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Prior stress promotes the generalization of contextual fear memories: Involvement of the gabaergic signaling within the basolateral amygdala complex

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ABSTRACT

Fear generalization occurs when a response, previously acquired with a threatening stimulus, is transferred to a similar one. However, it could be maladaptive when stimuli that do not represent a real threat are appraised as dangerous, which is a hallmark of several anxiety disorders. Stress exposure is a major risk factor for the occurrence of anxiety disorders and it is well established that it influences different phases of fear memory; nevertheless, its impact on the generalization of contextual fear memories has been less studied. In the present work, we have characterized the impact of acute restraint stress prior to contextual fear conditioning on the generalization of this fear memory, and the role of the GABAergic signaling within the basolateral amygdala complex (BLA) on the stress modulatory effects. We have found that a single stress exposure promoted the generalization of this memory trace to a different context that was well discriminated in unstressed conditioned animals. Moreover, this effect was dependent on the formation of a contextual associative memory and on the testing order (i.e., conditioning context first vs generalization context first). Furthermore, we observed that increasing GABA-A signaling by intra-BLA midazolam administration prior to the stressful session exposure prevented the generalization of fear memory, whereas intra-BLA administration of the GABA-A antagonist (Bicuculline), prior to fear conditioning, induced the generalization of fear memory in unstressed rats. We concluded that stress exposure, prior to contextual fear conditioning, promotes the generalization of fear memory and that the GABAergic transmission within the BLA has a critical role in this phenomenon.

1. Introduction

Anxiety related disorders are among the most prevalent psychiatric disorders affecting almost 30% of the population in the USA (Kessler and Wang, 2008). Pavlovian fear-conditioning procedures are considered valuable tools to gain insight in the neurobiology of anxiety (Mineka and Zinbarg, 2006). In fact, these disorders have been attributed to inappropriate behavioral outcomes following associative fear learning, such as excessive fear due to reduced fear inhibition and/or deficit in fear extinction as well as overgeneralization of fear (Milad and Quirk, 2012; Jovanovic and Ressler, 2010). From all of these maladaptive behavioral outcomes, the neurobiological underpinnings of fear generalization have been less studied.

Fear generalization occurs when a response previously acquired with a threatening stimulus/context, is transferred to a similar one (Lopresto et al., 2016; Luyten et al., 2016). This generalization serves as an adaptive function that allows an organism to respond rapidly to new stimuli related to a previously learned fear experience. However, it could be maladaptive when the stimuli, that do not represent a real threat, are then treated as dangerous (Lopresto et al., 2016; Lissek, 2012). For instance, patients with PTSD show an exaggerated reactivity to multiple neutral stimuli distantly related to the traumatic event, and this occurs even in contexts that confer safety (Duits et al., 2015; Dunsmoor et al., 2009). Furthermore, overgeneralization is thought to be implicated in the etiology of PTSD by proliferating anxiety cues signals in the individual's environment that increase and/or sustain anxiety symptoms (Lissek, 2012).

Beyond the well-characterized role of the amygdala in the formation, consolidation, and retrieval of associative fear memory (LeDoux, 2007; Janak and Tye, 2015), a recent report indicates that the amygdala is also a nodal structure for the generalization processes (Ghosh and Chattarji, 2015). Animals that discriminated a tone after a

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differential conditioning procedure had a proportion of neurons that responded selectively to this tone. However, the same cells started to respond to the non-reinforced tone when the animals were re-conditioned with a higher intensity footshock that produced fear generalization (Ghosh and Chattarji, 2015). In the same way, Rajbhandari and co-workers using c-fos to assess neuronal activity during generalization indicated that the activity of the basolateral amygdala (but not in the hippocampus), was highly associated with the level of behavioral fear generalization, suggesting that stronger basolateral amygdala activation is driving the generalization processes (Rajbhandari et al., 2016).

