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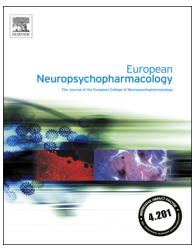

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Activation of ERK2 in basolateral amygdala underlies the promoting influence of stress on fear memory and anxiety: Influence of midazolam pretreatment

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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.

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),

−2.3 mm; lateral (L), ±5.0 mm; dorso ventral (DV), −6.2 mm and for the CeA: antero posterior (AP), −2.1 mm; lateral (L), ±4.0 mm; dorso ventral (DV), −5.8 mm, (Paxinos and Watson, 2007). The guide cannulas were secured in place using acrylic cement, and two stainless-steel screws were anchored to the skull. Animals were removed from the stereotaxic instrument to recover under a heat lamp and with close supervision. After surgery, animals received a subcutaneous injection of a penicillin/streptomycin suspension to reduce the risk of infections. Animals were gently handled every day, replacing missing dummy cannulas when necessary, and were allowed to recover from surgery for 5–7 days. Micro infusions were made using 33-gauge infusion cannulas that extended 2 mm beyond the guide cannulas implanted into the BLA or CeA. The infusion cannulas were connected via polyethylene tubing (PE 10, Becton Dickinson, MD) to a 10 μl micro syringe (Hamilton, Reno, NV) mounted on a microinfusion pump (Cole-Parmer® 74900-Series). The U0126 or VEH was infused at a flow rate of 0.25 μl/min over a period of 120 s then, after completion of the volume injection, the infusion cannulae were kept in place for an additional period of 60 s to allow diffusion of the drug.

2.5. Histological procedures

After the behavioral tests, rats were sacrificed using an overdose of chloral hydrate, and their brains removed and immersion-fixed in a 4% formalin solution. Frontal sections were cut in a cryostat (Leica, Nussloch, Germany). The injection sites and the extent of tissue damage caused by cannulation were examined under a light microscope. Only animals with proper cannula placements and tissue damage not exceeding the diameter of the cannulas were included in the study. Ten animals were discarded from this experiment.

2.6. Behavioral procedures

2.6.1. Conditioning apparatus

The conditioning chamber was made of white acrylic (20 cm × 23 cm × 20 cm) and had clear lids. The shock-grid floor consisted of 10 parallel stainless-steel grid bars, each measuring 4 mm in diameter and spaced 1.5 cm apart (center to center), connected to a shocker-scrambler unit to deliver shocks of defined intensities (Ugo Basile Biological Research Apparatus, Italy). Illumination was provided by a 2.5 W white light bulb, and the background noise was supplied by ventilation fans and the shock scrambler. The chamber was cleaned with 10% aqueous ethanol solution both before and after each session.

2.6.2. Contextual fear conditioning

The procedure used was similar to that previously described (Maldonado and Martijena et al., 2011). On the day of the experiment, rats were transported from the housing room, individually placed in the conditioning chamber and left undisturbed for a 3 min acclimatization period (pre-shock period), which was followed by a single footshock (0.3 mA; 3 s duration). Animals remained in the chamber for an additional 50 s (post-shock period). Testing for contextual fear conditioning, expressed as freezing, was performed 24 h later. One week prior to experiments, rats were handled daily for approximately 15 s each. The behavior of each rat was continuously videotaped in order to score freezing behavior during the pre-shock and post-shock period, and during the entire 5 min testing period. Freezing behavior was scored by a person who was unaware of the experimental conditions of each animal. Freezing, a reliable measure of learned fear, was defined as a total absence of body or head movement, except for that associated with breathing (Blanchard and Blanchard., 1969). For the intra-BLA and intra-CeA experiments, cannulated rats were conditioned with a single footshock of 0.5 mA × 3 s, in order to induce levels of conditioning similar to those exhibited by rats with systemic drug administration, because chronic cannulation tends to attenuate the expression of conditioned freezing (Fendt., 2001). Cannulated rats were tested for conditioned freezing as previously described.

2.6.3. Elevated plus maze

The elevated plus maze (EPM) consisted of a plus-shaped apparatus of black Plexiglas with two opposite open arms (50 cm × 10 cm) and two opposite closed arms (50 cm × 10 cm × 40 cm). The arms were attached to a central square (10 cm^2) and the whole apparatus was elevated 50 cm above the floor. The testing room was quiet and dimly lit, and animals were habituated to this room for at least 45 min before starting the tests. Rats were placed in the center of the maze facing an enclosed arm and the following scores were recorded for 5 min: number of entries into enclosed or open arms, and time spent in both open and enclosed arms. An entry was defined as when all four paws of the rat were inside an arm. The maze was cleaned with a 10% ethanol solution both before and after each trial. The time spent in open arms relative to the total time spent in both open and closed arms was used as an index of anxiety (Cruz et al., 1994; Martijena et al., 2002; Calfa et al., 2006). All rats were tested in the EPM 24 h after the restraint experience, since a number of reports (including those from our laboratory) have shown that changes in anxiety-like behavior induced by stress are still present one day after the aversive experience (Martijena et al., 1997; Bignante et al., 2010).

