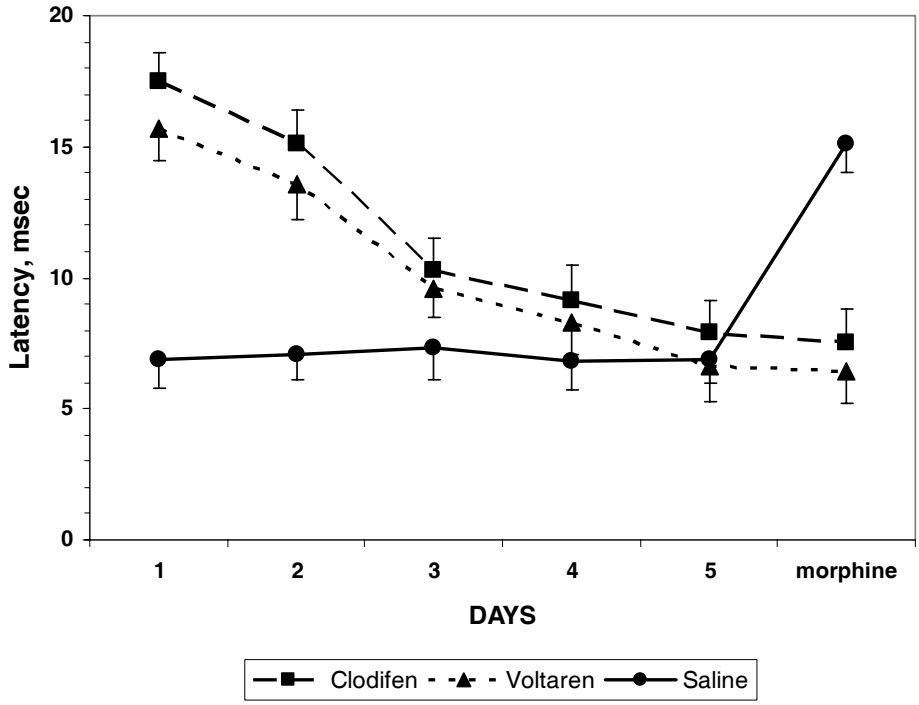

Fig. 3. Response latency in HP for five consecutive experimental days to bilateral microinjections into Ce of clodifen and voltaren following morphine.

eral administration of these NSAIDs differences were seen on the very first two days of experiments. The HP latency in the group of bilateral microinjections was stronger than in the unilateral group ($p<0.05$). Therefore, we were able to suppose that, when both sides of Ce (left and right) are together involved in fulfillment of the same task the magnitude of responses is more than when they worked separately. Therefore, the tolerance develops more slowly. In bilateral microinjections of these NSAIDs we also observed cross-tolerance effects to morphine as compared with controls (Fig. 3).

Discussion

The present study revealed that microinjection of clodifen and voltaren into the Ce induced antinociception in awake rats. This confirms our previous results obtained in the same experimental paradigm where we used TF test in rats with microinjections of other NSAIDs, as analgine, ketorolac and xefocam [16]. More importantly, our investigations [6,7,16] as well as of our colleagues [3,5] indicate that repeated microinjections of NSAIDs into the Ce and PAG induce a decrease in antinociceptive effectiveness reminiscent of that induced by opiates.

A large involvement of opioidergic mechanisms in tolerance effects of NSAIDs is surprising, because traditionally cellular and molecular actions of opioids have been considered as different from those of non-opioid analgesics. One interesting aspect of NSAIDs administration, however, emphasizes their similarities to opioid analgesics, namely induction of tolerance. Indeed, microinjection of metamizol, or LASA into PAG [19] or metamizol, ketorolac and xefocam into Ce, [16] progressively leads to a loss of their antinociceptive effects, i.e. produces tolerance. Furthermore, tolerance to these NSAIDs is accompanied by cross-tolerance to morphine [3,4,16] as if opioid analgesics had been repeatedly administered. Interestingly, tolerance to the effect of PAG-microinjected metamizol can–like tolerance to morphine–be reverted by microinjection of proglumide, a cholecystokinin antagonist, into the same PAG site [5]. The latter fact constitutes additional evidence that the PAG effects of non-opioid analgesics are similar to those of morphine.

Our results on tolerance effects with clodifen and voltaren microinjections into Ce confirm the suggestion that the mechanism of their tolerance must be realized through PAG triggering the descending pain control system on the dorsal spinal cord level [1] and suggest that Ce should be incorporated into current models of endogenous pain control circuitry [20].

Conclusions

These results show that alongside PAG and RVM the Ce is an important site of endogenous antinociceptive

system, which triggers the descending pain control mechanism and thus inhibits nociceptive transmission on the spinal cord level. On the other hand, our data confirm the results of other authors that NSAIDs are in close relation with endogenous opioids and the tolerance to these non-opioid drugs probably depends on opioid tolerance.

