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GLUTAMATERGIC MECHANISMS OF THE DORSAL PERIAQUEDUCTAL GRAY MATTER MODULATE THE EXPRESSION OF CONDITIONED FREEZING AND FEAR-POTENTIATED STARTLE

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Abstract—It is well known that excitatory amino acids induce unconditioned fear responses when locally injected into the dorsal periaqueductal gray matter (dPAG). However, there are only few studies about the involvement of excitatory amino acids mediation in dPAG in the expression of conditioned fear. The present series of experiments evaluates the participation of AMPA/Kainate and NMDA glutamatergic receptors of dPAG in the expression of conditioned fear, assessed by the fear-potentiated startle (FPS) and conditioned freezing responses. Wistar rats were subjected to fear conditioning to light. Twenty-four hours later, they received intra-dPAG injections of kainic acid or NMDA (AMPA/Kainate and NMDA agonists) and 1,2,3,4-Tetrahydro-6-nitro-2, 3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium salt hydrate (NBQX) or D(−)-2-Amino-7-phosphonoheptanoic acid (AP7) (AMPA/Kainate and NMDA antagonists) and were submitted to the FPS test. Conditioned freezing response was simultaneously measured. Effects of drug treatment on motor activity were evaluated in the open-field test. Intra-dPAG injections of glutamatergic agonists enhanced conditioned freezing and promoted pro-aversive effects in the FPS. Lower doses of the agonists had no effect or enhanced FPS whereas higher doses disrupted FPS, indicating a non-monotonic relationship between fear and FPS. The antagonist NBQX had no significant effects while AP7 decreased conditioned freezing but did not affect FPS. Both antagonists reduced the effects of the agonists. The obtained results cannot be attributed to motor deficits. The results suggest an important role of the AMPA/Kainate and NMDA mechanisms of the dPAG in the expression of conditioned freezing and FPS. © **2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: fear conditioning, dorsal periaqueductal gray matter, AMPA/Kainate receptor, NMDA receptor, fear-potentiated startle, conditioned freezing.

INTRODUCTION

It is well established that the midbrain periaqueductal gray matter (PAG) is a key structure coordinating behavioral and physiological patterns of defensive reactions that are critical for survival (Depaulis and Bandler, 1991; Behbehani, 1995; Bandler and Keay, 1996; Vianna and Brandão, 2003). Electrical stimulation of PAG in neurosurgical patients produces neurovegetative changes and feelings of fear, terror and impending death (Nashold et al., 1969). In laboratory animals, gradual increases in the electrical stimulation of the dorsal part of PAG (dPAG) induces arousal, freezing and escape behavior, together with changes in autonomic responses, comparable to those observed in situations such as the confrontation of a prey with predators (Spiegel et al., 1954; Brandão et al., 1982; Canteras and Goto, 1999; Martinez et al., 2006; Reimer et al., 2009). This defensive repertoire induced by electrical stimulation of dPAG can also be mimicked by pharmacological manipulation of this structure. For example, previous studies from this and other laboratories showed that microinjections of excitatory amino acids (EAA) into the dPAG produce freezing and behavioral activation (Bandler and Carrive, 1988; Ferreira-Netto et al., 2005).

Glutamate seems to play an important role in the pathogenesis of anxiety (Bergink et al., 2004). Riluzole, a glutamate-release inhibitor, has been found to decrease anxiety symptoms in patients with generalized anxiety disorder (Mathew et al., 2005). Former studies confirmed that all types of glutamatergic receptors are present in the dPAG (Albin et al., 1990; Tölle et al., 1993). Glutamatergic receptors can be classified into metabotropic and ionotropic ones. The latter can be divided into AMPA/Kainate and NMDA, according to their sensitivities to the agonists (Monaghan et al., 1989). EAA mechanisms acting through these distinct receptors could be related to different behavioral responses. Indeed, some evidences suggest that activation of AMPA/Kainate receptors would mediate freezing response while NMDA would be responsible for escape behavior in rats (Pandossio and Brandão, 1999; Ferreira-Netto et al., 2005).

While the involvement of EAA mechanisms of dPAG in defensive behavior is mainly supported by numerous evidence obtained by studies on unconditioned fear

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Abbreviations: ANOVA, analysis of variance; AP7, D(−)-2-Amino-7-phosphonoheptanoic acid; CS, conditioned stimulus; dPAG, dorsal periaqueductal gray matter; EAA, excitatory amino acids; FPS, fear-potentiated startle; KA, kainic acid; NBQX, 1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium salt hydrate; PAG, periaqueductal gray matter; US, unconditioned stimulus.

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responses, as predator–prey exposures or dPAG electrical or chemical stimulation (Brandão et al., 1982; Graeff et al., 1986; Dielenberg et al., 2001; Canteras, 2003; Ferreira-Netto et al., 2005), less is known regarding their involvement in conditioned fear responses. Therefore, the understanding of how the dPAG is called into action in conditioned fear would be of relevance to our better knowledge of the neurobiology of fear and anxiety. So, the present series of experiments evaluates the participation of AMPA/Kainate and NMDA glutamatergic receptors of dPAG in the expression of conditioned fear.

