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Research report

Role of homocysteic acid in the guinea pig (*Cavia porcellus*) anterior cingulate cortex in tonic immobility and the influence of NMDA receptors on the dorsal PAG

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ABSTRACT

Tonic immobility (TI) is an innate defensive behaviour elicited by physical restriction and postural inversion, and is characterised by a profound and temporary state of akinesis. Our previous studies demonstrated that glutamatergic stimulation of the dorsomedial/dorsolateral portion of periaqueductal gray matter (dPAG) decreases the duration of TI in guinea pigs (*Cavia porcellus*). Furthermore, evidence suggests that the anterior cingulate cortex (ACC) constitutes an important source of glutamate for the dPAG. Hence, in the current study, we investigated the effects of microinjection of the excitatory amino acid (EAA) agonist DL-homocysteic acid (DLH) and the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 into the ACC on the duration of TI in guinea pigs. We also assessed the effect of the NMDA receptor antagonist (MK-801) into the dorsal periaqueductal gray matter (dPAG) prior to DLH microinjection into the ACC on the TI duration in the guinea pig. Our results demonstrated that DLH microinjections into the ACC decreased the duration of TI. This effect was blocked by previous MK-801 microinjections into the ACC or into the dPAG. The MK-801 microinjections alone did not influence TI duration. These results provide the new insight that EAAs in the ACC can decrease the duration of TI. The mechanism seems to be dependent on the NMDA receptors present in the ACC and in the dPAG.

1. Introduction

Tonic immobility (TI) is an inborn defence response characterised by a dramatic and profound state of motor inhibition. It is elicited in a wide variety of species under fear circumstances and is associated with a brief period of physical restraint. TI is considered to be the last resource used by prey in a sequence of distance-dependent antipredator behaviours [30,43]. Its adaptive value has been associated with the state of akinesis, which seems to reduce the probability of continued predatory attack [45,51].

Results from our laboratory have showed the role of some of the neural substrates involved in TI modulation [13,14,16,36]. In this context, Ramos Coutinho et al. [42] demonstrated that the microinjection of DL-homocysteic acid (DLH), an excitatory amino acid (EAA) agonist, into the dorsolateral/dorsomedial portion of the periaqueductal gray matter (dPAG) produces a decrease in TI duration in the guinea pig. This effect was blocked by previous administration of MK-801 (NMDA receptor antagonist) into the same site. These results were expected since the dPAG seems to be strongly involved in the mediation of active strategies to cope with aversive situations [3,10].

Interestingly, the Ramos Coutinho et al. [42] study also raised questions concerning the origin of glutamatergic projections that could be capable of releasing EAA into the dPAG and causing early disruption of the TI duration in some specific situations. Beitz and Williams [5] used retrograde trace techniques combined with immunohistochemical techniques for assessing glutamate to demonstrate that the cingulate cortex sends one of the most robust projections of EAA to the dPAG in rats. Other detailed works demonstrated a high density of axons from the anterior cingulate cortex (ACC) to the dPAG in the rat [47] and monkey [2].

The ACC is part of the rostral limbic system, which is involved in modulation of several affective and motivational responses such as conditioned fear, anxiety, pain, vocalisation and posttraumatic stress disorder [8,11,18,26,31]. The extensive connections with the dPAG may be important in the regulation of these responses. In accordance with Floyd et al. [17], the projections between the ACC and dPAG comprised part of the circuitry engaged in generating active emotional coping in response to stress that has a strong psychological component. Taken together, these data provide support for our hypothesis that the ACC modulates TI responses by releasing EAA into the dPAG. In order to address this issue, the current study investigated the effects of DLH and MK-801 injection into the ACC on the TI duration in the guinea pig, as well as the effect of MK-801 microinjection into the dPAG prior to ACC activation by DLH on the TI duration in guinea pigs.

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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 ($n = 71$) were kept in Plexiglas-walled cages (56 cm × 37 cm × 39 cm, five animals per cage) in a room maintained 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. 069/2007) by the Ethical Committee for Animal Experimentation of the School of Medicine of Ribeirão Preto, University of São Paulo. All efforts were made to minimise animal suffering. The guinea pigs were selected for this study since they are considered moderately susceptible to tonic immobility [29].

2.2. Tonic immobility recording

In the present study, each animal was subjected to five control procedures of TI induction, and the duration of the episodes was recorded. Induction of TI was performed by holding the animal around the thorax with the hands, quickly inverting it and pressing it down into a V-shaped plywood trough (30 cm × 17 cm × 17 cm). The restraint and postural inversion maneuvers used in the laboratory are an attempt to simulate the preying condition. This restraining was applied by the hands of the experimenter and it was proportional to the resistance offered by the animal to the induction of TI. 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 an upright position. If the animal did not become motionless within 60 s, the episode was recorded as having zero duration. For group analyses, the mean of five episodes per animal was considered.

2.3. Surgical procedures

One day after the control TI episode, the animals were anaesthetised by intramuscular 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 below the interauricular line. A guide cannula (14 mm long and 0.6 mm outer diameter, prepared from a hypodermic needle) was implanted into the ACC (experiments 1 and 2) and into the dPAG (experiment 2). Stereotaxic coordinates for the ACC were 3.2–1.1 mm rostral to the bregma, 0.2–0.6 mm lateral to the midline, and 4.7 mm above the intraural line. The parameters for the dPAG were 9.4–9.8 mm caudal to the bregma, 0.6–0.8 mm lateral to the midline, and 3.2–3.4 mm above the intraural line. These references were obtained from the Rössner atlas [44] for guinea pigs. The guide cannula was lowered to a depth of 1 mm above the target region and fixed to the skull by means of autopolymerising resin and an additional anchoring screw. All animals were allowed to recover from surgery for 6–7 days. The choice of the ACC region as a site for microinjecting was the anterior region of the ACC which is more related with the modulation of responses involved in emotional contexts [15,53].

