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Research report

Opioidergic and GABAergic mechanisms in the rostral ventromedial medulla modulate the nociceptive response of vocalization in guinea pigs

Luis Felipe Souza da Silva[a], Márcio Ramos Coutinho[b], Leda Menescal-de-Oliveira[b,*]

[a] Veterinary Science Department, Center for Agrarian Sciences, University of Paraiba, 58397-000 Areia, Paraiba, Brazil
[b] Department of Physiology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes 3900, 14049-900 Ribeirão Preto, SP, Brazil

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ABSTRACT

Vocalization generated by the application of a noxious stimulus is an integrative response related to the affective-motivational component of pain. The rostral ventromedial medulla (RVM) plays an important role in descending pain modulation, and opiates play a major role in modulation of the antinociception mediated by the RVM. Further, it has been suggested that morphine mediates antinociception indirectly, by inhibition of tonically active GABAergic neurons. The current study evaluated the effects of the opioids and GABA agonists and antagonists in the RVM on an affective-motivational pain model. Additionally, we investigated the opioidergic–GABAergic interaction in the RVM in the vocalization response to noxious stimulation. Microinjection of either morphine (4.4 nmol/0.2 μl) or bicuculline (0.4 nmol/0.2 μl) into the RVM decreased the vocalization index, whereas application of the $GABA_A$ receptor agonist, muscimol (0.5 nmol/0.2 μl) increased the vocalization index during noxious stimulation. Furthermore, prior microinjection of either the opioid antagonist naloxone (2.7 nmol/0.2 μl) or muscimol (0.25 nmol/0.2 μl) into the RVM blocked the reduction in vocalization index induced by morphine. These observations suggest an antinociceptive and pro-nociceptive role of the opioidergic and GABAergic neurotransmitters in the RVM, respectively. Our data show that opioids have an antinociceptive effect in the RVM, while GABAergic neurotransmission is related to the facilitation of nociceptive responses. Additionally, our results indicate that the antinociceptive effect of the opioids in the RVM could be mediated by a disinhibition of tonically active GABAergic interneurons in the downstream projection neurons of the descending pain control system; indicating an interaction between the opioidergic and GABAergic pathways of pain modulation.

1. Introduction

Vocalization generated by the application of a noxious stimulus is an integrative response mediated by the activation of the limbic structures. In addition, this response is related to the affective-motivational component of pain [3,4,37]. In our laboratory, we have been using the nociceptive test of vocalization to study central mechanisms and neurotransmitters in pain modulation [9,13,30,32–34]. Furthermore, the vocalization test has proven to be useful in evaluating nociception in unanesthetized guinea pigs [9,13,30,32–34].

The rostral ventromedial medulla (RVM), which includes the nucleus raphe magnus and the adjacent ventral reticular formation, plays an important role in descending pain modulation [2,16]. Fields et al. [15] classified three distinct physiological classes of neurons in the RVM based on the temporal correlation of changes in their firing rates with the execution of the nocifensive reflexes. Thus the "on-cells" show a burst of activity just before the nociceptive reflex, while the "off-cells" pause just before such reflexes, and "neutral-cells" show no change during nociceptive reflexes [15]. Additionally, it was suggested that "on-" and "off-cells" participate in the facilitation and inhibition of the nociception, respectively [16].

The opiates play a major role in the modulation of antinociception mediated by the RVM. Correspondingly, studies have demonstrated μ-opiate binding sites [7] as well as opioid immunoreactive terminals in the RVM [6,35]. Additionally, the microinjection of morphine into the RVM induces antinociception, accessed by distinct nociceptive tests in rats [27,28,42]. Interestingly, direct action of opioids in the RVM is limited to the "on-cells", which show a decrease in activity [21,22]. Then, since the effect of opioid receptor agonists in the RVM is generally inhibitory [12,40], it has been suggested that morphine mediates antinociception indirectly, through inhibition of tonically active GABAergic neurons [16,21].

The role of GABAergic neurotransmission in the modulation of RVM neurons has been described in several articles [11,18,20].

* Corresponding author. Tel.: +55 16 602 3333; fax: +55 16 633 0017.
E-mail addresses: felipecf@yahoo.com.br (L.F.S. da Silva), marciorcoutinho@gmail.com (M.R. Coutinho), lmdolive@fmrp.usp.br (L. Menescal-de-Oliveira).

