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Conditioned fear is modulated by CRF mechanisms in the periaqueductal gray columns

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ABSTRACT

The periaqueductal gray (PAG) columns have been implicated in controlling stress responses through corticotropin-releasing factor (CRF), which is a neuropeptide with a prominent role in the etiology of fear- and anxiety-related psychopathologies. Several studies have investigated the involvement of dorsal PAG (dPAG) CRF mechanisms in models of unconditioned fear. However, less is known about the role of this neurotransmission in the expression of conditioned fear memories in the dPAG and ventrolateral PAG (vlPAG) columns. We assessed the effects of ovine CRF (oCRF 0.25 and 1.0 μg/0.2 μL) locally administered into the dPAG and vlPAG on behavioral (fear-potentiated startle and freezing) and autonomic (arterial pressure and heart rate) responses in rats subjected to contextual fear conditioning. The lower dose injected into the columns promoted proaversive effects, enhanced contextual freezing, increased the blood pressure and heart rate and decreased tail temperature. The lower dose of oCRF into the vlPAG, but not into the dPAG, produced a pronounced enhancement of the fear-potentiated startle response. The results imply that the PAG is a heterogeneous structure that is involved in the coordination of distinct behaviors and autonomic control, suggest PAG involvement in the expression of contextual fear memory as well as implicate the CRF as an important modulator of the neural substrates of fear in the PAG.

Introduction

The neuropeptide corticotropin-releasing factor (CRF) has been implicated in the regulation of the neuroendocrine reaction (Vale et al., 1981). Over the last decades, CRF has been shown to modulate the autonomic, immunologic, behavioral and cognitive responses to aversive stimuli (Bale and Vale, 2004). CRF receptors (CRF1 and CRF2) have been demonstrated to be broadly distributed in the structures that compose the limbic circuitry (De Souza et al., 1985; Hauger et al., 2006; Owens and Nemeroff, 1991; Swanson et al., 1983) and experimental evidence demonstrated that intracerebroventricular (i.c.v.) CRF administration increases anxiety-related behavior in rodents (Momose et al., 1999; Yang et al., 2006). CRF specifically influences the reaction to aversive stimuli when locally injected into structures such as the amygdala (Hubbard et al., 2007; Pitts and Takahashi, 2011), hippocampus (Pentkowski et al., 2009; Todorovic et al., 2007), bed nucleus of the stria terminalis (Sink et al., 2012; Walker et al., 2009) and periaqueductal gray (PAG) (Borelli and Brandão, 2008; Carvalho-Netto et al., 2007; Martins et al., 1997, 2000; Miguel and Nunes-de-Souza, 2011).

The PAG is a limbic structure that is considered to be a final pathway of the stress reaction (Brandão et al., 2005; Carrive, 1993). Anatomical and histological analyses have divided this structure into longitudinal columns parallel with the aqueduct, including the dorsal (dPAG, comprising dorsomedial and dorsolateral columns), lateral (lPAG) and ventrolateral (vlPAG) columns (Bandler and Shipley, 1994; Carrive, 1993; Keay and Bandler, 2001). In rodents, PAG electrical stimulation, lesions or pharmacological manipulation alters anxiety-like behaviors in several paradigms, such as open field (Borelli et al., 2004; Brandão et al., 1999; Coimbra et al., 2006), elevated plus-maze (Netto and Guimarães, 2004), conditioned-place aversion (Zanoveli et al., 2007), contextual conditioned fear (Kim et al., 1993; Walker and Carrive, 2003) and fear-potentiated startle (Reimer et al., 2012; Zhao et al., 2009).

Receptor autoradiography and binding studies in different central nervous system regions in the rat demonstrated that there is a substantial population of both CRF receptor subtypes within the different PAG columns (Merchenthaler, 1984; Swanson et al., 1983). In support of a CRF neurotransmitter role within the PAG columns, the CRF receptor localization corresponds well with the distribution of CRF-immunoreactive terminals in this structure (Steckler and Holsboer, 1999). Furthermore,

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perikarya with immunoreactive CRF were identified in the PAG, predominantly in its ventral portion, medial to the Edinger–Westphal nucleus and CRF fibers can also be seen coursing from the amygdala, bed nucleus of the stria terminalis and hypothalamus to the dPAG (Gray and Magnuson, 1992; Swanson et al., 1983). Also, Bowers et al. (2003) using an electrophysiological recording technique showed that CRF produced a dose dependent excitatory effect on PAG neurons, both in dorsal and in ventrolateral subdivisions.

Ovine CRF (oCRF) and cortagine, a selective CRF1 agonist, administered into dPAG produced anxiogenic-like effects in the mouse defense test battery and rat exposure test, which are animal models used to investigate mice defensive patterns in the presence of natural aversive stimuli (Carvalho-Netto et al., 2007; Litvin et al., 2007). Additionally, intra-dPAG injection of the CRF antagonist α-helical-CRF reduced anxiety-related behaviors in rodents subjected to the elevated plus-maze (Martins et al., 2000), whereas oCRF produced opposite responses (Borelli and Brandão, 2008). Although a number of studies has investigated the involvement of dPAG CRF mechanisms in models of unconditioned fear (Borelli and Brandão, 2008; Carvalho-Netto et al., 2007; Litvin et al., 2007; Miguel and Nunes-de-Souza, 2011), less is known about the CRF neurotransmission in the expression of conditioned fear memories in the PAG columns. To further investigate this issue, we assessed the effects of CRF locally administered into the dPAG and vlPAG on behavioral (fear-potentiated startle and freezing) and autonomic (arterial pressure and heart rate) responses in rats subjected to contextual fear conditioning. In this paradigm, electrical footshocks are paired with an initially neutral background context. After some pairings, the context evokes a conditioned fear reaction consisting of behavioral and autonomic responses including freezing, urination, increased arterial blood pressure and ultrasonic vocalization (Antoniadis and McDonald, 1999; Bouton and Bolles, 1979; Fanselow and Tighe, 1988; LeDoux et al., 1988; Resstel et al., 2006; Wöhr et al., 2005).