2.4. Experimental procedures

After recovery from surgery for 6–7 days, the different animal groups were subjected to a TI session with cannulas implanted into the ACC (experiment 1) and into the ACC plus dPAG (experiment 2) and then microinjected with the drugs and subjected to the TI procedure.

In experiment 1, we tested the effect of DL-homocysteic acid (DLH) and of the MK-801 microinjection into the ACC on TI duration. In this experiment, the animals for which the cannula was implanted in the ACC were assigned to one of four groups. In group 1 ($n = 8$), the animals were microinjected with saline (0.2 μl); in group 2 ($n = 11$), the animals were microinjected with DLH (30 nmol/0.2 μl); in group 3 ($n = 10$), the animals were microinjected with MK-801 (3.6 nmol/0.2 μl) followed 1 min later by DLH (30 nmol/0.2 μl); in group 4 ($n = 8$), the animals were microinjected with MK-801 (3.6 nmol/0.2 μl).

In experiment 2, we evaluated the role of the functional block of NMDA receptors within dPAG 1 min before the microinjection of DLH into the ACC on the TI duration. In this experiment, the animals were assigned to four groups. In group 1 ($n = 10$), the animals were microinjected with saline (0.2 μl) into dPAG previously to the DLH (30 nmol/0.2 μl) microinjection into the ACC; in group 2 ($n = 8$), the animals were microinjected with MK-801 (3.6 nmol/0.2 μl) into the dPAG prior to DLH (30 nmol/0.2 μl) microinjection into the ACC; in group 3 ($n = 8$), the animals were microinjected with MK-801 (3.6 nmol/0.2 μl) into the dPAG followed 1 min later by saline (0.2 μl) into the ACC; in group 4 ($n = 8$), the animals were microinjected with saline (0.2 μl) into the dPAG 1 min before the microinjection of saline (0.2 μl) into the ACC.

Microinjections were performed with a Hamilton microsyringe (5.0 μl) connected to a PE-10 polyethylene catheter, which in turn was coupled to a Mizzy needle segment (0.3 mm outer diameter; 1.0 mm longer than the guide cannula). In all 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.

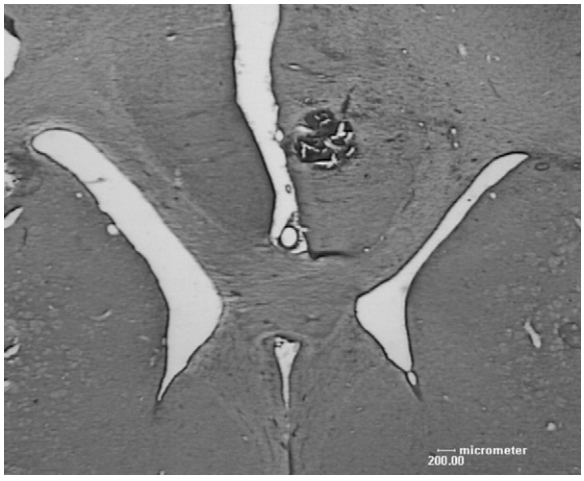

Fig. 1. Photomicrograph of a coronal section of the guinea pig's brain showing the microinjection site into the anterior cingulate cortex (ACC).

2.5. Drugs

The drugs used were DLH (DL-homocysteic acid from Aldrich) and MK-801 (maleate salt, from Sigma St. Louis, MO, USA), diluted in saline. The doses were based on studies by Ramos Coutinho et al. [42].

2.6. Histological analysis

Following all tests, the injection site was marked by microinjecting 0.2 μl of 2% pontamine sky blue dye. Each animal was deeply anaesthetised with sodium pentobarbital and then intracardially perfused with saline followed by 10% formalin. Brains were removed, maintained in formalin solution for 24 h, and cryoprotected in 30% sucrose for at least 48 h. Serial 40 μm coronal brain sections were cut using a cryostat (−21 °C), mounted on gelatin-coated slides, and stained with Cresyl Violet (0.25%; Sigma–Aldrich, St. Louis, MO, USA) to localize the positions of the microinjection sites according to the atlas of Rössner atlas [44]. The microinjection sites were evaluated by microscopic examination. Fig. 1 shows a photomicrography of a coronal section representing the microinjection site in ACC.

2.7. Statistical analysis

The TI results are reported as the mean ± standard error of the mean (S.E.M.) for the mean duration of five TI episodes. Data were analysed by a repeated-measures analysis of variance (ANOVA). The degree of freedom of the repeated measure (treatment) was corrected by the Huynh–Feldt ε parameter. The Duncan test was used to determine the difference between treatments, with the level of significance set at $P < 0.05$.

3. Results

3.1. Experiment 1

Microinjection of DLH (30 nmol/0.2 μl) into the ACC promoted a decrease in the duration of TI in guinea pigs and prior application of MK-801 (3.6 nmol/0.2 μl) blocked this response (Fig. 2A and B, respectively). The repeated-measures ANOVA indicated a significant difference between treatments ($F_{2,20} = 4.07$; $P = 0.033$) and a Duncan's post-test showed that treatment with DLH differed from the other treatments (control and sham), which did not differ from one another. In addition, microinjection with either MK-801 alone or saline did not alter the mean duration of TI when compared to the control and sham situations ($P > 0.05$; Fig. 2C and D, respectively). Fig. 2E shows a schematic representation of the microinjection sites in the ACC.

3.2. Experiment 2

Microinjection of DLH (30 nmol/0.2 μl) into the ACC 1 min after saline (0.2 μl) microinjection into the dPAG promoted a decrease in the duration of TI in guinea pigs. The ANOVA for repeated measures