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Involvement of opioid and GABA systems in the ventrolateral periaqueductal gray on analgesia associated with tonic immobility

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ABSTRACT

Ventrolateral periaqueductal gray (VL-PAG) contains key neuronal circuits related to the analgesic effect involved in integrated defensive behaviors such as immobility response (IR). The latter is characterized by a reversible state of motor inhibition that can be elicited in rats under several conditions including restriction of movements (tonic immobility: TI). It is known that IR-induced analgesia can be elicited by manipulations or drugs acting on the central nervous system (CNS) at different levels. The aim of this study was to assess the role of the opioid and the GABA systems in TI-elicited analgesia. After inducing TI in naïve rats by neck clamping, the analgesic effect was evaluated by the tail-flick (TF) test. Compared to the control group, rats with TI had increased TF latency evidencing an analgesic effect. An opioid receptor agonist and antagonist were injected systemically, as well as microinjected locally in VL-PAG, as well as $GABA_A$ receptor agonist and antagonist were microinjected into VL-PAG. Under both injection schemes, morphine increased TF latency and TI duration, while naloxone blocked TI-induced analgesia. Muscimol reduced TF latency and TI duration while bicuculline increased TF latency but not TI duration. This suggests that TI-elicited analgesia was mediated by opioids at different levels of the CNS especially in the VL-PAG by inhibition of intrinsic tonic GABAergic activity. There were no additive analgesic effects of morphine or bicuculline with tonic immobility, which probably means reach a certain upper limit under such conditions.

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1. Introduction

Some structures of the central nervous system (CNS), such as amygdala, hypothalamus, periaqueductal gray (PAG), rostral ventral medulla (RVM) and the spinal cord, play a key role in pain, analgesia and related behaviors. The midbrain central gray or PAG is the midline structure of the brainstem that surrounds the mesencephalic aqueduct from the posterior commissure to the rostral locus coeruleus (Beitz, 1994), being a large and prominent structure in the mammalian midbrain. The PAG is a major component of a descending pain inhibitory system (Basbaum and Fields, 1979), with the same relative size about 10% of the midbrain cross section in humans, cats and rats (Carrive, 1993).

It is well established that the PAG can be divided into different anatomic columns, each playing a distinct role in the mediation or modulation of integrated defensive behaviors (Bandler and Keay, 1996; Carrive, 1993; Depaulis et al., 1992; Fanselow et al., 1995). Whereas the dorsal and lateral PAG are responsible for more active behaviors (e.g. confrontational defensive reactions) and non-opioid analgesia, the ventrolateral PAG (VL-PAG) is involved in the evocation of more passive behaviors (e.g. quiescence, decreased responsiveness to the environment) and opioid analgesia (Bandler and Keay, 1996; Bandler and Shipley, 1994). The immobility response (IR), a behavior characterized by a reversible state of motor inhibition, is elicited by manipulation and restriction of movement of an animal. In nature, IR can be elicited in practically every animal species by a wide variety of stimuli (e.g., visual, auditory, olfactory and tactile).

Terminal defensive behavior to certain dangerous stimuli is characterized by antinociception, immobility, and some changes in autonomic parameters. As a reaction to deep unavoidable pain, chronic injury or defeat, animals often reduce their locomotor activity and become more solitary, as well as being generally less responsive to their environment (Depaulis et al., 1994). The hyporeactive immobility evoked electrically or pharmacologically throughout the caudal VL-PAG is similar to the immobility response or tonic immobility (TI) observed in young or adult rats after: (i) predator attack or related (Blanchard et al., 1990), (ii) defeat by conspecific aggression (Miczek et al., 1982), or (iii) certain kinds of somatosensory stimuli such as (a clip or bite on the back of the neck including pup pick-ups, mouthing and the lordosis reflex) (De la Cruz and Russek, 1987; Fleischmann and Urca, 1988). Direct activation of ventral PAG neurons produces immobility and antinociception (Morgan et al., 1998). Despite its importance there are

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few physiological, pharmacological, or behavioral reports about the confluence of analgesia and TI. It is possible that besides analgesic properties, morphine exerts a facilitatory action in the nucleus raphe magnus (NRM) in medulla on behaviors in which the inhibition of motor activity predominates, as is the case for TI (Da Silva and Menescal-de-Oliveira, 2007).

It has been shown that IR caused by neck clamping involves an analgesic effect in mice (Fleischmann and Urca, 1989; Miranda et al., 2006). However it is still unclear how key mediators, such as opioids and the GABAergic system, are involved in the confluence of analgesia and TI in the VL-PAG. Thus, the aim of the present study was to confirm the analgesic effect of TI through tail-flick (TF) test and explore the participation of these two mediators in the aforementioned brain region.

2. Material and methods

2.1. Animals and housing

66 Male Wistar rats weighing 250–300 g were obtained from our animal care facilities (Harlan Laboratories was the original source). The experimental protocol for the study was according to procedures established by The Guide for the Care and Use of Laboratory Animals of the Mexican Council for Animal Care (NOM-062-ZOO-1999). Every effort was made to alleviate any distress that might be experienced by animals during this set of experiments. We used the minimum number of animals required to attain the goals of the study.

Rats were isolated in individual cages one week before tests or stereotaxic surgery, under a normal dark/light cycle (lights on at 07:00), with controlled temperature (20–22 °C) and humidity (45–55%), and free access to food and water. The rats were returned to the individual cages for one week after surgery (recovery period). All behavioral testing was made between 10:00 and 16:00.