2.7. Western blotting

Animals were individually transferred to a separate room for sacrifice. Their brains were quickly removed and placed in an acrylic brain matrix (Stoelting CO., USA) on ice. Coronal brain slices of 2 mm containing the amygdala were prepared, and the BLA and CeA were micropunched (Maldonado et al., 2011). These punches were homogenized in RIPA's buffer [150 mM NaCl, 0.1% triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulphate (SDS), 50 mM Tris, pH 7.5] containing protease inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 100 μg/ml phenylmetylsulfonyl fluoride) and phosphatase inhibitor (1 mM Na_3VO_4, sodium orthovanadate), and centrifuged at 10,000*g* for 10 min at 4 °C. The samples were combined with an equal to a third part of the supernatant volume with Laemmli's buffer [2% SDS, 20% glycerol, 10% mercaptoethanol, 0.01% bromophenol blue, 125 mM Tris, pH 6.8], boiled at 100 °C for 5 min and stored at −80 °C until use. Aliquots of the supernatant were used for total protein quantification using the Bio-Rad Bradford Protein Assay Kit (Hercules, CA). Protein samples (30 μg/lane) of tissue homogenate were separated in 10% SDS-PAGE gel, and subsequently blotted to polyvinylidene fluoride membrane (BioRad). Blots were blocked with 5% nonfat dry milk diluted in TTBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, and then incubated overnight at 4 °C with an antibody that recognized phosphorylated ERK1/2 MAPK at Thr202 and Tyr204 (1:1000; Cell Signaling Technologies). These were then probed with a secondary antibody anti-rabbit conjugated to horseradish peroxidase (1:2500, Cell Signaling) for 2 h at room temperature and developed using enhanced chemiluminescence on X-ray film. The immunoblots with phosphorylation site-specific antibodies were subsequently stripped with NaOH 0.1 M, for 10 min, washed 3 times for 5 min with TTBS, and reblocked with 5% nonfat milk. Then, the blots were reprobed with anti-ERK1/2 MAPK antibody (1:2500; Cell Signaling Technologies) at 4 °C overnight. The next day these were probed with anti-mouse conjugated to horseradish peroxidase (1:2500, Cell Signaling) for 2 h at room temperature. α-Tubulin expression was used as the internal control to correct for any protein differences loading. The blots were previously stripped with NaOH 0.1 M for 10 min, and then were incubated with α-Tubulin antibody (1:2000 Sigma) at 4 °C overnight. The next day the membranes were subsequently incubated with the corresponding anti-mouse antibody (1:3000, Cell Signaling) for 2 h at room temperature.

Only film exposures that were in the linear range of the ECL reaction were used for quantification analysis. Although the anti-phospho and anti-total ERK1/2 antibodies used in the present study recognized both ERK1 and ERK2, p-ERK1 was not quantified because the p-ERK1 signals were often too faint and inconsistent to be accurately analyzed. The resulting film samples were scanned and analyzed with an image analysis program (GelPro32 Analyzer). The value of the relative optical density of each band corresponding to phospho ERK2 was normalized to the value of total ERK2 to demonstrate the phosphorylation level; the relative density of each band corresponding to total ERK2 was normalized to the value of α-Tubulin band to illustrate the protein expression level. The presented data were expressed as means±S.E.M. The optical density of each band was normalized with the total signal of each plot.

2.8. Statistical analyses

All results were expressed as the means±S.E.M, and analyzed by ANOVA. The source of the main significant effects or interactions was determined by Student's Newman Keuls and the Dunnet test. The significance level used for all statistical analyses was set at $p<0.05$.

3. Results

3.1. Experiment 1. Restraint stress induced different patterns of ERK2 activation in the BLA and CeA

The goal of this experiment was to analyze the time course of ERK2 activation in the BLA and the CeA following stress exposure. Thirty seven animals were randomly distributed into the NAÏVE or RES groups. Then, animals were sacrificed at 5, 30, 60 min or 24 h after the stressful experience. The experimental design resulted in the following experimental groups for the BLA: NAÏVE ($n=7$), RES 5 min ($n=7$), RES 30 min ($n=8$), RES 60 min ($n=8$) and RES 24 h ($n=7$), whereas for the CeA: NAÏVE ($n=6$), RES 5 min ($n=5$), RES 30 min ($n=6$), RES 60 min ($n=6$) and RES 24 h ($n=5$).