One of the most used techniques to measure conditioned fear is the fear-potentiated startle (FPS). In FPS, the acoustic startle response, a simple defense reaction which is elicited by sudden, intense acoustic stimuli, is increased in the presence of a stimulus that has been previously paired with shocks (Davis et al., 1993; Koch, 1999; Reimer et al., 2008; de Oliveira et al., 2011). Although some studies have already proposed a role for the PAG in the expression and modulation of the FPS, the results are to some extent contradictory. For example, lesions of PAG or intra-PAG microinjections of GABA-A agonists, can decrease FPS or have no effects (Fendt et al., 1996; Walker et al., 1997; Walker and Davis, 1997; Fendt, 2000; Reimer et al., 2008). On the other hand, it has been shown that electrical stimulation of the PAG or intra-PAG injections of GABA-A antagonists can enhance, decrease or leave FPS unchanged (Borowski and Kokkinidis, 1996; Fendt, 1998, 2000). Moreover, some works have demonstrated that it is possible to decrease the FPS by administrating kainic acid (KA) intra-PAG prior to the testing (Walker et al., 1997; Walker and Davis, 1997; Fendt, 2000).

The aforementioned data indicate that more studies are necessary to clarify the involvement of dPAG in the FPS. Indeed, these apparently contradictory effects could be due to the fact that moderate fear levels produce maximal startle responses, whereas higher fear intensity can decrease the FPS magnitude (Davis and Astrachan, 1978; Walker and Davis, 1997; Santos et al., 2005). Because of these effects, some additional measures, as the evaluation of the conditioned freezing, which is an important response to cues associated with footshock (Leaton and Cranney, 1990; Fendt and Fanselow, 1999), could ensure the actual fear state measured on FPS test. So, in the present study we used both the FPS and the conditioned freezing responses to assess the participation of AMPA/Kainate and NMDA glutamatergic receptors of dPAG in the expression of conditioned fear.

EXPERIMENTAL PROCEDURES

Animals

Two-hundred and thirty-seven naive male Wistar rats from the animal facility of the University of São Paulo, *Campus* of Ribeirão Preto, were used. The animals, weighing 270–300 g at the beginning of the experiments, were housed in groups of four in plastic boxes (33 × 17 × 40 cm). The rats were maintained under a 12 h/12 h light/dark cycle (lights on at 7:00 a.m.) with room temperature at 23 ± 1 °C. Food and water were available *ad libitum*. The experiments were carried out during the light phase of the cycle. All the procedures were approved by the Committee for Animal Care and Use of University of São Paulo at Ribeirão Preto (No. 11.1.308.53.9) which is based on the United States National Institutes of Health Guide for Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Surgery

A detailed description of surgical procedure has been provided previously (Reimer et al., 2008, 2009). Briefly, animals were anesthetized with ketamine/xylazine (100/7.5 mg/kg, intraperitoneal; Agener União, Embu-Guaçu, SP, Brazil) and fixed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The upper incisor bar was set at 3.0 mm below the interaural line, such that the skull was horizontal between *bregma* and *lambda*. Unilateral dPAG cannulation for drug injection was performed in the left hemisphere. In previous studies, we showed that unilateral microinjection into the dPAG is sufficient in changing defensive behavior (Reimer et al., 2008, 2009). Taking *lambda* as the reference point, the coordinates used were (Paxinos and Watson, 2007): anterior/posterior, +0.1 mm; medial/lateral, +1.9 mm; dorsal/ventral, −4.3 mm, angled medially 16°. At the end of surgery, animals received an injection of a polyvalent veterinary antibiotic (Pentabiótico, 0.2 ml, intramuscular; Fort Dodge, Campinas, SP, Brazil) and an injection of the anti-inflammatory and analgesic flunixin meglumine (Banamine, 2.5 mg/kg, subcutaneous; Schering-Plough, Cotia, SP, Brazil). Afterward, the rats were allowed 5 days to recover from the surgical procedure. The experimental design used in this study is described below and presented schematically in Fig. 1.

Fear-potentiated startle and conditioned freezing

Matching. To record the amplitude of the acoustic startle response, two separated stabilimeter devices were used simultaneously. The rats were placed into a wired-grid cage (16.5 × 7.5 × 7.5 cm) suspended within a PVC frame, which was firmly fixed to a response platform by four thumb screws. The floor of the cage consisted of six 3.2 mm diameter stainless steel bars spaced 1.0 cm apart. Each cage and platform was located independently inside ventilated sound-attenuating plywood-chambers (64 × 60 × 40 cm). The startle reaction of the rats generated a pressure on the response platform and analog signals were amplified, digitized and analyzed by a software (Startle Reflex, version 4.10; Med Associates, St. Albans, VT, USA) provided by the manufacturer of the equipment. The presentation and sequencing of the acoustic stimuli were also controlled by the same software and an appropriate interface (Med Associates). A loudspeaker located 10 cm behind the testing cage delivered both the startle stimulus (100 dB; 50 ms burst of white noise) and continuous background noise (55 dB). The startle reaction was recorded within a time window of 100 ms after the onset of the startle stimulus. Calibration procedures were conducted before the experiments to ensure statistically comparable startle magnitudes across the response platforms. A red light bulb (6 W) located inside the isolation chamber provided illumination for the camera. For the first 2 days, the animals were placed in the testing cage for 5 min of habituation period and afterward received a total of 30 startle stimuli with an interstimulus interval of 30 s. Each matching session was 20 min in duration, including habituation period. Matching sessions are intended to familiarize the animals with the test cage and decrease possible variations in the startle response. Animals were assigned to the different drug and control groups in a way that each group had similar average startle amplitude based on the last matching day.