There is a consensus about stress exposure being a major risk factor for the occurrence of anxiety disorders (American Psychiatric Association, 2013). Hence, the modulatory effect of stress on different phases of fear memory has been intensely studied (Perusini et al., 2016; Martijena and Molina, 2012; Roozendaal et al., 2009). A number of reports have revealed that a single stressful experience, prior to fear learning, promotes the emergence of robust emotional memories (Cordero et al., 2003; Rodriguez Manzanares et al., 2005). Moreover, a similar manipulation results in a memory trace resistant to the labilizalization/reconsolidation process (Bustos et al., 2010; Espejo et al., 2016) and retards the formation of the extinction memory (Akirav et al., 2009). At the neurobiological level, the behavioral sequelae of stressful experiences are closely linked to a reduced central GABAergic neurotransmission in the basolateral amygdala complex (BLA) (Martijena and Molina, 2012). In fact, it has been observed that the decrease in the inhibitory GABAergic control in the BLA has a major role in the stress-induced promoting influence on both formation of fear memory and induction of long-term potentiation in the BLA (Rodriguez Manzanares et al., 2005). Furthermore, the administration of midazolam (MDZ) in the BLA prior to stress, prevents the facilitating effect of stress on fear learning. Conversely, the intra-BLA administration of bicuculline (an antagonist GABA-A sites) emulates the facilitating effect of stress on fear learning and on the associated hippocampal structural plasticity (Rodriguez Manzanares et al., 2005; Giachero et al., 2013, 2015). Research made with GAD-65 knockout mice, which display a deficit in GABA content in the amygdala, exhibited amygdala hyperexcitability and a phenotype of pathological fear memory (Müller et al., 2014). For instance, using a differential fear conditioning paradigm, these animals express generalization of auditory fear memory to the neutral acoustic stimulus explicitly not paired with the shock (Bergado-Acosta et al., 2008).

Even though generalization is receiving great attention (Jasnow et al., 2017; Besnard and Sahay, 2016), no studies have addressed the effects of acute stress on the generalization of contextual fear memories, so far. In the current work, we have evaluated whether previous stress exposure promotes the generalization to a novel context which was not associated with the threatening stimulus (footshock). Moreover, we have analyzed the potential role of the GABAergic signaling in the BLA, in the generalization of these fear memories.

2. Methods

2.1. Animals

Adult male Wistar rats aged 70–80 days (280–320 g) from our breeding stock were housed in groups of 2–3 per cage (45 × 30 × 20 cm) with food and water ad libitum. All the animals were maintained on a 12 h light/dark cycle (with light from 7:00 a.m.) at 21–22 °C, following the protocols approved by the Animal Care Committee of the "*Facultad de Ciencias Químicas*", National University of Cordoba, which is consistent with the NIH Guide for the Care and Use of Laboratory animals. The number of animals used as well as their suffering were minimized as much as possible. All the experiments were conducted between 10 a.m. and 4 p.m.

2.2. Stress

The animals were stressed by immobilization in plastic restrainers under intense light for 60 min (S group), after which the rats were returned to the colony room. Control animals (NS group) were transferred in their own home cages to a separate experimental room, handled for 2 min, and then returned to the colony room.

2.3. Contextual fear conditioning and testing

2.3.1. Fear conditioning

The rats were individually placed into the conditioning chamber (cxt-A) and after 3 min of acclimatization (pre-shock period) 2 unsignaled scrambled footshocks (1 mA, 3 s duration and 30 s intershock interval) were given to them. After that, the animals were returned to the home cage.

2.3.2. Conditioning chamber (cxt-A)

The conditioning chamber was made of white acrylic (20 × 23 × 20 cm) with a transparent lid and it was connected to a scrambled shocker (Ugo Basile Biological Research Apparatus, Italy). The grid floor consisted of 10 parallel stainless steel grid bars, each measuring 1.5 mm in diameter and spaced 1.5 cm apart (center to center). The conditioning room was illuminated by a white fluorescent tube located on the ceiling, with a ventilation fan used to provide background noise (55 dB). The chamber was cleaned with a 70% aqueous ethanol solution before and after each session.

2.3.3. Generalization chamber (cxt-B)

The chamber was made of wood (33 × 25 × 33 cm) with black walls, a black rubber floor and a lid of transparent plastic. The box was illuminated with a faint yellow light located near the chamber, with a ventilation fan used to provide background noise (55 dB). The chamber was cleaned with tap water before and after each session.

It is important to notice that cxt-A and cxt-B are quite dissimilar and could be closer to what in other studies, that use 3 different contexts, are cxt-A and cxt-C (e.g. Luyten et al., 2016; Rajbhandari et al., 2016). Cxt-B used here was selected from pilot studies based on the behavioral performance of non-stressed rats that underwent fear conditioning which showed similar levels of freezing as the animals that were not conditioned.

2.3.4. Test of fear memory and generalization

Freezing, a commonly used index of fear in rats, was defined as a total absence of body or head movement except for that associated with breathing (Bolles and Collier, 1976). Freezing behavior was videotaped and analyzed offline by a person who was blind to the experimental condition of each animal. The measure of fear was quantified (in seconds) using a stopwatch and expressed as the percentage of the total time. The testing sessions, in cxt-B and cxt-A, had 5 min of duration and were performed on the same animals in different consecutive days (for a detailed explanation, see below). The generalization index was calculated for each animal by the ratio between the percentage of freezing in cxt-B divided by the percentage of freezing in cxt-A.

