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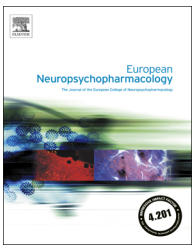

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Activation of ERK2 in basolateral amygdala underlies the promoting influence of stress on fear memory and anxiety: Influence of midazolam pretreatment

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Abstract
Exposure to emotionally arousing experiences elicits a robust and persistent memory and enhances anxiety. The amygdala complex plays a key role in stress-induced emotional processing and in the fear memory formation. It is well known that ERK activation in the amygdala is a prerequisite for fear memory consolidation. Moreover, stress elevates p-ERK2 levels in several areas of the brain stress circuitry. Therefore, given that the ERK1/2 cascade is activated following stress and that the role of this cascade is critical in the formation of fear memory, the present study investigated the potential involvement of p-ERK2 in amygdala subnuclei in the promoting influence of stress on fear memory formation and on anxiety-like behavior. A robust and persistent ERK2 activation was noted in the Basolateral amygdala (BLA), which was evident at 5 min after restraint and lasted at least one day after the stressful experience. Midazolam, a short-acting benzodiazepine ligand, administered prior to stress prevented the increase in the p-ERK2 level in the BLA. Pretreatment with intra-BLA infusion of U0126 (MEK inhibitor), but not into the adjacent central nucleus of the amygdala, attenuated the stress-induced promoting influence on fear memory formation. Finally, U0126 intra-BLA infusion prevented the enhancement of anxiety-like behavior in stressed animals. These findings suggest that the selective ERK2 activation in BLA following stress exposure is an important mechanism for the occurrence of the promoting influence of stress on fear memory and on anxiety-like behavior.
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1. Introduction

The facilitating influence of stress on fear memory formation has been supported by a substantial amount of behavioral and electrophysiological evidence (Rodriguez Manzanares et al., 2005; Isoardi et al., 2007; Roozendaal et al., 2009). Augmented

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behavioral responses to aversive stimuli, including freezing, has been reported in a number of different learning and memory paradigms in animals subjected to prior stressful experiences (Sananbenesi et al., 2003; Maldonado et al., 2011). Related to this, extensive data in humans and rodents suggest that stress effects on emotional processing and memory formation are largely mediated by the amygdala complex (LeDoux, 2000; Anglada-Figueroa and Quirk, 2005; Roozendaal et al., 2009; Brunetti et al., 2010; Diaz-Mataix et al., 2011), which is a heterogeneous collection of interconnected nuclei placed in the temporal lobe (LeDoux, 2003). Specifically, certain nuclei such as the Basolateral complex (BLA), which comprises the lateral, basomedial and basolateral nuclei, together with the Central nucleus (CeA), make essential contributions to the formation, storage, retrieval and expression of fear memory (Pitkanen et al., 1997; LeDoux, 2000; Maren, 2003; McGaugh, 2004; Pape, 2010).

Consistent with the behavioral effect of stress on fear memory, accumulating data sustains a facilitating influence of stress on the generation of long-term potentiation (LTP) in amygdala nuclei, a process suggested to be critical in learning and memory (Rodriguez Manzanares et al., 2005; Isoardi et al., 2007). Moreover, emotional arousal resulted in a persistent enhancement of the spontaneous firing rates of BLA neurons and stressful experience led to hyperexcitability of BLA neurons accompanied by depressed GABAergic inhibition (Rodriguez et al., 2005; Isoardi et al., 2007).

However, despite the well-documented behavioral and electrophysiological effects of environmental challenges on fear memory, relatively few studies to date have addressed the molecular mechanisms underlying the facilitating influence of stress on fear memory formation.

