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Lack of effects of clomipramine on Fos and NADPH-diaphorase double-staining in the periaqueductal gray after exposure to an innate fear stimulus

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Abstract

Lack of effects of clomipramine on Fos and NADPH-diaphorase double-staining in the periaqueductal gray after exposure to an innate fear stimulus — nitric oxide (NO) acts as a neurotransmitter in the rat dorsolateral periaqueductal gray (dlPAG), a midbrain structure that modulates fear and defensive behavior. Since defensive reactions can be alleviated by anxiolytic/anti-panic drugs, the present study tested the effect of clomipramine, a serotonin re-uptake inhibitor, on the activation of NO-producing neurons in the dlPAG of rats exposed to a live predator. Double staining was performed using Fos immunohistochemistry and NADPH-diaphorase as techniques to mark neural activation and to detect NO-producing neurons, respectively. Male Wistar rats received acute or chronic (21 days) injections of saline or clomipramine (10 or 20 mg/kg/day) and were exposed to a live cat. The animals exhibited a robust defensive reaction accompanied by an increase in the number of Fos- and double-stained neurons in the dlPAG, suggesting that cat exposure activates NO-producing neurons. Such effects were not significantly attenuated by clomipramine treatments. The intensity of fear reaction correlated with the intensity of neural staining in the dlPAG, regardless the drug treatment. Thus, the present results reinforce the hypothesis that NO may coordinate defensive responses in the dlPAG and indicate that this mechanism may not be modulated by a serotonin re-uptake inhibitor.

Keywords: Anxiety; Fear; Nitric oxide; Clomipramine; Periaqueductal gray

1. Introduction

The dorsolateral columns of the midbrain periaqueductal gray (dlPAG) are proposed to coordinate defensive reactions related to anxiety and panic disorders [1–4]. Experiments employing Fos protein as a marker of neural activation revealed an increased activity in this structure in rats exposed to threatening stimuli, such as a predator or its odor [5–9]. Accordingly, the behavioral effects of predator exposure are reduced after lesions of the PAG [10]. The possible neurotransmitters underlying these defensive reactions have been investigated by pharmacological experiments with anxiolytic and anti-panic drugs, such as benzodiazepines and serotonin (5-hydroxytryptamine, 5-HT) re-uptake inhibitors [6,11–13]. These drugs can prevent neuronal activation induced by cat odor or by conditioned stimulus in diverse brain regions related to fear and anxiety-like behavior, such as medial prefrontal cortex, amygdale, cingulated cortex, PAG, several hypothalamic nuclei and dorsal raphe [14–16]. Collectively, these structures are proposed as a neural system mediating aversive emotion and may constitute the neural basis of psychiatric disorders [3,4,6,13]. However, the effects of these drugs in Fos expression in the PAG induced by direct predator exposure have not been investigated. Also, it remains to be understood which neurotransmitters co-ordinate these responses.

One possible mediator of these defensive reactions is nitric oxide (NO). This gaseous neurotransmitter is synthesized by nitric oxide synthase (NOS) [17], an enzyme that can be identified and localized by immunostaining or by the nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) technique [18,19].

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These methods show that NOS is densely expressed in the dlPAG [19,20], pointing to physiological functions of NO in this structure. Accordingly, injection of a NO-donor into the dlPAG induces escape responses in rats, which can be attenuated by 5-HT receptor activation [21,22]. Moreover, an increase in NO production occurs in the dlPAG of rats exposed to a live cat [23]. Also, cat exposure induces Fos expression in NADPH-d positive neurons in this structure, indicative of an increase activity of NO-producing neurons [24]. Therefore, considering the relevance of NO for defensive reactions in the dlPAG and its possible interaction with 5-HT, the aim of this study was to investigate the effects of clomipramine, a 5-HT uptake inhibitor, on both Fos expression and Fos/NADPHd double staining in the dlPAG of rats exposed to a cat.

2. Material and methods

2.1. Animals

Male Wistar rats (180–200 g), obtained from the colony of pathogen-free rats maintained at the Pharmacy School of Ribeirao Preto, University of Sao Paulo, were housed in groups of four or five with free access to food and water in a temperature-controlled room (24 °C) with a 12 h light/dark cycle. An adult male cat (3 kg), kept at the animal farm of our University Campus with free access to food and water, was used throughout the study. A dummy cat, of approximately the same size of the live cat, was used as control.