2.4. Surgery and intracranial infusions

The rats were anesthetized with a mixture of ketamine (55 mg/kg, i.p.; Ketajects) and xylazine (11 mg/kg i.p.; Xyla-Jects) under aseptic conditions and were placed in a stereotaxic instrument (Stoelting, Wood Dale, IL) with the incisor bar set at 23.3 mm. Two stainless steel guide cannulas (22 gauge; length 12 mm) located in the BLA were used, following specific coordinates: anterior, − 2.8 mm; lateral, ± 5.0 mm; ventral, − 6.0 mm (Paxinos and Watson, 2009). The guide cannulas and a stainless-steel screw were fixed to the skull with acrylic cement. Smaller stainless cannulas were placed inside the guide cannulas to

prevent occlusion and were removed the day of injection. After surgery, the animals received a subcutaneous injection of a penicillin/streptomycin suspension to reduce the risk of infections. The animals were allowed to recover from surgery for 7 days before the behavioral procedures started. Microinfusions were made using 33-gauge infusion cannulas that extended 2 mm beyond the guide cannulas implanted in the BLA. The infusion cannulas were connected via polyethylene tubing (PE 10, Becton Dickinson, MD) to a 10 ml microsyringe (Hamilton, Reno, NV) mounted on a microinfusion pump (Cole-ParmerVR 74900-Series). After the completion of the experiment, animals were anesthetized with 16% chloral hydrate and then decapitated. After that, the brains were removed and placed in 4% paraformaldehyde in order to evaluate the injection site. Only those animals with adequate bilateral injection sites were considered for statistical analysis.

2.5. Drugs and administration

Bicuculline (BIC) was dissolved and diluted in sterile isotonic saline (SAL, 0.9% w/v) and bilaterally infused at a dose of 5 ng/0.5 μl per side, 20 min for intra-BLA administration before conditioning. The BIC dose selected well below doses that have been previously reported to induce convulsion and brain seizure activity (Turski et al., 1985; Sanders and Shekhar, 1991; Dickinson-Anson and McGaugh, 1997). Besides, this dose was previously reported to facilitate fear memory (Rodriguez Manzanares et al., 2005). Midazolam (MDZ) (GobbiNovag, Buenos Aires, Argentina) was diluted in sterile isotonic saline (SAL, 0.9% w/v) and a dose of 1 μg/0.5 μl per side was used for bilaterally intra-BLA administration 10 min before stress exposure.

2.6. Experimental design

2.6.1. Experiment 1

On day 1, all groups were exposed to cxt-B for habituation to the generalization test chamber. The rationale for this habituation was to diminish the novelty induced anxiety that could favor the expression of unspecific freezing during the test in cxt-B. In fact, in several pilot studies that we have done to set up the present protocol, we observed that this previous familiarization in cxt-B reduced the freezing levels during the generalization test in non-stressed rats that underwent fear conditioning. Accordingly, this was selected in order to have more sensitivity of the fear behavior in cxt-B. On day 2, half of the animals underwent the stress protocol. On day 3, half of the stressed and non-stressed animals were conditioned in cxt-A and the other half remained the same time in the training context without receiving the unconditioned stimulus. On day 4, all the animals were tested for 5 min in cxt-B for fear generalization assessment. On day 5, all the animals were exposed for 5 min in cxt-A for fear memory evaluation (Fig. 1A). In order to know if the stress induced generalization was long lasting, the same protocol was applied in another set of experiments but the animals were evaluated 7 days after fear conditioning in cxt-B and in ctx-A the following day.

2.6.2. Experiment 2

The protocol was the same as described above, except that on day 3 all the animals did not have the preshock period so that they received the first footshock as soon as they entered the conditioning box (cxt-A) and received the second shock 10 s later after which the animals were returned to the home cage (Fig. 2A).This protocol named “immediate shock deficit” is used to prevent the contextual fear memory formation (Fanselow, 1986; Landeira-Fernandez et al., 2006). Hence, it was applied to determine whether an associative memory was necessary for the generalization or if any increase in freezing could be due to an unspecific sensitization of the fear response.

2.6.3. Experiment 3

The protocol was the same described for experiment 1, except that the testing order in conditioning and generalized context was reversed (Fig. 3A). Hence, on day 4, the animals were tested for 5 min in cxt-A and the next day, they were tested in cxt-B. The rationale for this experiment was to examine if the order of presentation was relevant since previous reports have shown that the generalization of fear is greatly diminished if animals are exposed to the conditioning context before the generalization context (Huckleberry et al., 2016) while others have done a counterbalanced design and showed no order effects (Poulos et al., 2016). Furthermore, other researchers test first in the training context and then in a new context, observing increasing levels of freezing in the novel context (Baldi et al., 2004).