As shown in Figure 1C, the p-ERK2 levels in the BLA were increased at 5, 30, 60 min, and 24 h after the stress exposure in comparison with the NAÏVE group. This effect was confirmed by one-way ANOVA analysis [$F(4, 32)=6.23$, $p<0.001$] followed by a Dunnet test (*$p<0.05$) (Figure 1C). In contrast, stress induced an increase in the p-ERK2 levels only at 60 min after stress ($p<0.01$) compared with the NAÏVE group (Figure 1E). The results of this experiment suggest that the p-ERK2 levels from BLA were elevated in response to stress exposure at each time point evaluated. No changes in the total ERK levels were noted in any of the groups.

3.2. Experiment 2. MDZ pretreatment prevented ERK2 activation in the BLA following stress exposure

It has been suggested that MDZ administration prior to the environmental challenge prevents the facilitating influence of stress on fear memory formation (Rodriguez Manzanares et al., 2005; Maldonado et al., 2011). Therefore, if stress-induced activation of the ERK2 in the BLA is a prerequisite for the promoting effect of stress on fear memory, MDZ should

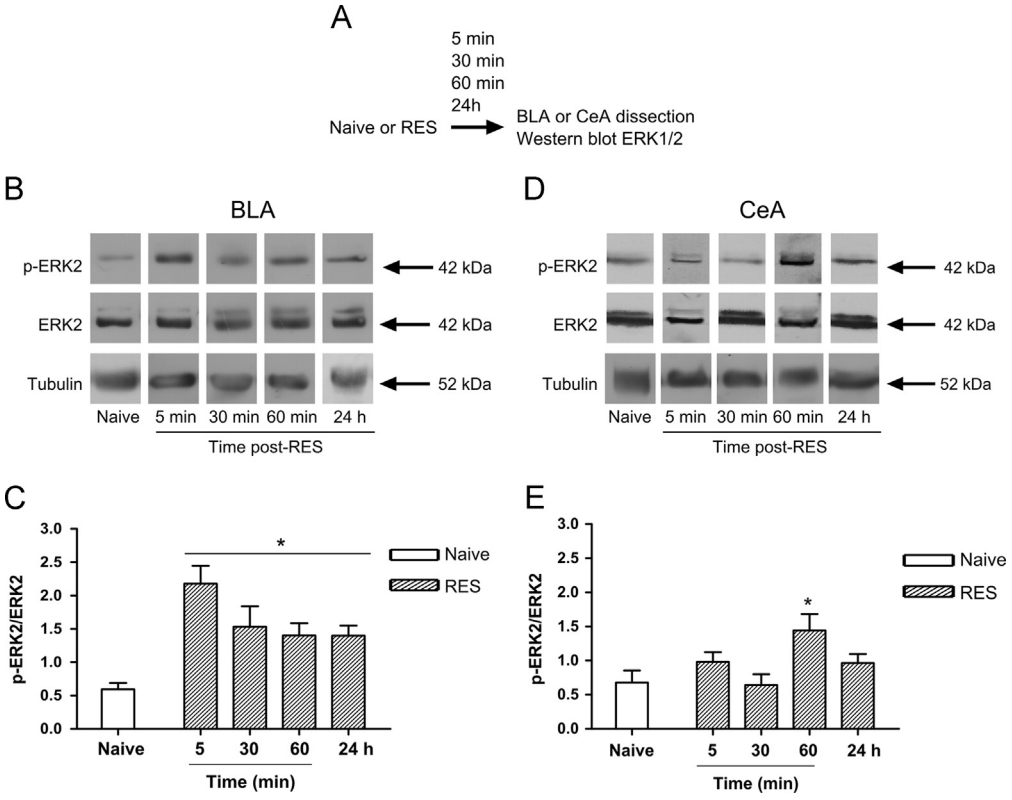

Figure 1 ERK2 activation in the BLA and CeA at different time points after acute stress exposure. (A) Experimental design. Top panels show representative inmunoblots of p-ERK1/2, total ERK1/2, and α-Tubulin in the BLA (B) and the CeA (D). In the bottom panels, bars represent the mean of p-ERK2/ERK2 of the relative optical density±S.E.M. *$p<0.05$ vs. Naïve group.

prevent the elevation of BLA p-ERK2 levels following stress. To explore this possibility, animals were administered with SAL or MDZ (1.5 mg/Kg, i.p.) 10 min before the restraint exposure. Five min after the stress, the animals were sacrificed to assay p-ERK2 levels in the BLA. Twenty five animals were randomly assigned to SAL or MDZ injections and later on subjected or not to the stressful experience. The experimental design resulted in the following groups: SAL-CON ($n=6$), SAL-RES ($n=7$), MDZ-CON ($n=6$) and MDZ-RES ($n=6$). Similar to the findings of experiment 1, stress resulted in a significant increase of p-ERK2 levels in BLA relative to unstressed rats (Figure 2A). As depicted in Figure 2 B, MDZ pretreatment prevented the p-ERK2 enhancement induced by restraint stress in the BLA. A two-way ANOVA revealed a significant effect of treatment [$F(1, 21)=5.44$ $p<0.05$] and drug x treatment interaction [$F(1, 21)=7.28$, $p<0.05$]. The *post hoc* Student Newman Keuls test showed that the p-ERK2 levels exhibited by SAL-RES were significantly different from the remaining groups ($^{*}p<0.05$).

