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Research report

Nitric oxide involvement and neural substrates of the conditioned and innate fear as evaluated in the T-maze test in rats

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Abstract

The L-arginine/nitric oxide (NO) pathways are widely distributed in the central nervous system (CNS) and have been implicated in the modulation of anxiety. The elevated plus-maze (ETM) is an animal test pharmacologically validated for the study of experimental anxiety in rats, designed to evaluate inhibitory avoidance (AVOID) learning and one-way escape (ESC) from open arms, thought to represent learned (conditioned) and innate (unconditioned) fear, respectively. The aim of the present study was to evaluate the effect of prior treatment with the NO-synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) on both AVOID and ESC behavior of rats in the ETM, when applied to different cerebral regions associated with defensive behaviors. Central treatment with L-NAME (50, 100, 400 and 800 nmol) did not impair the AVOID response through the trials and had no effect on the ESC behavior. Nevertheless, animals treated with L-NAME at 200 nmol into the lateral ventricle (LV), basolateral amygdala (BLA), dorsolateral periaqueductal gray (dlPAG) matter, lateral septal nucleus (LSN), but not in the bed nucleus of *stria terminalis* (BNST), displayed impaired AVOID2 in comparison to the control group. Thus, our results suggest that NO may underlie learned fear in the ETM via BLA, dlPAG and LSN, but not BNST. These results are compatible with the proposal that NO exerts a positive modulatory role on defensive reactions in rats, exerting among them an anxiogenic-like effect as evaluated in rats submitted to ETM.

Keywords: NO; Fear; T-maze; Basolateral amygdala; dlPAG; Lateral septum

1. Introduction

The gaseous messenger nitric oxide (NO) has been implicated in a wide range of behaviors. NO plays an important role in several brain functions and/or dysfunctions, including regulation of neuronal excitability, synaptic plasticity, long-term potentiation (LTP) and depression (LTD), neurotoxicity and neuroprotection [30,45,46,52]. NO is synthesized by a group of enzymes named NO-synthases (NOS), which catalyze the conversion of L-arginine (L-Arg) towards L-Citruline, producing NO as a by-product [38]. In the brain, NOS has been located to several regions of the limbic system involved in the control of anxiety states, such as the amygdaloid nucleus, dorsolateral periaqueductal gray (dlPAG) matter, bed nucleus of *stria terminalis* (BNST), lateral septal nucleus (LSN), hypothalamic paraventricular nucleus, and the hippocampus [2,8,14,26,33,41,48,58]. The literature shows that intra-dlPAG administration of NOS inhibitors induces anxiolytic-like effects in the elevated plus-maze (EPM) [25], whereas injection of NO-donors produces defensive reactions characterized by wild running and jumps [12,14]. These reactions were associated with intense neuronal activation (as measured by c-*fos* expression) of brain areas related to defensive behaviors, such as the anterior and medial hypothalamus, superior and inferior colliculus, raphe and amygdaloid nuclei [13]. Altogether, these results suggest that NO is a critical mediator in anatomical areas closely involved in the modulation of anxiety-like behaviors.

The elevated T-maze (ETM) is a validated animal test for the behavioral and pharmacological study of experimental anxiety in rats [24,37,56,62]. It is derived from the elevated plus-maze by excluding an enclosed arm and it is designed to evaluate, firstly, AVOID learning and, secondly, ESC from the open arms. These are thought to represent learned (conditioned) and innate (unconditioned) fear, respectively [24]. In the ETM, both AVOID and

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ESC measurements represent defensive behaviors motivated by the animal's fear of the open spaces of the maze, which present fearful/anxiogenic features to rodents [39].

The aim of the present study was to evaluate the effect of prior treatment with the Nω-nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor, on both AVOID and the ESC behavior of rats in the ETM, when applied to different cerebral regions containing NOS-positive neurons and associated with defensive behaviors.

2. Material and methods

2.1. Animals

Male Wistar rats obtained from the local breeding facilities, weighing approximately 300 g, were used in the present study. Animals were housed in a colony room in groups of five in polypropylene cages (49 cm × 34 cm × 16 cm) with a period of adaptation of 7 days with free access to food and water, under a light/dark cycle of 12 h (lights on 06:00 h). Conditions of animal housing and all experimental procedures followed the recommendations of the National Institutes of Health (1985) and were approved by the local Ethics Committee (#23080.020840//2003-01/UFSC).

2.2. Drugs

L-NAME was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in phosphate buffered saline (PBS, pH 7.4; Sigma Chemical Co.). The control group received only PBS by the same route and in equal volume.