2.6.4. Experiment 4

On day 1, all groups were exposed to cxt-B for habituation to the generalization test chamber. On day 2, the animals were administered with either BIC or SAL intra- BLA and 20 min later, half of them were conditioned in cxt-A whereas the other half remained the same time in this chamber without receiving the unconditioned stimulus. On day 3, all the animals were tested for 5 min in cxt-B for fear generalization assessment. On day 4, all groups were exposed in cxt-A for fear memory evaluation during 5 min (Fig. 4A). The rationale for the timing of BIC administration is based on previous data from our lab, showing that 24 h after stress the inhibitory currents mediated by GABAergic transmission in BLA are decreased and the excitability of the system is enhanced (Rodriguez Manzanares et al., 2005). This indicates that at the time that the stressed animal undergoes the fear conditioning training, the gabaergic inhibitory system is decreased. Therefore, in order to mimic this state pharmacologically, we used bicuculline 20 min before the conditioning, so that when the animals underwent a fear conditioning protocol they had the inhibitory currents reduced and the excitability of the system was enhanced.

2.6.5. Experiment 5

On day 1, all groups were exposed to cxt-B for habituation to the generalization test chamber. On day 2, animals were administered with either MDZ or SAL intra- BLA and 10 min later, all the animals underwent the stress protocol. On day 3, the rats were conditioned in cxt-A. On day 4, all the animals were tested in cxt-B for fear generalization assessment during 5 min. On day 5, the rats were exposed in cxt-A for fear memory evaluation during 5 min (Fig. 5A).

2.7. Statistical analysis

The experiments were analyzed by a two-way ANOVA or by a repeated-measure ANOVA, depending on the experimental design, followed by Newman-Keuls post hoc test. The generalization index was compared by unpaired *t*-test. The data were expressed as mean ± SEM.

3. Results

3.1. Stress exposure, prior to contextual fear conditioning, promotes fear generalization

In order to determine whether stress exposure favors fear generalization, a 2 × 2 design was used with stress and conditioning as factors. The protocol lasted five days, starting on day 1 with a period of habituation to the generalization test box (cxt-B). On day 2, one group of animals -randomly assigned- was stressed (SS) and another group was left undisturbed. On day 3, stressed and control rats underwent the contextual fear conditioning protocol (see Methods) in context A (cxt-A). Other groups of stressed and non-stressed animals were exposed to cxt-A without the unconditioned stimulus (US). On day 4, the levels of freezing behavior were evaluated in cxt-B to test fear memory generalization (during 5 min). On day 5, the animals were tested in cxt-A to evaluate fear memory in the conditioning context. Thus, the different groups of animals were as follows: no-SS/US: N = 9, SS/US: N = 11,

