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Research report

Rostral ventromedial medulla modulates nociception and tonic immobility behavior through connections with the A7 catecholaminergic region

Luis Felipe Souza da Silva [a], Norberto Cysne Coimbra [b], Leda Menescal-de-Oliveira [c,*]

[a] Veterinary Science Department, Center for Agrarian Sciences, University of Paraiba, 58397-000 Areia, Paraiba, Brazil
[b] Department of Pharmacology, Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP), Av. Bandeirantes 3900, 14049-900 Ribeirão Preto, SP, Brazil
[c] Department of Physiology, Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP), Av. Bandeirantes 3900, 14049-900 Ribeirão Preto, SP, Brazil

HIGHLIGHTS

- Rostral ventromedial medulla (RVM) projects directly to the A7 region.
- RVM cholinergic stimulation produces antinociception and reduces tonic immobility.
- Inhibition of A7 region interrupts behavioral and antinociceptive responses from RVM.
- A7 region is key site to the behavioral responses from cholinergic stimulation of the RVM.
- RVM receives projections from the PAG and cuneiform nucleus.

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ABSTRACT

Cholinergic stimulation of the rostral ventromedial medulla (RVM) produces antinociception and reduces the duration of tonic immobility (TI) behavior in guinea pigs. Previous studies indicated that cholinergic antinociception in the RVM is mediated through connections with the A7 catecholaminergic cell group (A7). In the current study, we tested the role of the A7 in both the antinociception and reduction of TI duration mediated by cholinergic stimulation of the RVM. In addition, we used biotinylated dextran amines (BDA) to evaluate the connections between the RVM and A7. The microinjection of the cholinergic agonist carbachol into the RVM produced antinociception and reduced TI behavior duration. These effects were blocked by prior administration of lidocaine to the A7. However, the microinjection of lidocaine into the A7 prior to saline injection into the RVM had no effect on either the nociceptive or TI responses. The microinjection of the neurotracer BDA into the RVM positively stained fibers and synaptic boutons in the A7, indicating that there are direct projections from the RVM to the A7. Taken together, our results indicate that the antinociception and reduction of TI behavior duration after cholinergic stimulation of the RVM depends on connections with the A7.

1. Introduction

Tonic immobility (TI) is an inborn defensive response, characterized by a profound state of physical inactivity and a lack of responsiveness to environmental stimuli [15]. The TI behavior is elicited by the prey, during a prey–predator interaction, with the purpose of interrupting the attack as prey movement can stimulate predator attack. In addition to the characteristic motor response, during TI, animals show an important antinociception response that permits the prey to stay immobile even during an attack. Further laboratory studies using rabbits or guinea pigs indicated that this analgesia is mediated by opioids [1,20]. With respect to the central modulation of TI and nociception, both responses seem to share sites, neurotransmitters and pathways. Previous studies have demonstrated that sites such as the periaqueductal gray (PAG), hypothalamus, amygdala and rostral ventromedial medulla (RVM) regulate both antinociception and TI defensive behavior responses [8,10,11,18,19,23,24,27].

Specifically, the RVM, which includes the nucleus raphe magnus and the adjacent ventral reticular formation, plays an important role in descending pain modulation [2,9,13]. It has recently been related to the regulation of behavioral responses as well [10,11,30]. Previous studies showed that the TI and nociceptive responses are under cholinergic, opioidergic and GABAergic modulation in the

Abbreviations: TI, tonic immobility; RVM, rostral ventromedial medulla; CCh, carbachol; A7, A7 catecholamine cell group; BDA, biotinylated dextran amines; VI, vocalization index; PAG, periaqueductal gray matter.

* Corresponding author. Tel.: +55 16 602 3333; fax: +55 16 3602 0221.
E-mail addresses: felipe@cca.ufpb.br (L.F.S. da Silva), nccoimbr@fmrp.usp.br (N.C. Coimbra), lmdolive@fmrp.usp.br (L. Menescal-de-Oliveira).

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RVM. The microinjection of opioid agonists into the RVM increased the duration of TI and produced antinociception in guinea pigs [9,11]. However, cholinergic stimulation of the RVM reduced the duration of TI and produced antinociception [10]. Interestingly, in this study, the dose of cholinergic agonist necessary to reduce TI duration was lower than the dose needed to induce antinociception, showing a higher sensitivity of the RVM to the modulation of defensive responses than to nociception during cholinergic stimulation.

With respect to cholinergic stimulation, a previous study indicated that analgesia induced by the microinjection of carbachol (CCh) into the nucleus raphe magnus was adrenergically mediated. The administration of an alpha-2 adrenergic antagonist into the spinal cord attenuated the analgesia induced by CCh in the nucleus raphe magnus [6]. Furthermore, in a subsequent study, the microinjection of tetracaine or cobalt chloride into the A7 catecholamine cell group (A7) blocked the analgesia mediated by cholinergic stimulation of the RVM [26]. This indicates that an antinociceptive effect of cholinergic stimulation of the RVM depends on projections to the catecholaminergic A7 cell group.

The current study was undertaken to evaluate whether the A7 region is an important site for the behavioral and antinociceptive responses mediated by cholinergic stimulation of the RVM in guinea pigs. In addition, we used neuronal tracers to evaluate the neuroanatomical connections between the RVM and A7.

