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Gabaergic mechanisms of hypothalamic nuclei in the expression of conditioned fear

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ABSTRACT

The amygdala, the dorsal periaqueductal gray (dPAG), and the medial hypothalamus have long been recognized to be a neural system responsible for the generation and elaboration of unconditioned fear in the brain. It is also well known that this neural substrate is under a tonic inhibitory control exerted by GABA mechanisms. However, whereas there is a growing body of evidence to suggest that the amygdala and dPAG are also able to integrate conditioned fear, it is still unclear, however, how the distinct hypothalamic nuclei participate in fear conditioning. In this work we aimed to examine the extent to which the gabaergic mechanisms of this brain region are involved in conditioned fear using the fear-potentiated startle (FPS). Muscimol, a GABA-A receptor agonist, and semicarbazide, an inhibitor of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD), were used as an enhancer and inhibitor of the GABA mechanisms, respectively. Muscimol and semicarbazide were injected into the anterior hypothalamus (AHN), the dorsomedial part of the ventromedial nucleus (VMHDM), the dorsomedial (DMH) or the dorsal premammillary (PMD) nuclei of male Wistar rats before test sessions of the fear conditioning paradigm. The injections into the DMH and PMD did not produce any significant effects on FPS. On the other hand, muscimol injections into the AHN and VMHDM caused significant reduction in FPS. These results indicate that injections of muscimol and semicarbazide into the DMH and PMD fail to change the FPS, whereas the enhancement of the GABA transmission in the AHN and VMHDM produces a reduction of the conditioned fear responses. On the other hand, the inhibition of this transmission led to an increase of this conditioned response in the AHN. Thus, whereas DMH and PMD are known to be part of the caudal-most region of the medial hypothalamic defensive system, which integrates unconditioned fear, systems mediating conditioned fear select the AHN and VMHDM nuclei that belong to the rostral-most portion of the hypothalamic defense area. Thus, distinct subsets of neurons in the hypothalamus could mediate different aspects of the defensive responses.

1. Introduction

The search for the subcortical organization of fear through the use of electrical stimulation of the brain dates back to the works of Hess and Brugger (1943) and Hunsperger (1956), which showed that electrical stimulation of the hypothalamus of the cat results in flight-like responses—or simply escape behavior—accompanied by autonomic responses. Chi and Flynn (1971) in an attempt to identify the neuroanatomical pathways underlying affective defense behavior placed lesions at sites in the medial hypothalamus from which affective defense was elicited and degenerating axons were traced to the anterior and posterior hypothalamus, midline thalamus and midbrain central gray. Several other studies have shown that the medial zone of the hypothalamus contains diverse well-defined nuclei that play a key role in the expression of the defense reaction (Canteras, 2002; Canteras, Ribeiro-Barbosa, & Comoli, 2001; Johnson & Shekhar, 2006; Keay & Bandler, 2001). The medial hypothalamus (MH), the amygdala, and the dorsal periaqueductal gray (dPAG) have long been recognized to be a neural system responsible for the generation and elaboration of aversive states in the brain (Brandão, Anseloni, Pandóssio, De Araújo, & Castilho, 1999; Graeff, 1990). Anatomical studies have revealed that these structures are heavily interconnected, suggesting a hierarchically organized brain defense system (Canteras et al., 2001; Graeff, 2004). Distinct defensive behaviors are evoked depending on the intensity and distance of the aversive stimuli and it has been proposed that proximal, distal and potential threats can activate the dPAG, MH and amygdala, respectively (Blanchard & Blanchard, 1988; Brandão, Vianna, Masson, & Santos, 2003). Unconditioned fear such as direct exposure to predators occurs with upregulated expression of Fos in specific sites of the MH. These sites include a circuit formed by the anterior hypothalamic nucleus (AHN), the dorsomedial portion of ventromedial nucleus (VMHDM), the dorsomedial hypothalamus (DMH) and the dorsal premammillary nucleus (PMD). This circuit is thought to integrate innate defensive

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responses to environmental threats (Canteras, 2002; Canteras, Chiavegatto, Ribeiro do Valle, & Swanson, 1997; Canteras & Swanson, 1992; Dielenberg, Hunt, & McGregor, 2001; DiMicco, Samuels, Zaretskaia, & Zaretsky, 2002; Shekhar & Keim, 1997). It is also well known that this neural substrate is under tonic inhibitory control exerted by GABA mechanisms (Brandão, Di Scala, Bouchet, & Schmitt, 1986; Di Scala, Schmitt, & Karli, 1984). However, the brain systems modulating non-innate defensive behaviors seem to be different and are related to the unconditioned or conditioned nature of the threat stimuli (Blanchard et al., 2003). Given the importance of the hypothalamus in generation of emotional states and in expression of defensive behaviors, an understanding of its extent and how it is called into action in conditioned fear would be of relevance to our understanding of the neurobiology of fear and anxiety (Brandão et al., 1999; Di Scala, Schmitt, & Karli, 1984; Millan, 2003). Conditioned fear has been used to explore the neural circuitry of fear and emotional learning (Kim & Fanselow, 1992; LeDoux, 2000).