One candidate signaling molecule involved in stress-induced facilitating effects might be the extracellular signal-regulated kinase (ERK) subfamily of mitogen-activated protein (MAP) kinases. In fact, the ERK1/2 signaling pathway mediates signals from the cell surface and receptors to nuclear targets, leading to changes in gene expression (Impey et al., 1999; Feld et al., 2005). It is well established that the ERK1/2 pathway plays a major role in synaptic plasticity, (Sweatt, 2001; Thomas, 2004), and several reports have indicated that ERK1/2 activation in the amygdala and hippocampus after fear conditioning is a key molecular event for fear memory consolidation (Atkins et al., 1998; Alonso et al., 2002; Pelletier et al., 2005; Schafe et al., 2008). Accordingly, previous reports have shown that the blockade of ERK1/2 phosphorylation by the MEK inhibitors, blocks memory consolidation in multiple paradigms, including fear memory (Schafe et al., 2000, 2001). In support of these evidences this inhibition also impaired the emergence of neural plasticity (Schafe et al., 2008). Finally, previous findings have shown a consistent enhancement of p-ERK2 expression in the BLA in the consolidation of contextual fear memory in stressed animals (Maldonado et al., 2011).

Acute stress results in the activation of the ERK1/2 signaling cascade in brain regions that are essential components of the neural circuitry orchestrating emotional responses (LeDoux, 2000; Meller et al., 2003). Given the importance of the ERK1/2 cascade in stress effects and in the formation of fear memory, the main aim of this study was to evaluate the role of the ERK2 in the BLA and the CeA nuclei in an attempt to establish a potential involvement of this molecular cascade in the processing of stress in both areas. Importantly, since the BLA also plays a pivotal role in the unconditioned behavioral responses induced by stressful experiences (Bignante et al., 2010), the second aim of this study was to explore the role of such ERK2 activation in this amygdale complex in the influence of stress on the anxiety-like behavior (Cruz et al., 1994).

2. Experimental procedure

2.1. Animals

Adult male Wistar rats (270–320 g) of our breeding stock were housed in standard laboratory Plexiglas cages (four per cage of dimension: 30 cm × 45 cm × 18 cm) with food and water ad libitum. All animals were maintained throughout the experiments in a 12 h light/dark cycle (lights on at 7:00 a.m.) with a constant room temperature of 21 ± 2 °C. Behavioral testing was performed during the light cycle between 10:00 a.m. and 2:00 p.m. Procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba. Efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Drugs and drug administration

MDZ (Gobbizolam, Gobbi Novag S.A., Argentina) was diluted in sterile saline (SAL, 0.9%, w/v) for intraperitoneal (i.p.) injection (1 ml/Kg), and administered at a dose of 1.5 mg/kg. SAL was used for control injections and administered i.p. in a volume of 1 ml/kg. The dose for systemic administration was selected based on previous reports (Rodriguez Manzanares et al., 2005).

U0126 (Sigma, St. Louis, MO, USA), was dissolved in 100% of dimethyl sulfoxide (DMSO, Sigma USA), stored at −20 °C and diluted in SAL before intracranial infusions. The U0126 and vehicle (VEH) solutions had a final concentration of 20% DMSO. Rats were given a bilateral intra-BLA or intra-CeA infusion of either 0.5 μl of U0126 (1 μg/side) or its vehicle (20% DMSO in SAL). The dose of U0126 was selected based on a previous report (Schafe et al., 2000) and on pilot experiments performed in our laboratory.

2.3. Stressor

The animals were transferred to the experimental room and placed for 30 min inside a plastic restrainer which was fitted close to the body, and did not allow the animal to move, with only the tail and the tip of the nose of the rat being free. No other subjects were present in the experimental room during stress exposure, and at the end of the stress session, the animals were returned to the colony room (RES Group). Control animals were transferred in their own home cages to a separate experimental room, handled for 2 min and then returned to the colony room (CON Group). This procedure was selected based on previous findings from our laboratory using a similar stress protocol to that used in the current study (Rodriguez Manzanares et al., 2005; Isoardi et al., 2007; Bustos et al., 2010).

2.4. Surgery and intracranial infusions

Under aseptic conditions, animals were anesthetized with an i.p injection of ketamine (55 mg/kg)-xylazine (11 mg/kg) and placed in a stereotaxic instrument (Stoelting, Wood Dale, IL). The scalp was incised and retracted, and the head position was adjusted to place bregma and lambda in the same horizontal plane, with the incisor bar set at −3.3 mm. Small burr holes were made to implant two stainless-steel guide cannulas (22 gauge; length, 12 mm) billaterally into the BLA using the following coordinates: antero posterior (AP),

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