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Furthermore, studies have shown that the RVM contains GABA binding sites [5], cell bodies and terminals containing GABA [36]. Microinjection of GABAergic receptor antagonists into the RVM produce antinociception, while GABA agonists facilitate nociceptive responses [11,20]. Additionally, the iontophoretic application of the $GABA_A$ receptor antagonist bicuculline into the RVM blocked tail flick related responses attributable to "off"-cell activity [19].

Prior studies have demonstrated an antinociceptive effect of either morphine [27,28,42] or bicuculline [11,17,20] in the RVM. However, none of these studies have evaluated the contribution of these drugs in the modulation of the affective-motivational component of pain. The current study was undertaken to evaluate the effects of the opioid and GABA receptor agonists and antagonists in the RVM on an affective-motivational pain model. Additionally, we investigated the effects of opioidergic–GABAergic interactions in the RVM on the vocalization response to noxious stimulation.

2. Materials and methods

2.1. Animals

Adult male guinea pigs (*Cavia porcellus*) weighing 400–500 g were obtained from the animal care facility of the Faculty of Medicine of Ribeirão Preto (FMRP). The animals ($n = 81$) were housed in plexiglass cages (56 cm × 37 cm × 39 cm, five animals per cage) at 24 ± 1° C, on a 12-h light cycle, with free access to water and food. The experiments were carried out in compliance with the recommendations of the Brazilian Association for Laboratory Animal Science (COBEA), which are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals. The experimental procedures were approved (Proc. no. 031/2004) by the Ethical Committee for Animal Experimentation of the School of Medicine of Ribeirão Preto, University of São Paulo. All efforts were made to minimize animal suffering.

2.2. Surgical procedures

The animals were anesthetized by intra-muscular injection of 40 mg/kg ketamine plus 5 mg/kg xylazine and placed in a stereotaxic apparatus (David-Kopf Instruments, USA) with the mouthpiece 21.4 mm ventral to the interauricular line, and a guide cannula (18 mm long and 0.6 mm outer diameter) was implanted into the RVM. The cannula was placed 2.0 mm dorsal to the RVM using the following coordinates of atlas of Rössner [43] for guinea pigs: 16.4 mm caudal to the bregma, 0.0 mm lateral to the midline and −0.3 mm below the intra-aural line. The guide cannula was fixed to the skull with autopolymerizing resin and anchored with an additional screw.

2.3. Nociceptive test

For the evaluation of nociception, the animals were submitted to a vocalization test. The vocalization test consists of the application of a peripheral noxious stimulus (electric shock) that provokes the emission of a vocalization response by the animal, which was interpreted as a manifestation of pain.

A pair of non-insulated electrodes (extension of 1.5 cm) was implanted into the subcutaneous region of the thigh. The animal was placed in an acrylic box lined with nylon foam in which some movement was possible. After 20 min of acclimation to the experimental situation, the electrodes were connected an electric stimulator that released three intermittent stimuli with pulses (AC current with square waves, 100-Hz frequency, and 0.5-ms duration) of varying intensity (0.6–4.0 mA) sufficient to induce audible vocalization, which is the most frequent response elicited by guinea pigs during nociceptive stimulation. Prior to testing for noxious stimulation, a control baseline measurement was performed to determine the smallest noxious stimulus necessary to produce a vocalization response. Three consecutive stimuli were applied, and the mean amplitude of vocalization was calculated during control periods (without saline or drug microinjection). Each animal was stimulated with the lowest intensity of electrical stimulus needed to produce the vocalization response. Vocalization was induced mainly during electric stimulation and only a small number of animals showed post-stimulus vocalization. The electrical stimulus (3-s duration) induced brief motor and vocalization responses that did not persist in the intervals between stimuli. After baseline testing was concluded the peripheral noxious stimulus was then applied at 5, 15, 30, 45 and 60 min after the different drug treatments.

Vocalization was recorded with an Aiwa DM-64 microphone connected to the pre-amplifier of a polygraph. In the polygraphic recording, peak amplitude was proportional to the intensity of animal vocalization. The peak amplitude of the graphic recording of vocalization was measured in millimeters, and the mean of each response was used for quantitative evaluation.

