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Tolerance Induced by Non-Opioid Analgesic Microinjections into the Central Nucleus of Amygdala of Rats

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ABSTRACT. Recent investigations have shown that in some brain areas, particularly, in the midbrain periaqueductal gray matter (PAG) and rostral ventro-medial medulla (RVM), the microinjection of non-opioid analgesics, metamizol, and lysine-acetylsalicylate, causes antinociception with some effects of tolerance. Our preliminary findings also have shown the same effects of tolerance in intraperitoneal injections of non-steroidal anti-inflammatory drugs (NSAIDs). The present study was designed to examine whether microinjection of analgine, ketorolac and xefocam into the central nucleus of amygdala (Ce) leads to the development of tolerance in rats, and to ascertain whether this nucleus is the pain-modulating pathway through PAG. Our investigation revealed that microinjection of NSAIDs into the Ce both unilaterally (the left side) and bilaterally produced antinociception as indicated by a latency increase in tail flick reflex (TF) compared to controls with saline, on the first experimental day for analgine ($p<0.001$), ketorolac ($p<0.001$), and xefocam ($p<0.001$) respectively. 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 TF latency was similar to that in the rats that received repeated injections of only saline. These results show that alongside with 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 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. *© 2008 Bull. Georg. Natl. Acad. Sci.*

Key words: *non-opioid tolerance, morphine cross-tolerance, tail-flick reflex.*

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. 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 preliminary findings also have shown the same effects of tolerance in intraperito-

neal (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 above-mentioned non-steroidal anti-inflammatory drugs (NSAIDs) involves 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 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 analgine, ketorolac and xefocam into the Ce leads to the development of tolerance in rats, and to ascertain whether Ce is the pain-modulating pathway through PAG.

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 (Small Parts, Inc., USA) was stereotaxically implanted unilaterally on the left side or bilaterally into the Ce amygdala by the atlas of Paxinos & Watson, 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. Analgine (1.5mg/3µl), derivate of pirazolon (metamizolum natricum, "Sanitas" Ltd, Lithuania), ketorolac (90µg/3µl), xefocam (1.2µg/3µl), 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, a proximal part of the tail was stimulated by focusing light from the electric bulb (30v, 400w) through the optical lens, and the latency of the TF was measured as an analogue signal by paper registration (Neuroscript EE208, Hellige, GmbH, Germany). A similar procedure was followed for the repeated microinjection of analgine, ketorolac, xefocam or saline for five consecutive days. On 5th day all animals received a Ce microinjection of morphine hydrochloride (3µg/2µl, "Laboratoires Stella", France) and TF 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.), 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 were used for statistical evaluations. The statistical software utilized was InStat 3.05 (GraphPad Software, Inc, USA). Statistical significance was acknowledged if $P<0.05$.

Results

Our investigation showed that microinjection of NSAIDs into the Ce unilaterally (the left side) produced antinociception as revealed by a latency increase in TF compared to controls with saline microinjected into the same nucleus, on the first experimental day for analgine ($p<0.001$) (Fig. 1A), ketorolac ($p<0.001$) (Fig. 2A), and xefocam ($p<0.001$) (Fig. 3A), respectively. 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 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 [16,17], 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. 1-3).

Bilateral microinjections into the Ce also increased the latency of TF compared to control rats on the first day for analgine (P<0.001) (Fig. 1B), ketorolac (P<0.001) (Fig. 2B), and xefocam (P<0.001) (Fig. 3B). Nevertheless, on the second day after repeated microinjections, this index began to decrease so as at the time of unilateral administration and on the fifth day of experiments, the TF latency was similar to that in the rats receiving bilateral injection of only saline. Between unilateral and bilateral administration of these NSAIDs differences were seen on the very first day of experiments. Latency of TF on the

