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Research report

Role of ventral hippocampal nitric oxide/cGMP pathway in anxiety-related behaviors in rats submitted to the elevated T-maze

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ABSTRACT

The L-arginine/nitric oxide (NO)/cGMP pathways have been implicated in the control of a variety of physiological mechanisms and are believed to participate in the modulation of anxiety in the CNS. The aim of this study was to investigate the effects of N^G-nitro-L-arginine-methyl-ester (L-NAME), a non-selective inhibitor of NO synthase (NOS); 7-nitroindazole (7-NI), a preferential inhibitor of neuronal NOS; and sodium nitroprusside (SNP), an NO donor, administered into the ventral hippocampus (VH) of rats submitted to the elevated T-maze (ETM). The ETM, an animal model derived from the elevated plus-maze, allows the measurement of two defensive behavioral responses in the same rat: inhibitory avoidance and escape. Results showed that L-NAME and 7-NI impaired the acquisition of inhibitory avoidance and prolonged escape latency in the ETM, suggesting an anxiolytic-like and panicolytic-like effect, respectively. SNP facilitated the acquisition of inhibitory avoidance without interfering with escape performance, suggesting an anxiogenic-like effect. Treatment with methylene blue did not alter *per se* any of the behavioral responses measured in the ETM, but blocked the effect promoted by SNP. Thus, altogether these results suggest that NO in the VH is critically involved in the modulation of defensive behavior of rats exposed to the ETM.

1. Introduction

There are several reports in the literature showing that the L-arginine (L-Arg)/nitric oxide (NO)/cGMP pathway is widely distributed in the limbic system and it has been implicated in the modulation of anxiety-related behaviors, supporting the involvement of NO as a modulator of experimental anxiety.

In this regard, Beijamini and Guimarães [6] reported that exposure to an elevated plus-maze activates nitric oxide synthase (NOS)-containing neurons in brain areas related to fear/anxiety. The acute inhibition of NO synthesis using a variety of antagonists promotes anxiolytic-like or anxiogenic-like effects depending on the animal, drug treatment and the methodology used to investigate the behavior [17,26,62,66]. In addition, it has been shown that the inhibition of cGMP degradation by sildenafil, a phosphodiesterase-5 inhibitor, can result in an anxiogenic-like effect, while the inhibition of cGMP production by methylene blue (MB) is anxiolytic [40]. Recently, we showed that NO may underlie learned fear in the elevated T-maze (ETM) via its actions in the basolateral amygdala, dorsolateral periaqueductal gray matter and lateral septal nucleus, but not in the bed nucleus of stria terminalis [12]. These results are in agreement with the proposal that NO exerts a positive modulatory influence on defensive reactions in rats, i.e. an anxiogenic-like effect as evaluated in rats submitted to the ETM.

The hippocampus is a brain structure involved in the modulation of several anxiety-related behaviors [32], in the innate defensive responses to various threatful stimuli [4,8,9,14,38]. Additionally, it is well-known that this structure expresses neuronal NOS (nNOS)-immunoreactive neurons [23,63,67]. Furthermore, there is considerable evidence suggesting that the hippocampus may be functionally differentiated into dorsal and ventral subregions [55], raising the possibility of a dual role in modulating both cognitive functions and defensive behaviors [25,48]. Thus, the dorsal part appears to be more involved in memory-related functions, whereas the ventral part is involved in anxiety-related phenomena (for a review, see [5]). However, this segregation is a controversial issue and several reports do not support this proposition [25,28].

Studies investigating the involvement of hippocampal NO in experimental anxiety have generated conflicting results, especially when the elevated plus-maze was used. Anxiogenic-like effects were found after intra-CA1 injection of the non-selective NOS inhibitors [N(ψ)-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine-methyl-ester (L-NAME)] [45,56]. On the other hand, an anxiolytic-like effect was found after L-NAME injection

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into the dentate gyrus of the dorsal hippocampus [24]. Moreover, the administration of 7-nitroindazole (7-NI), a preferential neuronal inhibitor of nNOS, and two NO donors [S-nitroso-N-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP)] into the ventral hippocampus did not change *per se* the behavioral parameters in this experimental paradigm [27].

In the present study, we investigated the experimental anxiety in rats evaluated in the ETM. The ETM is a behavioral procedure derived from the elevated plus-maze by sealing the entrance to one of the enclosed arms, and which allows the parallel measurement of responses related to both conditioned (inhibitory avoidance) and innate (one-way escape) types of fear in the same experimental subject. Based on extensive pharmacological validation, inhibitory avoidance and escape behavior in this test have been related to generalized anxiety and panic disorder, respectively [30,52]. The ETM was used in order to address the question of whether the ventral hippocampal L-Arg/NO/cGMP pathway is involved in the modulation of experimental anxiety. We used drugs with distinct actions on the L-Arg/NO/cGMP pathway: L-NAME and 7-NI, a non-selective and a preferential neuronal NOS enzyme inhibitor, respectively, methylene blue (MB), a non-selective inhibitor of soluble guanylate cyclase, and SNP, an NO donor.