The findings of the current experiment indicate that MDZ prior to restraint prevents the activating influence of stress on the pERK2 levels in the BLA.

3.3. Experiment 3. The blockade of ERK2 activation in the BLA prior to the restraint exposure prevented the facilitating influence of stress on fear memory formation

Based on the ability of the restraint experience to increase p-ERK2 levels in the BLA, we evaluated the functional consequences of blocking ERK2 activation on fear memory retention by means of local infusion of U0126 into the BLA prior to stress. Forty animals were randomly distributed into the NAÏVE or RES groups, and were either infused with U0126 or VEH into the BLA 30 min prior to stress exposure (Figure 3A). One day later, rats were subjected to the fear conditioning procedure, and the following day tested for fear retention. The experimental groups were: VEH-CON ($n=13$), VEH-RES ($n=9$), U0126-CON ($n=9$) and U0126-RES ($n=9$).

Figure 3B depicts the effect of ERK1/2 blockade in the BLA on fear memory. As expected, stressed animals infused with VEH exhibited an enhanced freezing response during testing. A two-way ANOVA analysis showed significant effects of stress [$F(1, 36)=5.59$; $p<0.05$] and of drug x stress interaction [$F(1, 36)=24.26$; $p<0.01$]. The post-hoc Student Newman Keuls test revealed that the VEH-RES group showed higher freezing levels compared with the remaining groups ($^{*}p<0.01$). In conclusion, intra-BLA infusion of U0126 prevented the facilitating influence of stress on fear memory. The BLA cannula placements are shown in Figure 3C.

3.4. Experiment 4. ERK2 in the CeA is not involved in the facilitating influence of stress on fear memory formation

To investigate a potential involvement of the ERK1/2 in the CeA in the facilitating effect of stress on fear retention, we explored the influence of the intra-CeA U0126 prior to stress. Thirty two animals were randomly distributed into the NAÏVE or RES groups and either infused with VEH or U0126 30 min prior to stress exposure. One day later, rats were subjected to the fear conditioning procedure, and the following day tested for fear retention. The final experimental groups were: VEH-CON ($n=8$), VEH-RES ($n=10$), U0126-CON ($n=6$) and U0126-RES ($n=8$).

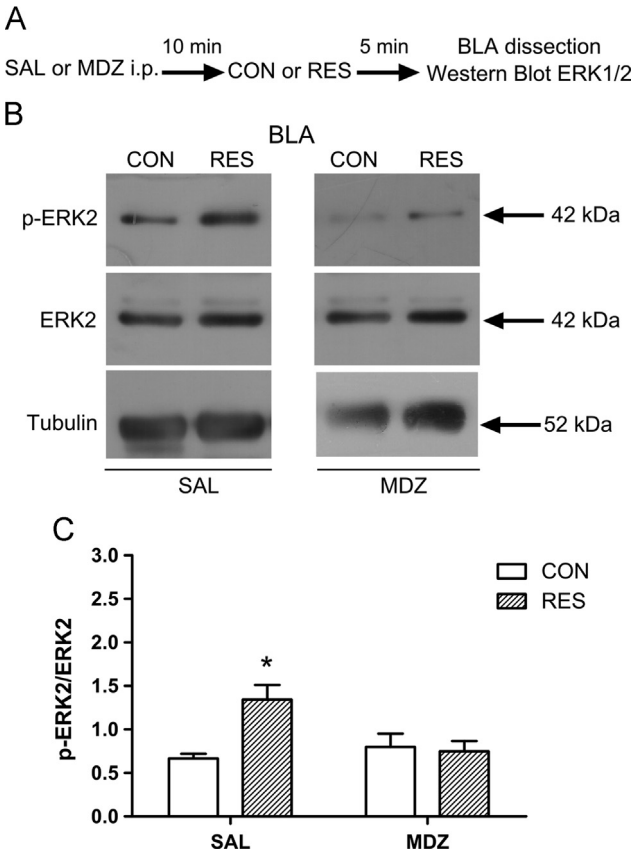

Figure 2 Influence of MDZ pretreatment on ERK2 activation induced by stress. (A) Experimental design. (B) Top panels show representative inmunoblots of p-ERK1/2, total ERK1/2, and α-Tubulin in the BLA. In the bottom panels, bars represent the mean of p-ERK2/ERK2 of the relative optical density ± S.E.M. $^{*}p<0.05$ vs. the remaining groups.