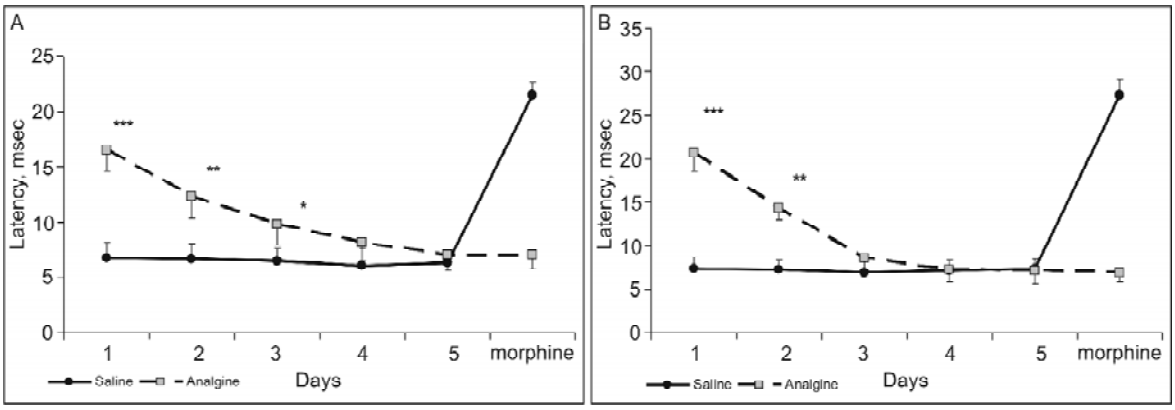

Fig. 1. Response latency in TF for five consecutive experimental days to analgine following morphine injections unilaterally (A) and bilaterally (B) respectively. In all figures, significance levels are: * - P<0.05, ** -P<0.01, *** -P<0.001.

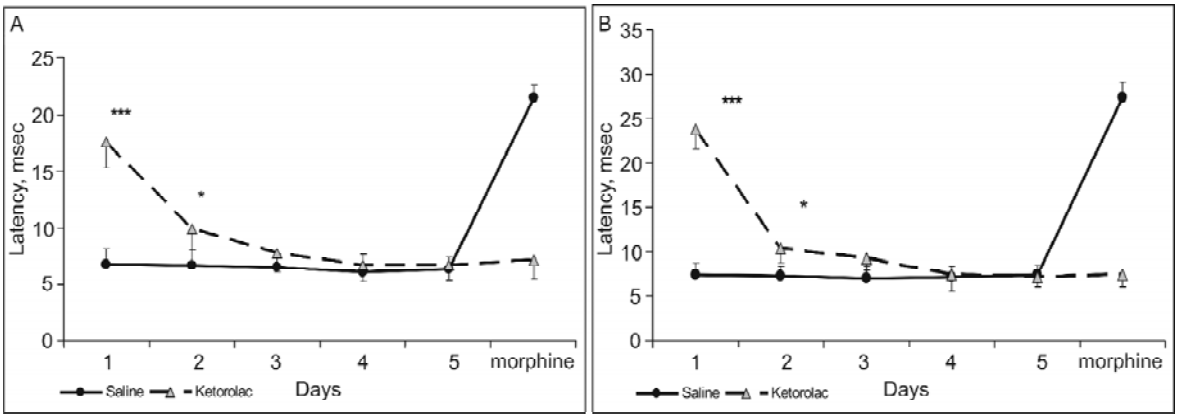

Fig. 2. Response latency in TF for five consecutive experimental days to ketorolac following morphine injections unilaterally (A) and bilaterally (B) respectively.

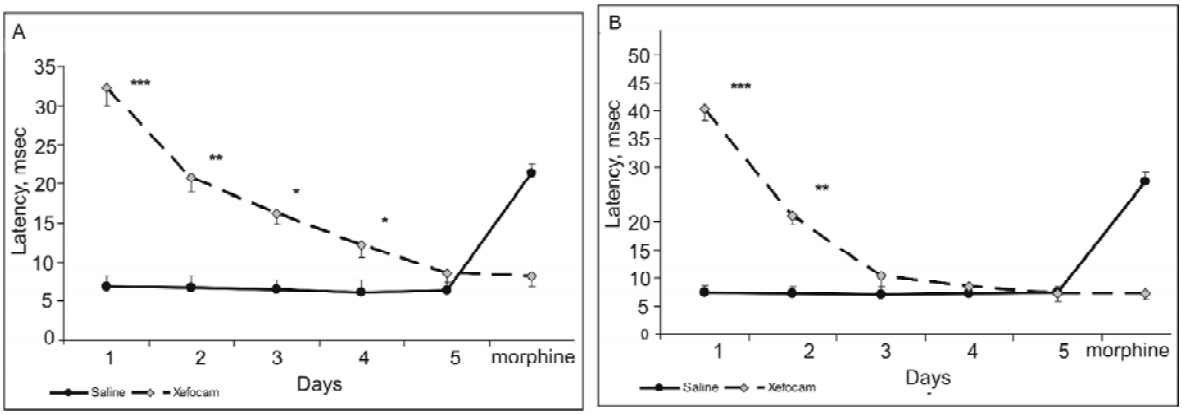

Fig. 3. Response latency in TF for five consecutive experimental days to xefocam following morphine injections unilaterally (A) and bilaterally (B) respectively.

first day in the group of bilateral microinjections was stronger than in the unilateral group ($p<0.01$). 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. In bilateral microinjections of these NSAIDs, we also observed cross-tolerance effects to morphine as compared to controls (Fig. 1-3).