Materials and methods

Ethics

The experiments reported in this article were performed in accordance with the recommendations of the Brazilian Society of Neuroscience and Behavior and complied with the United States National Institutes of Health Guide for Care and Use of Laboratory Animals. The procedures were approved by the Committee for Animal Care and Use, University of São Paulo (No. 125-2010 and 11.1.308.53.9). All efforts were made to minimize animal suffering and reduce the number of rats used.

Animals and surgical procedures

A total of 110 male Wistar rats from the animal house of the University of São Paulo Ribeirão Preto campus weighing 220–270 g were used in the study. The rats were housed in groups of four animals per cage with food and water available ad libitum in a temperature-controlled room (23 ± 1 °C) under a 12 h/12 h light/dark cycle (lights on at 07:00 AM) for 72 h.

Before the experimental sessions, the rats were anesthetized with ketamine/xylazine (100/7.5 mg/kg, intraperitoneal; Agener União, Embu-Guaçu, SP, Brazil) and fixed in a stereotaxic apparatus (David Kopf, Tujunga, CA, USA). The upper incisor bar was set 3.3 mm below the interaural line so that the skull was horizontal between bregma and lambda. After scalp anesthesia with 2% lidocaine, the skull was surgically exposed and a stainless steel guide cannula (10 mm length, 0.6 mm outer diameter, 0.4 mm inner diameter) was unilaterally implanted into the dPAG using lambda as the reference (angle of 16°; coordinates: anterior/posterior, 0 mm; medial/lateral, ±1.9 mm; dorsal/ventral: −4.1 mm) or vlPAG (angle of 18°; coordinates: anterior/posterior, −0.2 mm; medial/lateral, ±2.6 mm; dorsal/ventral: −3.3 mm) according to Paxinos and Watson (2005). The cannula was fixed to the skull with dental cement and two stainless steel screws. After surgery, the guide cannula was sealed with a stainless steel wire to prevent obstruction, and the rats received an intramuscular injection of penicillin G benzathine (Pentabiotic, 600,000 IU, 0.2 mL; Fort Dodge, Campinas, SP, Brazil) and a subcutaneous injection of the anti-inflammatory and analgesic Banamine (flunixin meglumine, 2.5 mg/kg [10 mg/mL, 0.2 mL]; Schering-Plough, São Paulo, SP, Brazil). After surgery, the rats were returned to their home cages in groups of four and were allowed to recover over a period of five days. To assess autonomic responses, one day prior to the test day, rats were anesthetized with ketamine/xylazine, and a catheter (4 cm PE-10 segment heat-bound to a 13 cm PE-50 segment; Clay Adams, Northridge, CA, USA) was inserted into the abdominal aorta through the femoral artery for cardiovascular recording. The catheter was tunneled under the skin, and exteriorized on the animal's dorsum.

Drug and infusion procedure

Ovine CRF (Sigma-Aldrich) was dissolved in 0.9% physiological saline shortly before use to achieve a final concentration of 0.25 μg or 1 μg/0.2 μL. Physiological saline served as the vehicle control. The drug was administered locally 5 min prior to testing. The doses and times of the injections were based on previous studies (Borelli and Brandão, 2008; Carvalho-Netto et al., 2007). Infusions were delivered using an infusion pump (Harvard Apparatus, Holliston, MA, USA) in a constant volume of 0.2 μL over 30 s. A thin dental needle (0.3 mm outer diameter) attached by polyethylene tubing to a 5 μL Hamilton syringe was introduced through each guide cannula. The injection needle extended 1.0 mm (dPAG) or 3.0 mm (vlPAG) below the ventral tip of the implanted guide cannula. The displacement of an air bubble inside the polyethylene tubing that connected the syringe to the injection needle was used to monitor the microinjections. The injection needles were left in place for 30 s after the end of the infusion to allow for diffusion.

Fear-potentiated startle test

The acoustic startle reflex is elicited by sudden, unexpected and intense auditory stimulation, and involves a series of rapid and phasic contractions of most of the skeletal muscles throughout the body. The rapidity of the primary response suggests that a relatively simple circuit mediates the startle reflex with few synapses interposed between the auditory nerve and the spinal motoneurons (Koch, 1999; Yeomans et al., 2002). The fear-potentiated startle paradigm has proven to be useful for analyzing neural systems involved in fear and anxiety. This test measures conditioned fear by an increase in the amplitude of the acoustic startle reflex in the presence of an explicit or contextual cue previously paired with a footshock (Davis et al., 1993; Grillon and Baas, 2003).

Fifty-eight rats were tested to examine the involvement of the PAG CRF mechanisms in the fear-potentiated startle response in a context previously paired with footshocks. Ovine CRF was injected into the dPAG or vlPAG before the test sessions. The choice of these columns was based on previous data from our laboratory showing that CRF mechanisms in the lPAG did not appear to be involved in the expression of conditioned fear responses (Borelli et al., unpublished data).

A wire-grid cage (16.5 × 7.5 × 7.5 cm) fixed to a response platform by four thumb screws was used as the test cage. The floor consisted of six stainless steel bars 3.0 mm in diameter spaced 1.5 cm apart. The cage and response platform were located inside a ventilated, sound-attenuating plywood chamber (64 × 60 × 40 cm). A loudspeaker located 10 cm behind the test cage delivered both the startle stimulus (100 dB, 50 ms burst of white noise) and continuous background noise (55 dB). The startle reaction of the rats generated pressure on