As expected, stressed rats with intra-VEH freeze significantly more than unstressed animals. As can be seen in Figure 4B, intra-CeA U0126 did not affect the increased freezing response induced by stress. A two-way ANOVA analysis revealed a significant effect of stress [$F(1, 28)=33.32$; $p<0.01$]. No other effects or interactions were detected. Regardless of the drug administered, the freezing levels exhibited by the stressed groups did not differ from each other and were significantly higher than their respective unstressed groups (Student Newman Keuls, $^{*}p<0.01$). Hence, the intra-CeA inhibitor does not prevent the promoting influence of stress on fear memory. The CeA cannula placements are shown in Figure 4C.

3.5. Experiment 5. The blockade of ERK2 activation in the BLA prior to restraint exposure attenuated the increased anxiety-like behavior in stressed animals

Previous experiments are consistent with the view that ERK pathway activation in the BLA is involved in the promoting

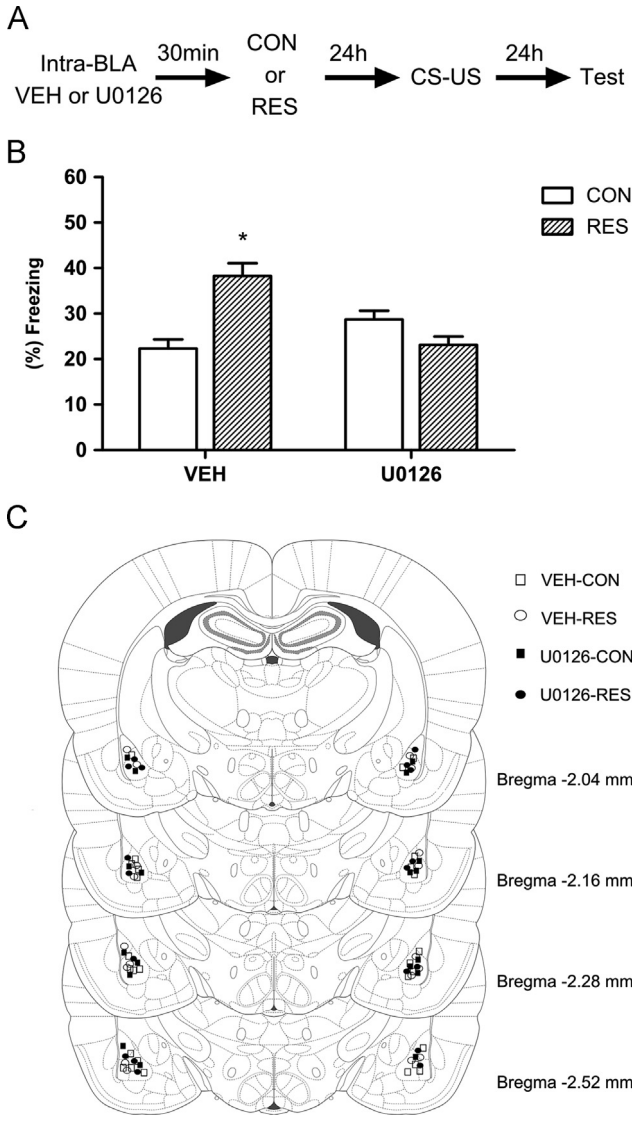

Figure 3 Effect of the intra-BLA infusion of U0126 prior to restraint session on subsequent fear memory formation. (A) Experimental design. (B) Bars represent the means ± S.E.M. of the percentage of time spent freezing during the test. $^{*}p<0.01$ compared with the remaining groups. (C) Schematic drawings of coronal sections of the rat brain show the location of the cannulas in the BLA, VEH-CON (□), VEH-RES (○), U0126-CON (■) and U0126-RES (●). These drawings were adapted from Paxinos and Watson (2007).

influence of stress on fear memory (Figure 3C). Next, we explored whether this activation in BLA was also functionally related to an increased anxiety-like behavior, an unconditioned behavioral response to stress. To address this goal, thirty two rats were either infused with VEH or U0126 into the BLA 30 min prior to stress exposure and one day later tested in the EPM (Figure 5A). The resulting groups were: VEH-CON ($n=9$), VEH-RES ($n=8$), U0126-CON ($n=7$) and U0126-RES ($n=8$). Prior restraint exposure induced a decrease in the percentage of time spent in the open arms of the EPM. This anxiogenic effect was not evident when stressed rats received the intra-BLA infusion of U0126 (Figure 5B). A two-way ANOVA analysis showed significant effects of U0126 treatment [$F(1, 28)=15.95$; $p<0.001$] and of U0126 treatment × stress interaction [$F(1, 28)=17.39$; $p<0.001$]. The post-hoc Student Newman Keuls revealed that the VEH-RES group exhibited a significant reduction in the time spent in open arms in comparison with the remaining groups ($^{*}p<0.01$). These evidences support the view that ERK2 activation in BLA is also involved in stress-induced enhancement of anxiety-like behavior. In addition, no significant effects on the number of closed arms entries was detected (VEH-CON = 7.11 ± 0.54, VEH-RES = 8.00 ± 0.38, U0126-CON = 8.57 ± 0.20 and U0126-RES = 6.68 ± 0.42), suggesting that the increased anxiety related behavior induced by restraint was not due to a reduction of exploratory activity. The BLA cannula placements are shown in Figure 5C.

