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Noradrenaline microinjected into the dorsal periaqueductal gray matter causes anxiolytic-like effects in rats tested in the elevated T-maze

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ABSTRACT

Aims: The dorsal periaqueductal gray matter (dPAG) is involved in the integration of behavioral and cardiovascular responses caused by fear and anxiety situations. Some studies suggest an involvement of noradrenergic neurotransmission in the dPAG in anxiety modulation, however, there is no evidence about its role in panic attacks. The goal of this work was to study the effect of NA microinjection in dPAG in rats submitted to the elevated T-maze (ETM).
Materials and methods: Male Wistar had a cannula implanted in the PAG where it was injected NA in the doses of 1, 3, 15, 45 nmol/50 nl or artificial cerebrospinal fluid previous the ETM test.
Key findings.: NA intra-dPAG decreased inhibitory avoidance behavior in the ETM without changing escape, indicating only an anxiolytic-like effect. Furthermore, the microinjection of NA did not change the general exploratory activity of the animals submitted to the open field test, suggesting that the anxiolytic-like effect is not due to an increase in exploratory activity.
Significance: The results indicate an involvement of noradrenergic neurotransmission in the dPAG in defensive reactions associated with generalized anxiety, but not as main mechanisms for the panic, in rats submitted to the elevated T-maze providing support for other research aimed at improving the treatment of generalized anxiety.

1. Introduction

Generalized anxiety and panic have a high prevalence throughout life [1,2]. Studies have indicated that noradrenaline (NA) has an important role in anxiety disorders [3,4]. Tricyclic antidepressants, monoamine oxidase inhibitors and selective noradrenaline re-uptake inhibitors facilitate noradrenergic activity and are prescribed for anxiety disorder treatment [5–7].

The periaqueductal gray matter (PAG) is a midbrain structure that receives noradrenergic pathways of the *Locus Coeruleus* (*LC*) and is involved in the integration of behavioral and autonomic responses in defensive reactions such as escape and inhibitory avoidance, which are associated to panic and generalized anxiety, respectively [8–10]. In addition, studies have showed the presence of noradrenergic terminals in the PAG [11–13].

Electrical stimulation of the dorsal region of the PAG (dPAG) has been considered a panic model, because its stimulation caused behavior related to this disorder in humans and rodents [14,15].

Previous studies described an anxiolytic-like effect of NA in the dPAG of rats submitted to the elevated plus maze (EPM) and to the Vogel punished licking test, both being animal models widely used for studying anxiety [16,17], suggesting a modulation by noradrenergic neurotransmission in the dPAG of anxiety-like behavior [4].

The elevated T-maze (ETM) is a behavioral model derived from the EPM that provides the measurement in the same rat of a generalized anxiety and a panic defensive response, i.e., inhibitory avoidance and escape, respectively [18,19]. Although the role of NA in anxiety modulation is known, few studies have investigated the involvement of noradrenergic neurotransmission in dPAG during defense reaction. So, the present work objected to evaluate the effects of NA microinjection in the dPAG in rats exposed to the ETM.

2. Methods

2.1. Animals

Male Wistar rats weighing 240–260 g (n = 35), from the Central Animal Care Unit of the University of Londrina (Paraná, Brazil) were used. Animals were housed in five animals per cage (cage size: 30 × 13 × 19.5 cm). The rats were kept in a room with standard laboratory conditions with free access to food and water, controlled room

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temperature (22 ± 1 °C), and a cycle of 12 h light/dark (lights on at 07:00 h). Procedures were performed with the approval of the Ethics Committee for Animal Experimentation of the State University of Londrina (n°15126.2013.99). All efforts were made to minimize animal suffering.

2.2. Surgery

Animals were anesthetized with tribromoethanol (250 mg/Kg, i.p.; Aldrich Chemical Co. Inc., Milwaukee, USA) and fixed to a stereotaxic apparatus (Insight, Ribeirão Preto, BR). A stainless steel guide cannula (23G; 11 mm long) was implanted 1 mm above the injection site according to Pelosi et al. [4]. Stereotaxic coordinates used to cannula implantation in the dPAG were: anteroposterior = +2.7 mm from, the interaural line, lateral = +2.5 mm from the sagittal suture, vertical = −4.7 mm deep from the, skull with a lateral inclination of 26° [4]. A tight-fitting mandrel of 0.2 mm diameter was kept inside the guide cannula to prevent its occlusion during the recovery period. As a prophylactic measure at surgery, animals received an intramuscular injection of 0.2 ml of benzylpenicillin (Pentabiotico®, Fontoura-Wyeth, SP, Brasil, 80,000 UI, intramuscular) and the non-steroidal analgesic flunixina meglumina (Banamine®, Schering Plough, Brazil; 2.5 mg/kg, sc).

The behavioral test was carried out 7 days after the surgery; animals were taken to the experimental room in their home cages, 1 h before testing.

2.3. Microinjection into the dorsal periaqueductal gray area

Microinjections were performed with a thin needle (185 μm outside diameter, 33G) introduced through the guide cannula until its tip was 1.0 mm below the end of the guide cannula. The volume (0.05 μL) was injected using a hand-driven microsyringe (1 μL, KH7001, Hamilton Co., Reno, Nevada, USA) connected to the 33G injection needle by a segment of PE-10 tubing (Clay Adams Sparks, Maryland, USA) and controlled by checking the air bubble inside the PE-10 tubing. To avoid reflux, the injection needle was left in place for 30 s after the end of each injection. NA (1; 3, 15 and 45 nmol/0.05 μL) or vehicle (0.05 μL) was administered 1 min before the test and each animal received only once microinjection.