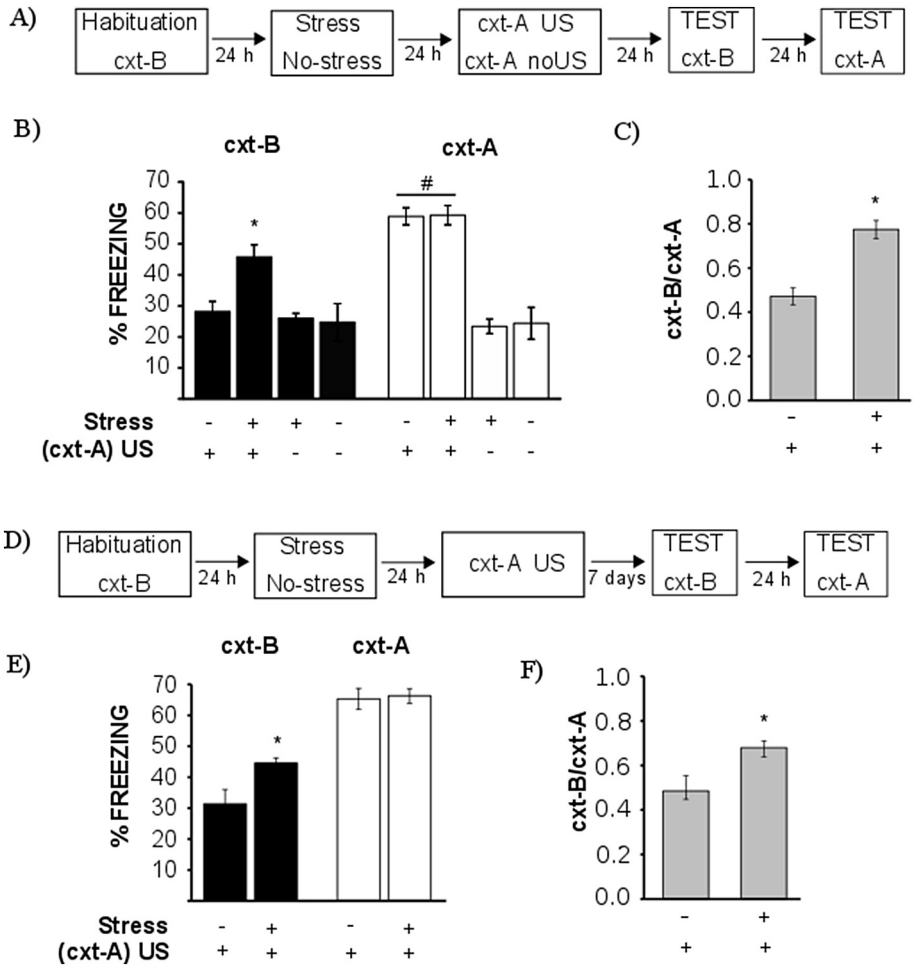

Fig. 1. Acute stress exposure previous to a contextual fear conditioning promotes fear generalization. A. Schematic representation of the experimental design. B. Freezing levels of the animals during the generalization test (cxt-B) and the fear memory test (cxt-A). In cxt-B, conditioned animals with previous stress showed higher levels of freezing behavior compared to all the other groups *($p < 0.01$). In cxt-A, stressed and non-stressed conditioned animals showed significant higher levels of freezing than the other control groups #($p < 0.001$). C. Generalization index (% freezing cxt-B/cxt-A) was significantly higher in stressed conditioned animals than in non-stressed conditioned animals *($p < 0.001$). D. Schematic representation of the experimental design for animals evaluated 1 week after fear conditioning. E. In cxt-B, conditioned animals with previous stress showed higher levels of freezing behavior *($p < 0.01$). F. Generalization index was significantly higher in stressed conditioned animals than in non-stressed conditioned animals *($p < 0.02$). Data are expressed as mean ± SEM (Fig. A–C: no-SS/US: N = 9, SS/US: N = 11, SS/no-US: N = 10, no-SS/no-US: N = 8; Fig. E–F: no-SS/US N = 10, SS/US N = 11). SS: stress, US: unconditional stimulus (footshocks).

SS/no-US: N = 10, no-SS/no-US: N = 8). Fig. 1A shows a schematic representation of the experimental design.

The animals that were conditioned and had a stress experience showed an increase in the freezing levels in cxt-B on day 4 as compared to the conditioned animals without previous stress exposure, and to the animals that were not conditioned, independently from the stress experience (Fig. 1B). A two- way ANOVA (stress × conditioning) with a within factor (repeated measurement cxt-B, cxt-A) revealed a significant effect of stress × conditioning × context interaction ($F(1, 34) = 4.59$, $p < 0.039$). Newman-Keuls post hoc test indicated that freezing levels observed in cxt-B in stressed animals that underwent fear learning were significantly higher than those shown by conditioned animals without prior stress ($p < 0.01$) and to the other control groups, stressed and non-stressed animals which did not receive the US in cxt-A (Fig. 1B). On the other hand, when the behavioral performance in cxt-A was analyzed, both stressed and non-stressed animals that underwent fear conditioning showed significantly higher levels of freezing as compared to non-conditioned animals (between comparison, $p < 0.001$, both) and when compared with their own performance in cxt-B (within comparison, non-stressed $p < 0.001$; stressed

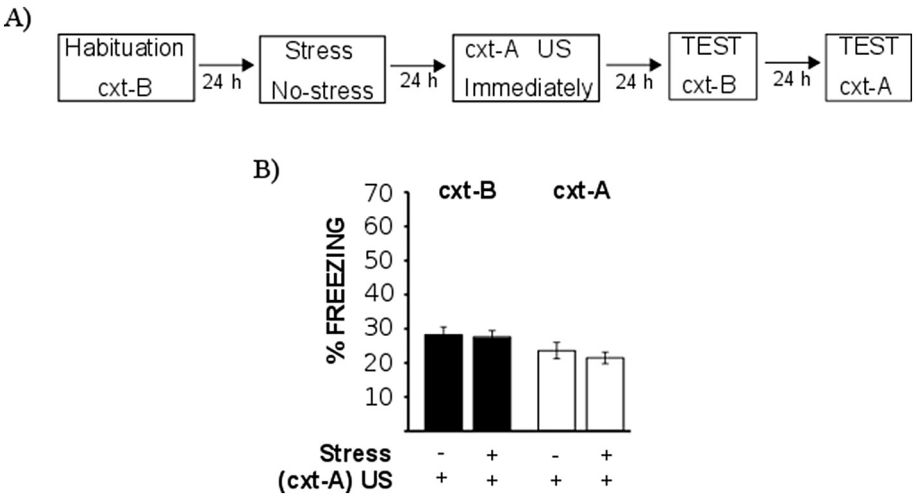

Fig. 2. Stress does not induce fear generalization following immediate footshock deficit. A. Schematic representation of the experimental design. On day 3 animals underwent an immediate shock protocol. B. Freezing levels of the animals during the generalization test (cxt-B) and the fear memory test (cxt-A). There were not differences between groups. Data are expressed as mean ± SEM (no-SS/US: N = 10, SS/US: N = 9). SS: stress, US: unconditional stimulus (footshocks).