4. Discussion

There is compelling evidence showing that stress activates the ERK1/2 signaling cascade in different brain regions (Zheng et al., 2007; Sweatt., 2001; Meller et al., 2003). Consistent with these findings, the present study showed that a single restraint experience promotes the activation of ERK2 in both nuclei of the amygdala. However, there were remarkable differences between the BLA and the CeA regarding the temporal pattern of ERK2 activation and also in terms of its temporal persistence. The activation in the BLA peaked at 5 min with this increase lasting at least one day after stress, whereas the ERK2 phosporylation was elevated in CeA only at the 60 min time point and then returned to basal levels. This persistence of ERK2 phosphorylation in the BLA is relevant in light of previous findings showing that stress induced a robust promoting influence on fear retention in procedures where fear training was conducted one day after stress (Maldonado et al., 2011).

Therefore, at the time of the learning trial and the resulting consolidation process, an elevated p-ERK2 level in BLA was still evident as shown by the present results, suggesting that the facilitating influence of stress on fear memory paralleled the long-lasting (24 h) stress-induced elevation of p-ERK2 levels in BLA. Since the activation of the ERK1/2 signaling pathway in BLA is a key molecular event for fear memory consolidation (Schafe et al., 2000), our findings suggest that the stress-induced enhancement of p-ERK2 in BLA is an important component in the facilitating effect of stress on fear memory consolidation. In contrast, the current findings showed no elevation of p-ERK2 in CeA one day after stress exposure (at the time of fear memory consolidation). Therefore, if the activation of ERK2 is a relevant event for the occurrence of fear memory consolidation, as previously suggested, the involvement of the ERK signaling in CeA in the promoting influence of stress on fear memory formation seems less likely. In fact, the heightened level of p-ERK2 described at the 60 min time point in the CeA could reflect neural activation via projections from the BLA.

Our behavioral findings with local infusion in both amygdaloid nuclei seem to support this argument. The increased fear memory of stressed rats with either intra-BLA VEH or intra-CeA VEH observed in this study is consistent with the widespread notion that emotionally arousing events result in robust and persistent aversive memories (Shors, 2001;

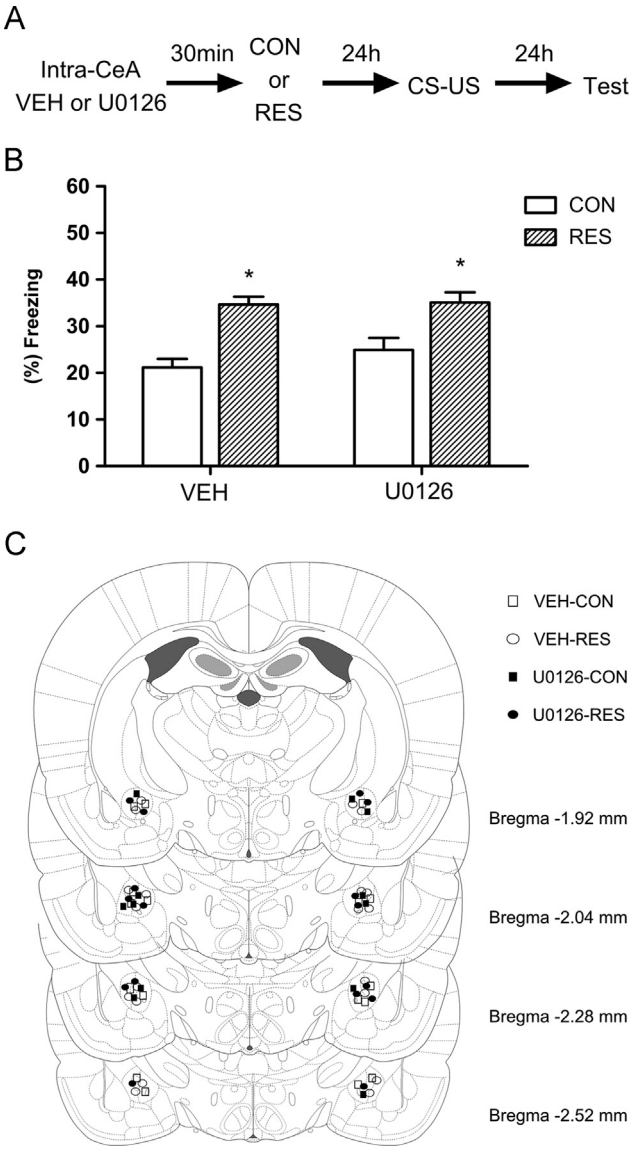

Figure 4 Effect of intra-CeA infusion of U0126 prior to restraint session on subsequent fear memory formation. (A) Experimental design. (B) Bars represent the means ± S.E.M. of the percentage of time spent freezing during the test. *$p < 0.01$ vs. the respective CON group. (C) Schematic drawings of coronal sections of the rat brain show the location of the cannulas in the BLA, VEH-CON (□), VEH-RES (○), U0126-CON (■) and U0126-RES (●). These drawings were adapted from Paxinos and Watson, 2007.

