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Amygdaloid involvement in the defensive behavior of mice exposed to the open elevated plus-maze

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ABSTRACT

Previous studies have shown that the exposure to an open elevated plus maze (oEPM, an EPM with all four open arms) elicits fear/anxiety-related responses in laboratory rodents. However, very little is known about the underlying neural substrates of these defensive behaviors. Accordingly, the present study investigated the effects of chemical inactivation of the amygdala [through local injection of cobalt chloride ($CoCl_2$: a nonspecific synaptic blocker)] on the behavior of oEPM-exposed mice. In a second experiment, the pattern of activation of the basolateral (BLA) and central (CeA) nuclei of the amygdala was assessed through quantification of Fos protein expression in mice subjected to one of several behavioral manipulations. To avoid the confound of acute handling stress, 4 independent groups of mice were habituated daily for 10 days to an enclosed EPM (eEPM) and, on day 11 prior to immunohistochemistry, were either taken directly from their home cage (control) or individually exposed for 10 min to a new clean holding cage (novelty), an eEPM, or the oEPM. An additional group of mice (maze-naïve) was not subjected to either the habituation or exposure phase but were simply chosen at random from their home cages to undergo an identical immunohistochemistry procedure. Results showed that amygdala inactivation produced an anxiolytic-like profile comprising reductions in time spent in the proximal portions of the open arms and total stretched attend postures (SAP) as well as increases in time spent in the distal portions of the open arms and total head-dipping. Moreover, Fos-positive labeled cells were bilaterally increased in the amygdaloid complex, particularly in the BLA, of oEPM-exposed animals compared to all other groups. These results suggest that the amygdala (in particular, its BLA nucleus) plays a key role in the modulation of defensive behaviors in oEPM-exposed mice.

1. Introduction

Animals exhibit various neurovegetative (e.g., increased blood pressure, defecation) and behavioral (e.g., freezing, flight) responses when confronted with innate or learned danger stimuli. These defensive responses can be detected through animal tests for anxiety. For instance, rodents exposed to predators [1,2], novelty [3,4] or open environments [5–9] display a range of defensive reactions (e.g., escape, avoidance, freezing and antinociception).

The open elevated plus maze (oEPM) is a type of highly aversive environment that has been used to study the neurobiology of fear-induced antinociception [8,10–12] and defensive behaviors [9]. This new paradigm is similar to that described by Lister (1987) [6], except that it comprises four open arms, instead of two open and two closed arms. Exposure of mice to the oEPM enhances plasma corticosterone titers [8] and elicits defensive behaviors such as stretched attend postures, flat-back approach, and head dipping [9]. Furthermore, systemic injection of alprazolam, a potent benzodiazepine [13], attenuates defensive behavior in mice exposed to this test, suggesting that the procedure is sensitive to anxiolytic/panicolytic drugs [9]. At present, very little is known about the neural substrates that modulate the defensive behavior of animals exposed to the oEPM. However, it has been reported that chemical lesions of dorsal or ventro-lateral portions of the periaqueductal grey matter (dPAG or vlPAG), components of midbrain defense circuitry [14], do not change oEPM-induced antinociception [12,15], a type of fear/anxiety-induced pain inhibition.

Cellular and functional studies have repeatedly demonstrated that the amygdaloid complex plays a crucial role in the modulation of fear

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and anxiety behaviors. Previous findings have shown that this forebrain area receives and processes aversive stimuli [16,17][e.g.,16,17]. The amygdala comprises a set of nuclei, with complex inter- and intra-nuclear connections and which are subdivided into three main groups: the basolateral, corticomedial and centromedial groups [review: 17]. Previous studies have shown that these amygdaloid subnuclei are differentially involved in the modulation of defensive behaviors [16,18,19]. For example, electrolytic lesion specifically of the central nucleus (CeA) and basolateral nucleus (BLA) of the amygdala attenuate fear-related responses [review: 17]. Furthermore, Tanimoto et al. [19] demonstrated that bilateral chemical lesions of the BLA or CeA decrease conditioned place aversion in rats induced by plantar injection of formalin. In addition, cellular imaging of immediate early genes (IEG), such as c-fos, zif268, and arc, has also been used to map the neuronal substrate involved in behavior [20–23]. The protein Fos has been the most commonly used IEG marker of neuronal activity in behavioral research, including studies on fear and anxiety [24]. For instance, increased Fos expression has been identified in the amygdala of animals exposed to a conventional EPM [25–29]. Together, this body of evidence suggests that the amygdala might play a role in the modulation of defensive reactions displayed by mice during the exposure to the oEPM.

Although the amygdala is a bilateral structure, previous studies have revealed a degree of functional lateralization in the modulation of emotional responses in animals [30] and humans [31]. For example, Baker and Kim (2004) [30] found that the right amygdala exerts a strong role in the control of contextual fear conditioning in rats. Briefly, electrolytic lesions of the right (but not the left) amygdala decreased freezing behavior in rats exposed to the fear-associated context. In contrast, Tran and Greenwood-Van Meerveld (2012) [32] showed a lack of hemispheric lateralization of the CeA in the control of corticoid-mediated anxiety-like behavior assessed in rats exposed to the EPM. These findings suggest the potential importance of hemispheric lateralization in the role of the amygdala in the modulation of defensive behavior of animals exposed to threatening situations, such as the oEPM.