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ადამიანისა და ცხოველთა ფიზიოლოგია

ამიგდალას ცენტრალურ ბირთვში არაოპიოიდების მიკროინიექციებით გამოწვეული ტოლერანტობის შესწავლა hot plate ტესტით

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(წარმოდგენილია აკადემიის წევრის თ. იოსელიანის მიერ)

უკანასკნელი მონაცემების თანახმად, ტკივილის მოდულაციის სისტემა შედგება შუა ტვინის რუხი ნივთიერებისა (რნ) და მოგრძო ტვინის როსტრალური ვენტრო-მედიალური (რვმ) უბნისაგან. თავის მხრივ, ნუშისებრი სხეული, ანუ ამიგდალა, ღებულობს რა ფართო შესავლებს ჰიპოკამპიდან და ახალი ქერქიდან, წარმოადგენს რნ-ის აფერენტების ძირითად წყაროს. ბაზო-ლატერალურ ამიგდალაში ოპიოიდების აგონისტების მიკროინიექციით გამოწვეული ანალგეზია იბლოკება რნ-ში ლიდოკაინით განპირობებული ინაქტივაციით ან ოპიოიდების ანტაგონისტების ინიექციით. ამასთან ერთად ბაზოლატერალური ამიგდალა პროეცირდება ამიგდალას ცენტრალურ ბირთვში (აცბ), რომელიც თავის მხრივ ფართოდ უკავშირდება რნ-ს. გარდა ამისა, აცბ ღებულობს ტკივილის, ანუ ნოციცეპტურ შესავალს როგორც პირდაპირ ზურგის ტვინიდან, ასევე არაპირდაპირ - ტვინის ხიდის პარაბრაქიული ბირთვიდან. სხვა ავტორები მიუთითებენ აცბ-ს მონაწილეობაზე ენდოგენური ტკივილის მოდულატორულ წრეში. ეს ბირთვი მნიშვნელოვანი უნდა იყოს მორფინის სისტემური შეყვანით გამოწვეული კუდის მოქნევის რეფლექსის (კმრ) დათრგუნვაში. წარმოდგენილი შრომის მიზანია გარკვევა საკითხისა, აცბ-ში არაოპიოიდური კლონიდინისა და ვოლტარენის მიკროინიექცია იწვევს თუ არა მათ მიმართ ტოლერანტობას და რამდენად არის აცბ დაკავშირებული ტკივილის მოდულატორულ სისტემასთან. ამისთვის ჩვენ გამოვიყენეთ ე.წ. **hot plate** ტესტი.

ცდები ტარდებოდა 200-250 გრამის თეთრ ვირთაგვებზე, რომელთაც სტერეოტაქსულად აცბ-ში ჩაენერგებოდათ 12 მმ სიგრძის კანულები უნილატერალურად (მარცხენა მხარეს) და ბილატერალურად. ცხოველს ვათავსებდით გაცხელებულ ფირფიტაზე (55°C) აღვრიცხავდით თათების მოცილების ფარულ პერიოდს სპეციალური ხელსაწყოთი. საკონტროლო ცდებში ვახდენდით ფიზიოლოგიური ხსნარის ინიექციას. მონაცემები მუშავდებოდა სტატისტიკურად ვარიაციული სტატისტიკისა და ტუკეი-კრამერის ტესტით.

მიღებულმა შედეგებმა აჩვენა, რომ აცბ-ში არაოპიოიდური, არა-სტეროიდული ანთების საწინააღმდეგო წამლების (ასასწ) ინიექცია, როგორც უნილატერალურად, ისე ბილატერალურად, იწვევს მკვეთრად

გამოხატულ ანტი-ნოციცეფციას როგორც კლოდიფენისთვის ისე ვოლტარენისთვის, საკონტროლო ჯგუფთან შედარებით, მაგრამ შემდგომი ოთხი დღის განმავლობაში აღნიშნული წამლების განმეორებითი მიკროინიექციებისას აღინიშნება მათი მოქმედების დაქვეითება ანუ ტოლერანტობა, ხოლო კროს-ტოლერანტობა მორფინის მიმართ. ბილატერალური ინიექცია იწვევს ანალგეზიის უფრო ძლიერ ეფექტს და ამასთან უფრო ნელა მიმდინარე ტოლერანტობას უნილატერალურთან შედარებით ($P<0.05$). წარმოდგენილმა გამოკვლევებმა დაადასტურეს ჩვენი ადრინდელი შედეგები, როდესაც ვახდენდით ასასწ-ის - ანალგინის, კეტოროლაკისა და ქსეფოკამის ინტრაპერიტონეალურ ინიექციასა, და აგრეთვე ამ პრეპარატების აცბ-ში მიკროინიექციას. უფრო მეტიც, ჩამოთვლილი ასასწ-ის მიმართ ტოლერანტობა იცვლება კროს-ტოლერანტობით მორფინის მიმართ, თითქოსდა ოპიოიდური პრეპარატებით მოქმედებას ვახდენდეთ. ეს ფაქტები მიუთითებს გარკვეულ კავშირზე არაოპიოიდურ და ოპიოიდურ წამლებს შორის. კერძოდ, ასასწ თავის მოქმედებას უნდა ახორციელებდეს ენდოგენური ოპიატების შუამავლობით.

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