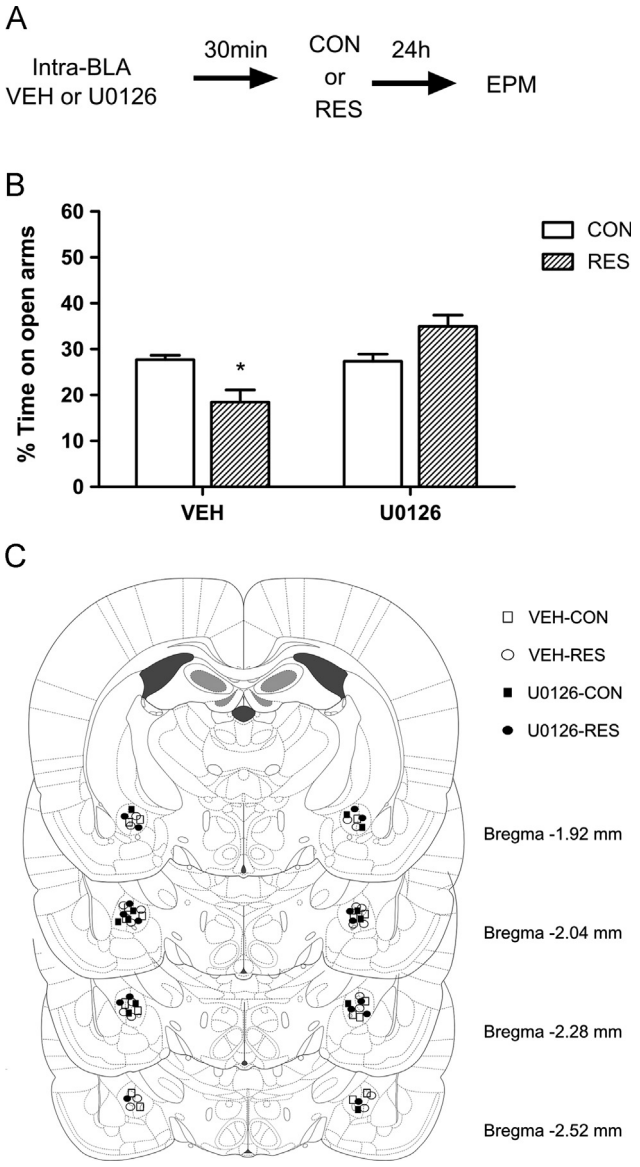

Figure 5 Effect of intra-BLA infusion of U0126 prior to restraint in the anxiety-like behavior on the EPM. (A) Experimental design. (B) Data represent the mean ± S.E.M. of the percentage of time spent in the open arms relative to total time spent in all four arms, *$p < 0.01$ vs. all other groups. (C) Schematic drawings of coronal sections of the rat brain show the location of the cannulas in the BLA, VEH-CON (□), VEH-RES (○), U0126-CON (■) and U0126-RES (●). These drawings were adapted from Paxinos and Watson (2007).

Roozendaal et al., 2009) and with earlier data from this laboratory, using a similar type of stressor to that used in the present study (Rodriguez Manzanares et al., 2005; Bignante et al., 2010). Moreover, the current findings showed that the intra-BLA infusion, but not following the local infusion into the adjacent CeA, of the MEK inhibitor prior to stress mitigated the stress-induced memory strengthening. Therefore, the fact that the stress-induced prolonged ERK activation is selective for the BLA and that the ameliorating influence is only effective after intra-BLA U0126 support the idea that the activation of the stress-induced ERK signaling pathway in BLA is crucial for the enhanced fear retention exhibited by animals exposed to the environmental challenge. Finally, the selective role of BLA in the stress-induced promoting influence on fear memory discards that this effect could be due to a non-specific consequence of the aversive experience or to a diffusion of the drug.