2.2. Drugs

The following drugs were employed in the assays: morphine sulfate (MOR), a non-selective opioid receptor agonist (Mallinckrodt®, St Louis, MO, USA); naloxone hydrochloride (NAL), an unspecific opioid receptor antagonist (Tocris®, Minneapolis, MN, USA); muscimol hydrobromide (MUS), a $GABA_A$ receptor agonist; and bicuculline methiodide (BIC), a $GABA_A$ receptor antagonist (the latter two from Sigma®, St Louis, MO, USA).

2.3. Experiment 1. Behavioral testing. Measuring the analgesia elicited by tonic immobility

Naïve rats (n = 18) were tested with TF, TI and a combined TF/TI test. Al three tests were given to the same animal in random order with a three-minute intertrial interval. TF with a tail-flick test meter (Columbus Instruments®, Columbus, OH, USA), after restricting movement of the rat with an air permeable plastic container and waiting until the animal was tranquil. TI was elicited by clamping the neck with two 5 cm rubber tipped alligator clips, one on the dorsal and the other on the ventral part of the neck. Each clip exerting 1300 g/cm^2 of force on approximately 1 cm^2 of neck skin. The animal was then inverted to a supine-lateral position and gently maintained in this posture until it stopped struggling (if applicable) and remained immobile. The duration of TI was measured from the time the experimenters hand was removed until the animal recovered the prone position, with a maximum of 180 s (Miranda et al., 2014; Sandoval et al., 2011).

We assessed TI elicited-analgesia with the combined TF/TI test. Briefly, TI was induced over the surface of tail-flick test meter apparatus, placing the tail over the light beam. This procedure leaves the animal immobile without using a plastic container, and at the same time allows for the evaluation of the TF (see video clip: MORTF-TI). After each test, rats were left in a plastic cage in the same laboratory during 3 min to reduce further influence of the environment. Meanwhile, the surfaces of the table and tail-flick apparatus were cleaned with a 10% ethyl alcohol solution.

2.4. Experiment 2. Effect of systemic opioids on the analgesia elicited by tonic immobility

To different groups of rats (n = 6–7) an injection was administered of MOR intraperitoneally (ip) at a dose of 20 mg/kg of body weight. NAL subcutaneously (sc) at a dose of 5 mg/kg, or isotonic saline solution (the vehicle: VEH) for each group using same volume and injection pathway. Behavioral tests (TF, TI and TF/TI) were conducted 0 and 60 min after the injection (see Section 2.3).

2.5. Experiments 3 and 4: effect on the analgesia elicited by tonic immobility exerted by opioids and the GABAergic system in the VL-PAG

For the experiments 3 and 4, the animals were anesthetized with sodium pentobarbital (35 mg/kg, ip Pfizer®) and a guide cannula aimed at the VL-PAG was implanted: anterior −7.5 mm, lateral +0.5 mm, and ventral 7.0 mm with a 15° angle, using a stereotaxic frame (World Precision Instruments® Sarasota FL mod: 502650) and coordinates from Atlas of Paxinos and Watson (1986). The guide cannula was held in place to the skull with two screws and dental acrylic, and with a stylet inserted into the guide.

One week after stereotaxic surgery, the drugs were injected into the VL-PAG via the cannula (31-gauge × 10 mm) which extended 1 mm beyond the tip of the guide cannula. The injection cannula was connected to a 1-μL syringe (Hamilton Co., Reno, NV, USA) with PE 20 tubing filled with sterile water. The drugs were microinjected through the cannula for 60 s, and for another 60 s the injection cannula was set to avoid backflow of the drug. Meanwhile any struggling movement of a rat was gently restricted and calm movements were allowed. The stylet was returned to guide cannula after all behavioral tests.

The drugs employed were MOR (5, 10 and 20 μg/0.5 μL; n = 7–9), NAL (5, 10 and 20 μg/0.5 μL; n = 5–8), MUS (1, 2 and 3 nmol/0.5 μL; n = 5–8), BIC (0.02, 0.2 and 3 nmol/0.5 μL; n = 5–9), or an isotonic saline solution as VEH in an equivalent volume randomly assigned. Each animal received from one to four microinjections into VL-PAG of the corresponding drug or VEH with 48 h separating each test session (thus modifying the 24 h period of Morgan and Clayton, 2005). MOR and NAL were employed for experiment 3, while BIC and MUS were used for experiment 4.

TF and TF/TI tests were used with each animal (in random order) 2 min after every drug microinjection, with a 3 min intertrial interval. All experiments were videotaped to enable review.

2.6. Histology

After the behavioral experiments, animals were overdosed with a lethal injection of sodium pentobarbital (90 mg/kg, ip). Intracardiac formaldehyde (4%) perfusion was followed by removal of the brain, which was then placed in formaldehyde (15%). This organ was sectioned coronally (100 μm) with vibratome (World Precision Instruments®, Sarasota FL), dyed with Cresyl Violet (Sigma®, St Louis, MO) and viewed under a microscope (Nikon® SM-10) to locate the microinjection site (using the coordinates of Paxinos and Watson, 1986). For experiments 3 and 4, data was considered only from animals with a verified location of the cannula in the VL-PAG.

2.7. Data analysis

All data in the text and figures, unless otherwise stated, are presented as the mean ± SEM. For experiment 1, the Student's t-test was performed to compare differences considering TF, TI, and TF/TI. For experiment 2, three-way repeated measures analysis of variance

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