Discussion

The present study revealed that microinjection of analgine, ketorolac and xefocam into the Ce induced antinociception in awake rats. This confirms our previous results obtained in rats where these NSAIDs were given i.p., or other authors' using microinjection into the PAG. More importantly, our investigations [6,7] 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.

The 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 [18] or into Ce, progressively leads to a loss of their antinociceptive effects, i.e. produces tolerance. Furthermore, tolerance to metamizol or LASA is accompanied by cross-tolerance to morphine [3,4] 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 analgine, ketorolac and xefocam 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 the current models of endogenous pain control circuitry [19].

Conclusions

These results show that alongside of 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 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 მიკროინექციებით გამოწვეული ტოლერანტობა

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(წარმოდგენილია აკადემიკოს თ. ონიანის მიერ)

უკანასკნელი მონაცემების თანახმად, ტკივილის მოდულაციის სისტემა შედგება შუა ტვინის რუხი ნივთიერებისა (რნ) და მოგრძო ტვინის როსტრალური ვენტრო-მედიალური (რვმ) უბნისგან. თავის მხრივ, ნუშისებრი სხეული, ანუ ამიგდალა, ღებულობს რა ფართო შესავლებს ჰიპოკამპისა და ახალი ქერქიდან,

წარმოადგენს რნ-ის აფერენტების ძირითად წყაროს. ბაზოლატერალურ ამიგდალაში ოპიოიდების აგონისტების მიკროინექციით გამოწვეული ანალგეზია იბლოკება რნ-ში ლიდოკაინით განპირობებული ინაქტივაციით ან ოპიოიდების ანტაგონისტების ინექციით. 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 ნაშრომის მიზანია გარკვევა საკითხისა, იწვევს თუ არა აცბ-ში არაოპიოიდური ანალგეტიკების, ანალგინის, კეტოროლაკისა და ქსეფოკამის მიკროინექცია მათ მიმართ ტოლერანტობას და რამდენად არის აცბ დაკავშირებული ტკივილის მოდულატორულ სისტემასთან.

ცდები ტარდებოდა 200-250 გრამის თეთრ ვირთაგვებზე, რომელთაც სტერეოტაქსულად აცბ-ში ჩაენერგებოდათ 12 მმ სიგრძის კანულები უნილატერალურად (მარცხენა მხარეს) და ბილატერალურად. კუდის პროქსიმალურ ნაწილზე ფოკუსირებული სხივით ვახდენდით გაღიზიანებას და კმრ-ს ფარული პერიოდი განისაზღვრებოდა როგორც ტკივილის შეგრძნების პარამეტრი. საკონტროლო ცდებში ვახდენდით ფიზიოლოგიური ხსნარის ინექციას. მონაცემები დამუშავდა სტატისტიკურად ტუკეი-კრამერის ტესტით.

მიღებულმა შედეგებმა უჩვენეს, რომ აცბ-ში არა-ოპიოიდური, არა-სტეროიდული ანთების საწინააღმდეგო წამლების (ასასწ) ინექცია, როგორც უნილატერალურად, ისე ბილატერალურად, იწვევს მკვეთრად გამოხატულ ანტი-ნოციცეფციას სამივე პრეპარატისთვის, საკონტროლო ჯგუფთან შედარებით ($P<0.001$). მაგრამ შემდგომი ოთხი დღის განმავლობაში აღნიშნული წამლების განმეორებითი მიკროინექციებისას აღინიშნება მათი მოქმედების დაქვეითება, ანუ ტოლერანტობა, ხოლო კროს-ტოლერანტობა - მორფინის მიმართ. ბილატერალური ინექცია იწვევს უფრო ძლიერ ეფექტს უნილატერალურთან შედარებით ($P<0.05$). წარმოდგენილმა გამოკვლევებმა დაადასტურეს ჩვენი ადრინდელი შედეგები, როდესაც ვახდენდით აღნიშნული ასასწ-ის ინტრა-პერიტონეალურ ინექციას, და აგრეთვე სხვა ავტორთა მონაცემებმა, რომლებიც აწარმოებდნენ მეტამიზოლისა (ანალგინი) და აცეტილსალიცილის ინექციას რნ-ში. უფრო მეტიც, ჩამოთვლილი ასასწ-ის მიმართ ტოლერანტობა იცვლება კროს-ტოლერანტობით მორფინის მიმართ, თითქოსდა ოპიოიდური პრეპარატებით მოქმედებას ვახდენდით. ეს ფაქტები მიუთითებენ გარკვეულ კავშირზე არაოპიოიდურ და ოპიოიდურ წამლებს შორის.

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