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. The animals ($n = 70$) 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 experimental procedures were carried out in compliance with the recommendations of the Brazilian Association for Laboratory Animal Science (COBEA) and 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 with intramuscular injection of 40 mg/kg ketamine plus 5 mg/kg xylazine and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) with the mouthpiece 21.4 mm ventral to the interauricular line. A guide cannula (18 mm long, 0.6 mm outer diameter) was implanted into the RVM or bilaterally in the A7. The cannula was placed 2.0 mm dorsal to the sites using the following coordinates of the atlas of Rossner [29] for guinea pigs: 16.5 mm caudal to the bregma, 0.0 mm lateral to the midline and −0.3 mm below the intra-aural line to the RVM; 9.8 mm caudal to the bregma, 2.4 mm lateral to the midline and 1.8 mm above the intraaural line to the A7. The guide cannula was fixed to the skull with autopolymerizing resin and anchored with an additional screw.

2.3. Tonic immobility recordings

In the present study, each animal was submitted to five control maneuvers of TI induction, and the duration of the episodes was recorded. The induction of TI was carried out by holding the animal around the thorax, quickly inverting it and pressing it down into a V-shaped plywood trough (30 cm × 17 cm × 17 cm). The pressure applied by the hands of the experimenter was proportional to the resistance offered by the animal to the restraining maneuver. When the animal stopped moving, the experimenter slowly withdrew his hands, and a chronometer was activated to measure the duration (in seconds) of the response, which ended when the animal resumed the upright position. If the animal did not become motionless within 60 s, the episode was recorded as having zero duration. For group analysis, the mean of five episodes per animal was considered. It was established that the animals serve as their own controls in the same trial group, i.e., the mean of TI episodes in the control situation was compared with the means of the sham and trial situations.

2.4. Nociceptive test

For the evaluation of nociception, the animals were submitted to a vocalization test. The nociceptive 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 to an electrical 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 the 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 electrical 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 concluded, the peripheral noxious stimulus was applied 5, 15, 30, 45 and 60 min after the various drug treatments.

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

2.5. Neuroanatomical procedures

For the labeling study, five guinea pigs were implanted with guide cannulae placed 2 mm dorsal to the RVM according to the coordinates of the atlas of Rossner [29]. Three days after surgery, the animals received a microinjection of 0.2 μl of the non-fluorescent biotinylated dextran amines (BDA: 3000 MW, Molecular Probes, USA). The microinjection was performed with a Hamilton microsyringe (10 μl) connected to PE-10 polyethylene tubing attached to a Mizzy needle segment (0.3 mm outer diameter; 2.0 mm longer than the guide cannula). The BDA was microinjected over a period of 60 s, and the needle was left in place for an additional 120 s to avoid reflux. After four days of microinjection, the animals were deeply anesthetized with sodium pentobarbital and perfused intracardially with saline followed by 4% paraformaldehyde (Sigma) in 0.1 M phosphate buffer (pH 7.4). The brains were removed and fixed in paraformaldehyde (Sigma) for 4 h and transferred to 30% sucrose for two days. After cryostat sectioning (40 μm thickness), BDA labeling was visualized with a standard avidin-biotinylated horseradish peroxidase method with a nickel-intensified peroxidase 3,3′-diaminobenzidine dihydrochloride reaction. After incubation, the sections were thoroughly washed in 0.1 M phosphate buffer (pH 7.4), mounted on gelatin-coated glass slides, and counterstained using the Nissl method.

2.6. Drugs

The drugs used in this study were carbachol (0.65 and 2.7 nmol, Sigma) and 2.0% lidocaine diluted in saline and biotinylated dextran amines (BDA: 3000 MW, Molecular Probes, USA) diluted in phosphate buffer. The doses were based on previous studies [10,17].

2.7. Experimental procedures

After five days of recovering from surgery, the animals were submitted to the TI or vocalization tests, as previously described. The animals were divided into eight experimental groups.

For TI experiments each animal was tested for TI duration before the surgery (control), after surgery but with no treatment (sham) and after microinjection of different drugs treatment into RVM. In group 1 ($n = 11$), the animals were injected with saline into the A7, followed 10 min later by CCh (0.65 nmol) injection into the RVM and TI testing. In group 2 ($n = 8$), the animals were injected with lidocaine into the A7, followed 10 min later by CCh (0.65 nmol) injection into the RVM and TI testing. In group 3 ($n = 7$), the animals were injected with lidocaine into the A7, followed 10 min later by saline injection into the RVM and TI testing. In group 4 ($n = 9$), the animals were injected with saline into the A7, followed 10 min later by saline injection into the RVM and TI testing. In groups 5 ($n = 9$), 6 ($n = 8$), 7 ($n = 7$) and 8 ($n = 6$), the animals were subjected to the same drug protocols used in groups 1, 2, 3 and 4, respectively, and tested for nociceptive behavior with the vocalization test. However, the CCh dose used for the vocalization nociceptive test was 2.7 nmol.

The microinjections were performed with a Hamilton microsyringe (10 μl) connected to 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.