Here, we investigated (i) the effects of chemical inactivation (through local injection of cobalt chloride ($CoCl_2$), an nonspecific synaptic blocker [33]) of the amygdala on the behavior of mice exposed to the oEPM; and (ii) the pattern of neuronal activation of the left and right amygdaloid complex and of the BLA and CeA subnuclei through quantification of Fos protein expression in mice exposed to the oEPM versus a number of important control conditions, including an enclosed EPM (eEPM, an EPM with four enclosed arms; control situation) and a non-maze novel environment (new cage). Basal Fos protein expression was also recorded in maze-naïve mice. This study therefore sought to clarify the role of the amygdala in the modulation of the defensive behavior of animals exposed to the oEPM and to further explore this new paradigm as a potential tool for research on the neurobiology of fear/anxiety.

2. Materials and methods

2.1. Subjects

Subjects were adult male Swiss mice (Univ. Estadual Paulista – UNESP, SP, Brazil) weighing 25–35 g at testing. They were housed in groups of 5 per cage (33 × 15 × 13 cm) and maintained under a normal 12 h light cycle (lights on at 7 a.m.) in a temperature (23 ± 1° C) controlled environment. Food and water were freely available, except during the brief test periods. All mice were experimentally naive, and experimental sessions were performed during the light phase of the cycle (09.00h-16.00 h). The experimental protocols were approved by the Research Ethics Committee of the School of Pharmaceutical Sciences of the Universidade Estadual Paulista – UNESP (CEUA number 14/2014).

2.2. Drug

Cobalt chloride ($CoCl_2$; Sigma-Aldrich, Brazil) was dissolved in 0.9% physiological saline solution which alone served as control solution. The dose of 1.0 mM of $CoCl_2$ was based on previous studies [34,35], and the total volume of injection within the amygdala was 0.1 μL.

2.3. Surgery and microinjection

Mice were bilaterally implanted with 7-mm stainless-steel guide cannulae (26-gauge; Insight Equipamentos Científicos Ltda) under ketamine and xylazine anaesthesia (100 mg/kg and 10 mg/kg i.p.). Guide cannulae, targeted 1.0 mm dorsal to the amygdala, were fixed to the skull using dental acrylic and jeweler's screws. Stereotaxic coordinates for the amygdala, based on Paxinos and Franklin (2001) [36] and a previous study in our laboratory [37], were 1.1 mm posterior to bregma, 3.1 mm lateral to the midline and 3.7 mm ventral to the skull surface. A dummy cannula (33-gauge stainless-steel wire; Fishtex Indústria e Comércio de Plásticos Ltda), inserted into each guide-cannula at the time of surgery, served to reduce the incidence of occlusion. At the end of the stereotaxic surgery, each mouse received an intramuscular injection of penicillin-G benzathine (Pentabiotic, 56.7 mg/kg in a 0.1 mL volume; Fort Dodge, Campinas, SP, Brazil) and a subcutaneous injection of the anti-inflammatory analgesic Banamine (3.5 mg/kg flunixin meglumine, Intervet Schering–Plough, Rio de Janeiro, RJ, Brazil, in a volume of 0.3 mL).

Five days after following surgery, solutions were injected into the amygdala by microinjection units (33-gauge stainless steel cannulae; Insight Equipamentos Científicos Ltda), which extended 1.0 mm beyond the tip of each guide cannula. Each microinjection unit was attached to a 2 μL Hamilton microsyringe via polyethylene tubing (PE-10), and administration was controlled by the experimenter at a rate of 0.1 μL (volume injected) over a period of approximately 20 s. The microinjection procedure consisted of gently restraining the animal, removing the dummy cannulae, inserting the injection units, infusing the solution, and keeping the injection units in situ for a further 60 s. Confirmation of successful infusion was obtained by monitoring the movement of a small air bubble in the PE-10 tubing.

2.4. Apparatus

The open elevated plus-maze (oEPM) is closely similar to the standard EPM (EPM) described by Lister (1987) [6] except that it has four open (30 × 5 × 0.25 cm) arms, raised 38.5 cm above floor level on a wooden pedestal. Each arm of the oEPM was divided into two sections, which were designated as proximal (10 × 5 cm) and distal [medial and end of the arms (20 × 5 cm)] portions relative to the central square (5 × 5 cm) [for details, see 9]. In Experiment 2, amygdaloid c-fos activation in response to oEPM exposure was contrasted with exposure to an enclosed elevated plus-maze (eEPM; four enlcosed arms; 30 × 5 × 15 cm; raised 38.5 cm above floor level) and an unfamiliar, clean home cage (33 × 15 × 13 cm).

2.5. Procedure

All behavioral tests were conducted during the light phase of the light/dark cycle, under the illumination of a 100-W light bulb (50 Lux on the floor of the apparatus).

2.5.1. Experiment 1

2.5.1.1. Effects of amygdala inactivation on the behavior of mice exposed to the oEPM. In order to investigate the role of the amygdala in the modulation of defensive behavior of mice exposed to the oEPM, each mouse received a bilateral intra-amygdala injection of saline or $CoCl_2$ (1.0 mM) and, after 10 min, was individually placed on the central