2.2. Protocols

The protocols were carried out according to the Brazilian Society of Neuroscience and Behavior guidelines for care and use of laboratory animals, and all efforts were made to minimize animal suffering. The animals received chronic (21 days) intraperitoneal injections of saline (1 mL/kg) or clomipramine chloridrate (RBI, USA) at the doses of 10 or 20 mg/kg/day. An additional group received saline for 20 days followed by acute clomipramine in the last day. The doses were selected due to their efficacy in attenuating panic-like behaviors in rats [25,26]. Thirty minutes after the last injection, the rats were placed in a rectangular arena (80 cm × 22 cm × 50 cm) with Plexiglas walls and a metal grid floor. This observation box was divided into two compartments by a metal grid wall. During the experimental session each rat was placed in the compartment opposite to the live or dummy cat. The box was designed to comfortably contain the cat and to provide space enough for measuring the presence of rats proximal or distal to the cat-compartment. The rat compartment was divided into two parts (close and distant to the cat compartment) by an imaginary line. The rational is that when the live cat was not present, the rats have no preference for any part of the box, whereas they escape to the distal part and avoid the proximity of the cat compartment when a live cat was present.

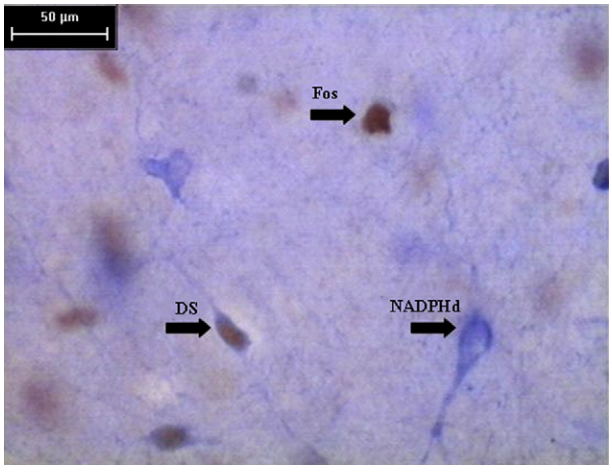

Fig. 1. Photomicrography showing NADPHd, Fos and double-stained (DS) neurons in the dlPAG of rats.

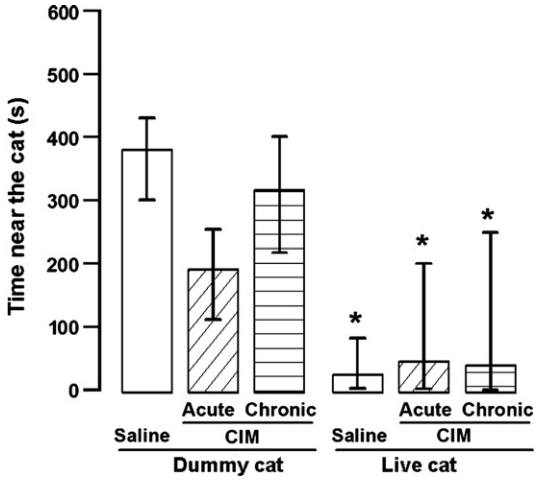

Fig. 2. Time spent near the cat compartment by rats treated with saline or acute or chronic (21 days) clomipramine (10 mg/kg/day, CIM) and exposed to a dummy cat or to a live cat. Each bar represent the median ± interquartil range (*$p < 0.05$ compared to the saline-dummy cat; Mann–Whitney; $n = 6$ per group).

The experiments were conducted between 8:00 a.m. and 12:30 p.m. During 3 days before the experiments, the animals were daily handled by the experimenter for 5 min and habituated to the observation box for 10 min. In the fourth day, the animals were exposed to the live or a dummy cat for 10 min. After each trial the observation box was carefully cleaned with an alcohol solution. To prevent eventual cat smell interference, exposure to the live cat always followed that of the dummy cat. All sessions were videotaped and later analyzed with the help of Ethovision (Version 1.9, Noldus, The Netherlands) software. The program detected the animal position in the observation box and calculated the time spent in the area near to the cat compartment.

Two hours after exposure to the observation box the animals were anaesthetized with an overdose of urethane and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4). Brains were removed and post fixed over 2 h in paraformaldehyde and stored for at least 30 h in 30% sucrose for cryoprotection. Coronal sections (40 µm) were obtained in a cryostat in duplicate. The sections were first processed for c-Fos immunohistochemistry, as previously described [24,27]. Briefly, tissue sections were washed