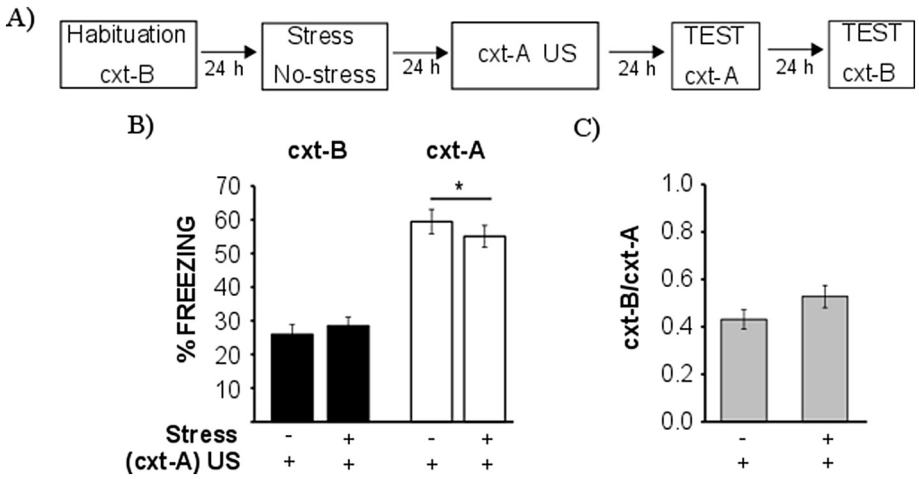

Fig. 3. Retrieval of the memory in the original context prevents the facilitatory effects of stress on fear generalization. A. Schematic representation of the experimental design. B. Freezing levels of the animals during the generalization test (cxt-B) and the fear memory test (cxt-A). In cxt-B, conditioned animals with or without previous stress showed similar levels of freezing behavior. In cxt-A, stressed and non-stressed conditioned animals showed significant higher levels of freezing compared to the own freezing levels in cxt-B *($p < 0.001$). C. Generalization index (% freezing cxt-B/cxt-A) was not significantly different. Data are expressed as mean ± SEM (no-SS/US: N = 14, SS/US: N = 15). SS: stress, US: unconditional stimulus (footshocks).

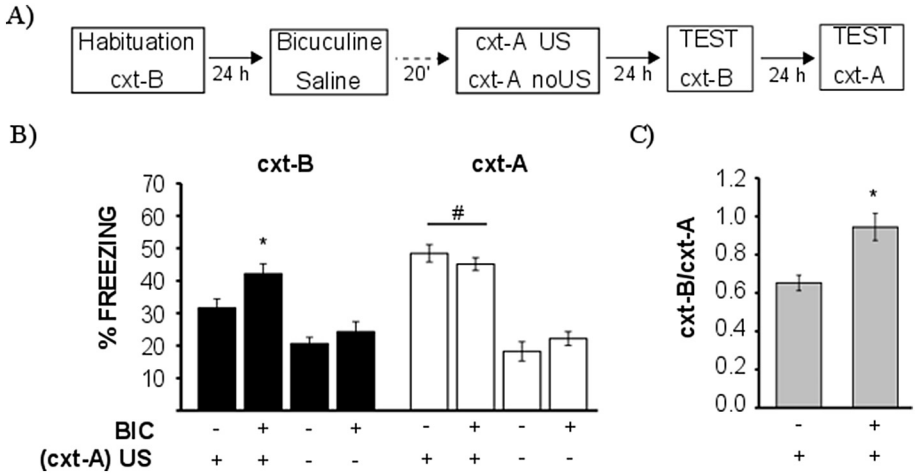

Fig. 4. Microinjection of bicuculline into the basolateral amygdala complex mimics stress induced facilitation of contextual memory generalization. A. Schematic representation of the experimental design. B. Freezing levels of the animals during the generalization test (cxt-B) and the fear memory test (cxt-A). In cxt-B, conditioned animals with previous BIC infusion showed higher levels of freezing behavior compared to all the other groups *($p < 0.04$). In cxt-A, BIC and SAL administered animals that were conditioned showed significantly higher levels of freezing compared to SAL and BIC administered animals that were not conditioned #($p < 0.001$). C. Generalization index (% freezing cxt-B/cxt-A) was significantly higher in BIC/US than in SAL/US *($p < 0.003$). Data are expressed as mean ± SEM (SAL/US: N = 11, SAL/no-US: N = 8, BIC/US: N = 11, BIC/no-US: N = 8). SAL: saline, BIC: bicuculline, US: unconditional stimulus (footshocks).