2.3. Stereotaxic surgery

Animals were anesthetized with a mixture of ketamine and xylazine (v/v; 1 ml/kg body weight) and stainless steel guide cannula (22 gauge) were unilaterally implanted from the bregma in the lateral ventricle (LV, AP = +0.8 mm, ML = ±1.5 mm, DV = −2.5 mm), basolateral amygdala (BLA, AP = −2.8 mm, ML = ±4.8 mm, DV = −7.5 mm), bed nucleus of *stria terminalis* (AP = −0.4 mm, ML = ±1.5 mm, DV = −5.5 mm), dorsolateral periaqueductal gray matter (AP = −7.6 mm, ML = ±1.9 mm; DV = −2.0 mm, at an angle of 22° with sagital plane) and the lateral septal nucleus (AP = −0.2 mm, ML = ±1.0 mm, DV = −2.8 mm). Each cannula was fixed with polyacrylic cement anchored to the skull with stainless steel screws and was plugged with stainless steel stylets. The experiments were performed after a 1-week post-operative recovery period.

2.4. Drugs and central administration procedures

During the drug administration, animals were gently held and a removable injector was inserted into the guide cannula, extending 1 mm beyond the guide tip, except for dlPAG, where the injector needle was 3.2 mm longer than the guide cannula. The injector was linked to a 5 μl Hamilton syringe and a volume of 0.5 μl was injected over a 30 s period. The volume of injection was selected according to previous literature data [25]. The injector remained in the guide cannula for an additional 30 s after the infusion.

2.5. Elevated T-maze

The maze was made of Plexiglas as described by Viana et al. [56] and modified by Calixto et al. [4]. It had three arms of equal dimensions (50 cm length × 10 cm width × 0.25 cm height), with one of them enclosed by clear lateral walls (40 cm high), and two other open arms, surrounded by a Plexiglas ledge of 1 cm height, elevated 50 cm above the floor. The enclosed arm was perpendicular to the two opposed open arms, in such a way as to form a "T". The apparatus was placed in a small, closed room lit by a 15 W red light that provided 3 lux in both open and closed arms.

2.6. Experimental procedures

A group of animals was assigned to receive either L-NAME (50, 100, 200, 400, 800 nmol), or the equivalent volume of PBS. Five minutes after the drug administration into the LV, each animal was placed at the end of the enclosed arm and the time required to leave the arm with all four paws was recorded and used as the baseline latency (BL; 300-s cut-off time). After the enclosed arm exit, the animal was allowed to freely explore the maze for 10 s, after which they were gently withdrawn from the maze and placed inside a polypropylene cage (30 cm × 20 cm × 15 cm) for 30 s. The animal was returned to the end of the enclosed arm for two successive trials; and the time elapsed to leave the arm was recorded again, as described for BL (Avoidance 1, AVOID1, and Avoidance 2, AVOID2). Thirty seconds after the AVOID2 measurement, the animal was placed at the end of the left open arm and the time required to enter the enclosed arm with all four paws was recorded (one-way escape, ESC). The same procedure was used for the other regions using two selected doses of L-NAME (50 and 200 nmol). After each animal, the maze was cleaned with wet (alcohol 10%, v/v) and dry cloths. Any animal that fell off the maze was excluded from the experiment. All experiments were carried out between 13:00 and 17:00 h. An experienced observer blind to the treatments remained within the room during the experiment.

2.7. Histological analysis

At the end of the experimental procedures, all rats were sacrificed with an overdose of pentobarbital and then transcardially perfused with saline solution (NaCl 0.9%) followed by 10% formalin. The brains were removed immediately and post-fixed in the same fixative solution containing 20% sucrose. Brains were then frozen, and 30 μm thick serial sections were cut in the frontal plane. The sections were mounted on gelatin-coated slides, and stained with thionin. Injection sites were localized under the light microscope with no knowledge of the behavioral data. All animals with mismatched injections were discarded from the statistical analysis (less than 5%).

2.8. Statistical analysis

All values are expressed as means ± S.E.M. Data were analyzed by two-way ANOVA with repeated measures, with treatment as the independent factor and trials (BL, AVOID1 and AVOID2) as the repeated measure. ESC was analyzed by one-way ANOVA. When appropriate, both AVOID and ESC parameters were compared by the Student–Newman–Keuls' *post hoc* test. Differences were considered significant at $p < 0.05$. All tests were performed using the *Statistica* version 6.0® software package and the graphics were drawn with the GraphPad Prism version 4.0® Software.

3. Results

3.1. Lateral ventricle (LV)

Fig. 1A shows the BL, AVOID1, and AVOID2 behavior of rats treated with either PBS or L-NAME (50–800 nmol) injected into the LV. Two-way repeated measures ANOVA revealed a significant effect of treatment ($F_{5,44} = 3.056$; $p < 0.019$), trials ($F_{5,44} = 65{,}212$; $p < 0.0001$) and interaction treatment × trial ($F_{5,44} = 2{,}203$; $p < 0.025$). Student–Newman–Keuls' test showed that the AVOID2 latency was significantly higher in the groups treated previously with either PBS ($p < 0.001$) or L-NAME (50, 100, 400 and 800 nmol; $p < 0.001$), but not L-NAME 200 nmol, compared to the respective BL ($p > 0.05$). Treatment with L-NAME 200 nmol displayed impaired AVOID2 ($p < 0.05$), and promoted an increase in the ESC latency when compared to the control group (PBS; $F_{5,44} = 3.13$; $p < 0.05$), although the other doses did not promote any significant changes in ESC (Fig. 1B).