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) for a 7-day period of adaptation 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 (# 0250/CEUA and 23080.020840//2003-01/UFSC).

2.2. Drugs

N^G-nitro-L-arginine-methyl-ester (L-NAME; 50, 200 or 400 nmol) was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in phosphate buffered saline (PBS, pH 7.4; Sigma Chemical Co., St Louis, MO, USA). 7-Nitroindazole (7-NI; 10 or 20 nmol), sodium nitroprusside (SNP; 80 nmol), and methylene blue (MB; 10 nmol) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The solutions were prepared immediately before use in PBS, except for 7-NI, which was dissolved in 5% DMSO in PBS. The control group received only PBS, or 5% DMSO plus PBS, by the same route and in the same volume. The doses were chosen based on previous studies [12,22,27,57].

2.3. Stereotaxic surgery

Animals were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) and stainless steel guide cannulas (22 gauge) were unilaterally implanted 3.0 mm above the ventral portion of the hippocampus (VH, AP = −4.5 mm, ML = ±5.5 mm, DV = −5.0 mm). Each cannula was fixed with polyacrylic cement anchored to the skull with stainless steel screws and was plugged with a stainless steel stylet. Animals underwent a 1-week post-operative recovery period before experiments were performed.

2.4. Central administration procedures

For drug administration, animals were gently held and a removable injector was inserted into the guide cannula, extending 3 mm beyond the guide tip. The injector was linked to a 5-μL Hamilton syringe and a volume of 0.5 μL was injected over a 30-s period. This injection volume was selected according to data in the literature [12,33]. The injector remained in the guide cannula for an additional 30 s to allow for diffusion of the injected substances away from the tip.

2.5. Elevated T-maze

The maze was made of Plexiglas as described by Viana et al. [65] and modified by Calixto et al. [13]. It was elevated 50 cm above the floor and 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 1 cm in height. 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 only by a 15-W red light that provided 3 lux in both open and enclosed arms.

2.6. Experimental procedures

2.6.1. Experiment 1

To evaluate the behavioral effects of the intra-VH injection of L-NAME and 7-NI, a non-selective and a preferential inhibitor of the nNOS enzyme, respectively, animals were assigned to receive 0.5 μL of PBS, L-NAME (50, 200 or 400 nmol) or 7-NI (10 or 20 nmol). Five minutes after the last drug administration, 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). Once they had exited the enclosed arm 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, to give avoidance 1 (AVOID 1, and avoidance 2 (AVOID 2). Thirty seconds after the AVOID 2 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 maze was cleaned with wet (alcohol 10%, v/v) and dry cloths after each animal. 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.

2.6.2. Experiment 2

To evaluate the behavioral effects of intra-VH injection of MB, a non-selective inhibitor of the soluble enzyme guanylate cyclase, and SNP, a donor of NO, animals were divided in four groups receiving two injections of 0.25 μL of each drug as follows: PBS + PBS, PBS + SNP 80 nmol, MB 10 nmol + PBS or MB 10 nmol + SNP 80 nmol. Five minutes after the last drug administration, animals were submitted to the behavioral test, as described in Experiment 1.

2.7. Histological analysis

At the end of the experimental procedures, animals received a 0.5-μL microinjection of Evans Blue in the VH. 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 Giemsa (Sigma–Aldrich). 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.

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, AVOID 1 and 2) as the repeated measure. Escape latency (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 graphs were drawn with the GraphPad Prism version 4.0® Software.

3. Results

3.1. Histology

A representative photomicrograph of an injection site in the VH is shown in Fig. 1. Few animals (less than 5% in total) presented misplaced injection sites (outside the VH).

3.2. Experiment 1

Fig. 2A shows BL, AVOID 1, and AVOID 2 behaviors of rats treated with either PBS, or L-NAME (50, 200 or 400 nmol), injected into the VH. Two-way repeated measures ANOVA revealed a significant effect of treatment ($F_{3,93} = 8.10$; $p < 0.001$), trials ($F_{2,93} = 32.58$; $p < 0.001$) and in the interaction treatment × trial ($F_{6,93} = 3.85$; $p < 0.05$). Student–Newman–Keuls' test showed that the AVOID 2 latency was significantly higher in the groups previously treated with either PBS ($p < 0.05$) or L-NAME (50 and 400 nmol; $p < 0.001$), in comparison to the respective BL ($p < 0.05$). Treatment with L-NAME 200 nmol decreased AVOID 2 ($p < 0.05$; Fig. 2A) and promoted an increase in ESC latency when compared to the control group ($F_{3,30} = 4.30$, $p < 0.05$; Fig. 2B).