$p < 0.02$) (Fig. 1B). This means that even stressed animal that exhibited higher freezing in cxt-B did not reach the freezing level displayed in the paired cxt-A. This is further reflected in the generalization index, the ratio between the percentages of freezing displayed in cxt-B vs cxt-A. This index is 0.47 ± 0.04 for the non-stressed animal which increased up to 0.77 ± 0.05 in those animals subjected to prior stress (*t*-test $p < 0.001$, Fig. 1C). In order to see if the stress induced facilitation of fear generalization was long lasting, another set of animals were evaluated 1 week after fear conditioning (Fig. 1D). Again, stressed animals showed a significant increase in the freezing levels in cxt-B which was 45 ± 2% compared to 31 ± 5% in non-stressed animals ($p < 0.01$, after ANOVA, stress × context interaction $F(1, 19) = 4.72$, $p < 0.05$). Non-significant differences were found in the freezing levels in cxt-A the following day (no-SS/US: 65 ± 3%, N = 10 vs SS/US: 66 ± 2%, N = 11, $p > 0.8$, Fig. 1E). Accordingly, the generalization index was 0.49 ± 0.07 in non-stressed animals and increased to 0.68 ± 0.03 in the stressed animals (*t*-test $p < 0.02$, Fig. 1F). The current data indicate that a single stress episode, prior to fear conditioning, promotes the generalization of the contextual fear memory.

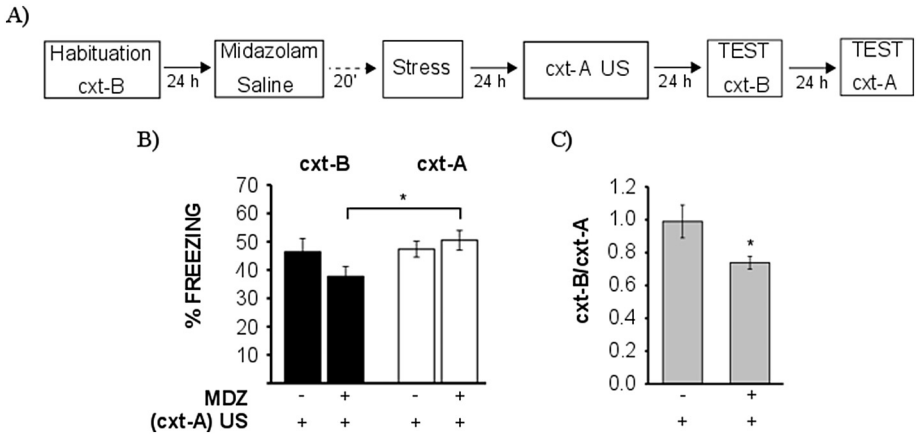

Fig. 5. Microinjection of midazolam into the basolateral amygdala complex reduces the stress induced facilitation of contextual memory generalization. A. Schematic representation of the experimental design. B. Freezing levels of the animals during the generalization test (cxt-B) and the fear memory test (cxt-A). In cxt-B, MDZ and SAL animals differences did not reach significance, but the intragroup comparison (cxt-A vs cxt-B) among MDZ treated animals indicated that freezing levels in cxt-B was significantly lower than freezing level in cxt-A *($p < 0.003$) while freezing scores in SAL administered rats were not different in both contexts ($p = 0.75$). C. Generalization index (% freezing cxt-B/cxt-A) was significantly lower in MDZ/US than in SAL/US *($p < 0.03$). Data are expressed as mean ± SEM (SAL/US: N = 10, MDZ/US: N = 11). SAL: saline, MDZ: midazolam, US: unconditional stimulus (footshocks).