This study was an attempt to explore the neural substrates underlying fear conditioning, and to further assess the gabaergic mediation of conditioned fear in the MH, specifically in the AHN, VMHDM, DMH, and the PMD nuclei using the fear-potentiated startle (FPS) paradigm. The startle reflex is a whole-body response reflex, which consists of a skeletal muscle contraction in response to a sudden and unexpected burst of noise—known as the acoustic startle reflex (Brown, Kalish, & Farber, 1951; Davis & Astrachan, 1978; Davis, Falls, Campeau, & Kim, 1993; Davis, Gendelman, Tischler, & Gendelman, 1982; Kock, 1999). Fear-potentiated startle reflects a conditioned response to a fear-eliciting stimulus, and it is one of the most common paradigms used to study the biological basis of emotion, as well as learning and memory in fear conditioning experiments. In this paradigm, an emotionally neutral stimulus, such as a tone, light, or context, is paired with an aversive unconditioned stimulus (US)—e.g. a footshock. As a result, the neutral stimulus becomes a conditioned stimulus (CS) that elicits conditioned fear responses when subsequently presented alone during the expression phase of the experiment. When the startle-inducing noise is shown in the presence of a CS, the startle response is enhanced. FPS is mediated by the amygdala and its projections to the deep layers of the superior colliculus and deep mesencephalic nucleus of the rostral midbrain and, subsequently, to the primary startle reflex circuit in the brain stem (Davis, 1992; Davis et al., 1993; Hitchcock & Davis, 1986; Zhao & Davis, 2004). The FPS method has been considered to be a valid and reliable tool for measurement of anxiety, based on extensive investigations analyzing several of its behavioral, physiological, and pharmacological aspects (Davis et al., 1993; Kock, 1999; Silva, Gárgaro, & Brandão, 2004; Yeomans & Frankland, 1996). As for the Pavlovian conditioning studies that have typically used footshock paired with a specific environment or cue, much of this research has suggested key roles for the amygdala and hippocampus in the acquisition, consolidation, and retrieval of memories associated with fear (Fendt & Fanselow, 1999; Gray & McNaughton, 2000; LeDoux, 2000; Maren, 2001; McGaugh, 2004).

In order to investigate the participation of gabaergic mechanisms of the hypothalamic nuclei described above in conditioned fear, muscimol—a GABA-A receptor agonist—and semicarbazide—an inhibitor of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD)—were used as enhancer and inhibitor of the GABA mechanisms, respectively (Brandão et al., 1986). The concentration of muscimol used in this study has been considered to produce fear-reducing effects through interaction with GABA-A receptors (Cooper, Bloom, & Roth, 2001; Nobre & Brandão, 2004). On the other hand, an important role for tonic inhibition mediated by endogenous GABA was clearly implied by the ability of semicarbazide to reduce the local GABA transmission (Borelli, Ferreira-Netto, Coimbra, & Brandão, 2005; Brandão et al., 1986).

2. Materials and methods

2.1. Animals

One hundred and eighty-two naive male Wistar rats from the animal house of the Campus of Ribeirão Preto of the University of São Paulo were used. The animals, weighing 250–280 g each, were housed in groups of four per cage under a 12:12 dark/light cycle (lights on at 07:00 h) at 22 ± 1 °C, and they were given free access to food and water. The experiments were carried out during the light phase of the cycle and they were performed in compliance with the recommendations of SBNeC (Brazilian Society of Neuroscience and Behavior), which are based on the US National Institutes of Health Guidelines for Care and Use of Laboratory Animals.

2.2. Surgery

The animals were anesthetized with tribromoethanol (250 mg/kg, i.p.) and fixed in a stereotaxic frame in flat skull position (David Kopf, USA). Lidocaine (20 mg/ml, 1 ml) was injected around the surgical field as a local complement to general anesthesia. One stainless-steel guide-cannula (14 mm in length, o.d. 0.6 mm, i.d. 0.4 mm) was implanted in the forebrain, aimed at the anterior hypothalamus (AHN), the dorsomedial hypothalamus (DMH), the ventromedial hypothalamic nucleus, dorsomedial part (VMHDM), or the dorsal premmamillary nucleus (PMD). The upper incisor bar was set at −3.3 mm below the interaural line (skull horizontal between bregma and lambda). The guide-cannula was introduced vertically at the right side of the brain, using the following coordinates with the bregma serving as the reference for each plane: AHN ($N = 44$)—antero-posterior (AP), 1.5 mm; medio-lateral (ML), 0.6 mm; dorsoventral (DV), 8.8 mm; DMH ($N = 46$)—AP, 3.2 mm; ML, 0.5 mm; DV, 8.4 mm; VMHDM ($N = 48$)—AP, 2.9 mm; ML, 0.5 mm; DV, 9.3 mm; PMD ($N = 44$)—AP, 3.9 mm; ML, 0.5 mm; DV, 9.2 mm. It was fixed to the skull by means of acrylic resin and two stainless-steel screws. At the end of the surgery, each guide-cannula was sealed with a stainless-steel wire to protect it from obstruction. In addition, the animals received an intramuscular injection of Pentabiotic (60,000 IU, 0.2 mL; Fort Dodge) and a subcutaneous injection of the anti-inflammatory and analgesic Banamine (flunixin meglumine, 2.5 mg/kg (10 mg/mL, 0.2 mL). Afterward, the rats were allowed a period of 1 week to recover from the surgical procedure.

3. Apparatus and procedure

3.1. Fear-potentiated startle

3.1.1. Matching

To record the amplitude of the acoustic startle response, two separated stabilimeter devices were used simultaneously. The rats were placed in a stabilimeter, which consisted of a wire-mesh cage (16.5 × 7.5 × 7.5 cm) suspended within a PVC frame, which was firmly placed on a response platform by four thumb-screws. The floor of the stabilimeter consisted of six 5.0 mm diameter stainless-steel bars spaced 1.5 cm apart. The stabilimeter and platform were located inside a ventilated plywood sound-attenuating chamber (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 with software (Startle Reflex, version 4.10; Med Associates Inc., VT) provided by the manufacturer of the equipment. The presentation and sequencing of the acoustic