2.4. Drugs

The drugs used in this study were morphine sulfate (opioid agonist; Sigma), naloxone hydrochloride (opioid antagonist; Sigma), bicuculline methionide ($GABA_A$ receptor antagonist; Sigma) and muscimol ($GABA_A$ receptor agonist; Sigma) diluted in saline. The doses were based on previous studies [10,30].

2.5. Experimental procedures

After five to seven days of recovery from surgery, the different experimental groups were submitted to a vocalization test, as previously described. The animals were divided into ten experimental groups.

In group 1 ($n = 8$), the animals were injected with saline as vehicle control. In groups 2 ($n = 6$) and 3 ($n = 8$), the animals were microinjected in the RVM with 2.2 and 4.4 nmol/0.2 μl of morphine, respectively. In group 4 ($n = 7$), to test the effect of opioid antagonists and agonists on the modulation of nociception, the animals were microinjected in the RVM with naloxone (2.7 nmol/0.2 μl) followed 10 min later by morphine (4.4 nmol/0.2 μl). In group 5 ($n = 8$), the animals were microinjected in the RVM with naloxone (2.7 nmol/0.2 μl) followed 10 min later by saline. In groups 6 ($n = 10$) and 7 ($n = 8$), the animals were microinjected in the RVM with 0.2 and 0.4 nmol/0.2 μl of bicuculline, respectively. In groups 8 and 9, different doses of muscimol (0.25 nmol; $n = 7$ and 0.50 nmol/0.2 μl; $n = 10$) were microinjected into the RVM. In group 10 ($n = 9$), the animals were microinjected in the RVM with muscimol (0.25 nmol/0.2 μl, a dose that has no effect per se) followed 10 min later by morphine (4.4 nmol/0.2 μl) to determine whether the effect of morphine depends on GABAergic inhibition.

Microinjections were performed with a Hamilton microsyringe (10 μl) connected to a PE-10 polyethylene tubing, attached to a Mizzy needle segment (0.3 mm outer diameter; 2.0 mm longer than the guide cannula). In all of the experimental groups, a volume of 0.2 μl was microinjected over a period of 1 min, and the Mizzy needle was left in place for an additional 40 s to avoid reflux.

2.6. Histological analysis

After the behavioral tests were completed, the injection site was identified by microinjecting 0.2 μl of 2% pontamine sky blue dye. Each animal was deeply anesthetized with sodium pentobarbital and perfused intracardially with saline followed by 10% formalin. The brains were removed and fixed in 10% formalin. Routine histological procedures were used for tissue sectioning, and the stained tissues were observed under a light microscope to determine the location of the stimulation sites according to the atlas of Rössner [43].

2.7. Statistical analysis

The results of the amplitude of vocalization were transformed into a vocalization index (VI) using the following formula: VI = (vocalization mean − control value)/control value. Data are reported as mean VI ± SEM and were analyzed statistically by repeated measures of two-way analyses of variance (ANOVA) followed by the Duncan post hoc test. The level of significance was set at $p < 0.05$.

3. Results

Microinjection of morphine (4.4 nmol/0.2 μl) into the RVM decreased the VI during painful stimulation, suggesting an antinociceptive effect (Figs. 1A and 2C). This response remained during the entire 60 min of testing and was blocked by prior administration of naloxone 2.7 nmol/0.2 μl into the same site (Figs. 1A and 2D). However, microinjection of naloxone followed by saline did not modify the VI (Figs. 1A and 2E). Two-way ANOVA demonstrated an interaction between time and treatment ($F_{(20,160)} = 2.156$; $p = 0.005$), and the post hoc test showed morphine 4.4 nmol treatment at 5 min was statistically different from saline, morphine 2.2 nmol and naloxone treatment followed by saline treatments ($p < 0.05$). At 15 min, morphine 4.4 nmol differed statistically from other treatments ($p < 0.05$), and at 30 and 45 min, the morphine treatment was different from saline, naloxone followed by morphine and naloxone followed by saline groups ($p < 0.05$). At 60 min, the morphine 4.4 nmol group differed from saline, morphine 2.2 nmol and naloxone followed by saline groups ($p < 0.05$).

The two doses of bicuculline 0.2 and 0.4 nmol/0.2 μl into the RVM produced short (5 min) and long (60 min) duration decreases in the VI, respectively (Figs. 1B and 2F and G). Two-way ANOVA demonstrated an interaction between time and treatment ($F_{(10,115)} = 2.46$; $p = 0.01$), and the post hoc test showed that at 5 min