2.4. Behavioral testing

ETM: Consisted of three equal sized wooden arms (50 cm long by 12 cm wide), elevated 50 cm above the ground. One of the arms was surrounded by side walls (40 cm high) and was arranged perpendicular to the two other arms, which had no walls. To prevent animals from falling off, the open arms were delimited by a 1 cm protective. The whole apparatus was elevated 50 cm above the floor [20–22].

On the fourth and fifth days after surgery, animals were gently handled for 5 min; on the sixth day animals were pre-exposed to one of the open arms of the ETM for 30 min, on the purpose to reduce its latency to leave this arm on a later trial and perform the escape task sensitive to chronic drugs treatment [22].

On the seventh day, the ETM test was conducted. Initially, the session consisted of placing the rat at the distal end of the enclosed arm facing the intersection of the arms and the time taken by the animal to leave this arm with the four paws was recorded; the inhibitory avoidance task was evaluated for three consecutive measurements (baseline latency, avoidance 1 and avoidance 2); following 30 s, the same animal was placed at the end of the same open arm pre-exposure 24 h before the experiment, and the latency to escape with four legs from this arm was measured for three consecutive times (escape 1, 2 and 3) at 30 s inter-trial intervals. A cut-off time of 300 s was established for the avoidance and escape latencies.

Thirty seconds after being tested in the ETM, each animal was placed for 5 min in the open-field for the evaluation of locomotion.

Open field: Each rat was individually placed in the center of a wooden square arena (60 × 60 cm surrounded by 30 cm high walls; marked with nine squares of 20 × 20 cm). The number of crossed lines was measured for 5 min. Exploratory activity in the ETM and open field was videotaped and later analyzed.

Luminosity at the level of the T-maze arms and open-field was 60 lx and room temperature was kept at 22 ± 11 °C by an air conditioner that also produced background noise. After each experimental session, the models were cleaned with 5% ethanol to minimize olfactory cues. The tests were done between 8:00 and 13:00 h.

2.5. Drugs

Noradrenaline chloride (Sigma, St. Louis, Missouri, USA); Vehicle: artificial cerebrospinal fluid (NaCl 100 mM; Na_3PO_4 2 mM; KCl 2.5 mM; $MgCl_2$ 1.0 mM; $NaHCO_3$ 27 mM; $CaCl_2$ 2.5 mM; pH = 7.4); Anesthetics: Tribromo-cloro-ethanol (Aldrich Chemical Co. Inc., Milwaukee, USA) and urethane (Sigma, St. Louis, Missouri, USA); Antibiotic: streptomycins and penicillins (Pentabiótico, Fontoura-Wyeth, São Paulo, Brazil); Anti-inflammatory: flunixina meglumina (Banamine, Schering Plough, New Jersey, USA).

2.6. Histology

At the end of behavioral tests, animals were anesthetized with urethane (1.25 g/kg i.p., SIGMA, St. Louis, Missouri, USA) and 0.05 μL of 1% Evans Blue dye was injected into the brain as a marker of the injection site. Animals were then submitted to intracardiac perfusion with 0.9% NaCl followed by 10% formalin. Brains were removed and post fixed for 48 h at 4 °C and serial 40-μm-thick sections were cut with a cryostat (CM1900, Leica, Wetzlar, Germany). Brain sections were stained with 0.5% cresyl violet for light microscopy analysis. The sites of microinjection were identified in serial sections with the help of the rat brain atlas [23].

2.7. Statistical analysis

Two-way repeated-measures ANOVA analysis (Two-way ANOVA) was used, with treatment (doses) as the independent and trials (baseline, avoidance 1 and 2, or escape 1 to 3) as the dependent factor; when appropriate, one-way ANOVA followed by the post-hoc was used. The number of crossings in the open field was analyzed by one-way ANOVA followed by the Bonferroni post-test. Data were expressed as Mean ± SEM (standard error of mean). The significance level was set at $p < 0.05$.

3. Results

3.1. Effect of noradrenaline microinjected in the dPAG on inhibitory avoidance in the ETM

The Two-way ANOVA analysis showed a significant effect on trial [$F_{(2,68)} = 38$; $p < 0.05$], treatment [$F_{(4,34)} = 4.9$; $p < 0.05$] and treatment × trial interaction [$F_{(8,68)} = 4.7$; $p < 0.05$). The post-hoc test revealed that 45 nmol/nl of NA intra dPAG significantly decreased the inhibitory avoidance 2 ($p < 0.05$) compared to the control group, indicating anxiolytic-like effect (Fig. 1).

3.2. Effect of noradrenaline microinjected in the dPAG on escape performance in the ETM

The statistical analysis showed a significant effect on trial [$F_{(2,68)} = 4.4$; $p < 0.05$], but no treatment [$F_{(4,34)} = 1.1$; $p > 0.05$] and treatment × trial interaction [$F_{(8,68)} = 0.9$; $p > 0.05$]. The post-hoc test showed no significantly effect of NA intra dPAG compared to the control group ($p > 0.05$; Fig. 2).