A substantial number of reports have revealed that stress exposure often results in the enhancement of anxiety-like behavior in paradigms designed to assess anxiety (Martijena et al., 1997; Calfa et al., 2006; Bignante et al., 2010). In agreement, the present behavioral results show an excessive

anxiety in the EPM in previously stressed animals with intra-BLA VEH infusion. The pharmacological blockade of the ERK activation by intra-BLA U0126 infusion prevented the increased anxiety-like behavior induced by the stressful experience, indicating that the ERK activating influence of stress in BLA also plays a major role in the unconditioned emotional response to environmental demands. In fact, stress-induced enhancement of BLA neuronal activity is a prerequisite for the occurrence of excessive anxiety (Bignante et al., 2010). There are contradictory findings regarding the involvement of the ERK pathway in the emergence of anxiety. Inhibition of ERK phosphorylation in the medial prefrontal cortex prior to EPM testing resulted in "normal" anxiety and blocked the anxiety-induced c-FOS expression in this area in animals previously exposed to a stressful procedure (Ailing et al., 2008). On the contrary, disrupted ERK signaling by deleting components of ERK2 was associated with elevated anxiety (Ailing et al., 2008). However, it should be pointed out that there are remarkable procedural differences between these reports and the current study. In fact, in our behavioral procedure the intra-BLA U0126 was performed prior to stress exposure and one day later such animals were exposed to the EPM testing, thus blocking stress-induced ERK activation in BLA prevented the subsequent anxiety enhancement in the EPM.

Collectively, the above results confirm that the BLA emerges as the principal region that orchestrates the emotional behavior following stress exposure (Roozendaal et al., 2009). Consistent with this view, a growing number of evidence have shown that the BLA is critical for emotional regulation and to assign emotional value to environmental information (LeDoux, 2000; Rodriguez Manzanares et al., 2005). Despite the fact that the CeA is the major output of the amygdaloid complex and that under certain conditions this nucleus may play a role in the regulation of motivational and emotional states independent of BLA, a substantial number of evidence indicates that the BLA plays a crucial role in the generation of fear memory and in anxiety regulation (Bignante et al., 2008; Rodriguez Manzanares et al., 2005). Hence, the involvement of the ERK signaling in the CeA area seems not to be essential for the stress-induced influence on both fear learning and anxiety.

The involvement of GABA neurotransmission of the amygdala complex in the negative emotional state induced by stress has been well established (Rodriguez Manzanares et al., 2005; Isoardi et al., 2007). For instance, stress results in both a reduced chloride uptake mediated by GABA-A sites and a decline in the benzodiazepine binding and $\alpha 1$ expression mRNA in BLA (Liu and Glowa, 2000; Rodriguez Manzanares et al., 2005). Besides, previous reports have shown that stress exposure, similar to the one used in the present study, reduced GABAergic inhibitory mechanism in BLA resulting in an unmasked activation of glutamatergic projecting neurons (Isoardi et al., 2007). Interestingly, stress and pharmacologically treatments that results in a decrease of GABAergic neurotransmission in BLA enhanced fear memory formation (Rodriguez Manzanares et al., 2005). Consistent with such findings, it was reported that blocking GABA-A sites with systemic administration of bicuculine enhanced p-ERK1/2 levels in other brain regions such as the hippocampus and the Prefrontal cortex (Zheng et al., 2007). In contrast, activation of the GABA-A sites by MDZ prior to the environmental challenge prevented stress-induced enhancement of anxiety (Bignante et al., 2010). In addition, MDZ (either intra-BLA or systemically) administration attenuated both the promoting influence of stress on fear memory and the reduction of the threshold induction of LTP in BLA, a neural plasticity process associated with learning and memory (Rodriguez Manzanares et al., 2005).

Therefore, if stress-induced activation of the ERK signaling in BLA is a prerequisite for the promoting effect of stress on fear memory as previously indicated, a logical prediction from all this evidence suggest that the administration of a positive modulator of GABA-A sites prior to environmental challenge should prevent the elevation of pERK2 levels in the BLA observed following stress. In line with this prediction, our results showed that MDZ prior to stress attenuated the enhancement of p-ERK2 levels in BLA. These findings therefore support the view that the activation of ERK2 in the BLA is relevant to the effect induced by stress on fear memory consolidation. In addition, previous data have shown that MDZ pretreatment is also capable of attenuating other molecular changes in the BLA elicited by a stressful stimulus identical to that used in the current study (Bignante et al., 2010).

To summarize, the present study provides evidence that the ERK1/2 signaling pathway in BLA plays a critical role in the stress-induced promoting influence on the formation of fear memory and on the increased anxiety-like behavior.

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Contributors

N.M. Maldonado carried out most of the experiments, undertook the statistical analyses and wrote a first draft of the paper. P.J. Espejo collaborated with the western blotting experiments. V.A. Molina designed this study, analyzed the data and wrote the manuscript. I.D. Martijena contributed to the interpretation of data and to the writing of the manuscript. All authors have approved the final manuscript.

Conflict of interest

The author (s) declared no conflicts of financial interest or potential with respect to the research, authorship, and/or publication of this article.

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