3.2. Stress does not induce fear generalization following immediate footshock

In order to minimize novelty- induced freezing, we developed a protocol that starts with a pre-exposure to cxt-B so that the generalization test box is not a novel context. However, it could be argued that a single pre-exposure session is not sufficient to minimize fear to novelty. Thus, increased freezing in cxt-B, in conditioned stressed animal, could be an unspecific fear sensitized response due to the sum of stressors that increase the levels of anxiety in these animals. In order to address this issue, we performed experiments identical to the one mentioned above, but on day 3, the unconditional stimulus (footshock) was delivered immediately after placing the animal in the conditioning box (cxt-A). This procedure is known as "immediate footshock deficit" (The groups were as follow, no-SS/US: N = 10, SS/US: N = 9). Fig. 2A shows a schematic representation of the experimental design. When this procedure was used, low levels of freezing in cxt-B were evident in stressed rats. In fact, a similar freezing behavior was observed in stressed and control animals (ANOVA stress × context interaction, $F(1, 17) = 0.16$, $p = 0.69$), indicating that the higher level of freezing observed in experiment 1 (Fig. 1) was not due to a sensitized response to prior stressors. In fact, when tested in cxt-A, neither stressed nor control animals showed more freezing than the basal level of fear response, indicating that they were incapable of forming an associative memory of the context where they were subjected to the US. Altogether, this strongly suggests that the increase of fear in cxt-B, in animals that underwent a contextual fear conditioning with a stress history, is a generalization of an associative memory formed in cxt-A.

3.3. Retrieval of the memory in the original context prevents the facilitatory effects of stress on fear generalization

Previous reports have shown that the generalization of fear is greatly diminished if animals are exposed to the conditioning context before the generalization context (Huckleberry et al., 2016). Hence, we tested if stress-induced facilitation of fear generalization was dependent on the testing order, i.e. conditioning context first vs generalization context first (Fig. 3A). When stressed rats were exposed first to cxt-A and then to cxt-B they showed similar freezing levels in cxt-B as those shown by non-stressed conditioned animals (the groups were as follow, no-SS/US: N = 14, SS/US: N = 15). The two way ANOVA indicated no stress effect ($F(1, 27) = 0.06$, $p = 0.81$) nor interaction (stress × context, $F(1, 27) = 3.08$, $p = 0.09$) effect. On the other hand, the freezing levels in cxt-A were significantly higher than in cxt-B, in both stressed and non-stressed animals (context effect: $F(1, 27) = 235.22$, $p < 0.001$), confirming that conditioned animals acquired a fear memory of the paired context (Fig. 3B). These data presumably suggest that retrieval of the memory in the original context makes the memory more precise so that stress no longer facilitates the generalization of fear memory. Accordingly, the generalization index was relatively similar in both groups (non-stress 0.43 ± 0.04 vs stress 0.53 ± 0.05) and it was not significant (*t*-test $p > 0.08$, Fig. 3C).

3.4. Modulation of GABA-A receptors in the basolateral amygdala complex is critical for the facilitatory effect of stress on fear memory generalization

It has been suggested that the promoting effect of restraint on fear memory formation is associated to a decrease in GABAergic inhibitory control in the BLA which, in turn, enhances the glutamatergic output (Rodriguez Manzanares et al., 2005; Isoardi et al., 2007). Under this consideration, we administered Bicuculline (BIC), an antagonist GABA-A sites, into the BLA in order to emulate the stress effect on fear generalization (the groups were as follow, SAL/US: N = 11, SAL/no-US: N = 8, BIC/US: N = 11, BIC/no-US: N = 8, Fig. 4).

An ANOVA indicated a drug × conditioning × context interaction ($F(1, 34) = 6.69$, $p = 0.01$). Post hoc comparisons specified that BIC administered animals that were conditioned showed a significant increase in freezing response in cxt-B compared to conditioned SAL administered animals ($p < 0.04$) and non-conditioned BIC administered animals ($p < 0.002$) or SAL ($p < 0.001$). When tested in cxt-A, BIC and SAL animals showed a significant increase in freezing compared to non-conditioned animals ($p < 0.001$). When the animals that underwent fear conditioning were compared between cxt-A and their own performance in cxt-B, SAL injected animals showed a significant increase in the freezing level in cxt-A ($p < 0.001$). However, BIC administered animals were not different ($p > 0.27$), indicating that the response is similar in both contexts. Accordingly, the generalization index showed a significant increase in the BIC injected animals as compared to SAL administered animals (SAL = 0.65 ± 0.04, BIC = 0.94 ± 0.07; *t*-test $p < 0.003$, Fig. 4C). Thus, the blockade of GABA-A sites in BLA before fear conditioning was able to induce a fear generalization similar to that produced by stress (experiment 1). Based on that, we predicted that the intra-BLA administration of midazolam (MDZ), which increases the inhibitory activity through GABA-A receptor prior to the stress experience, should prevent the stress-induced facilitating effect on fear generalization (the groups were as follows: SAL/US: N = 10, MDZ/US: N = 11, Fig. 5). In fact, the generalization index in SAL administered animals during the stress session was 0.99 ± 0.01 and it was significantly reduced in MDZ injected animals 0.74 ± 0.04 (*t*-test $p < 0.03$). A two-way ANOVA analysis indicated a significant interaction between drug and context ($F(1, 19) = 7.91$, $p = 0.01$). Even though posthoc comparison between MDZ and SAL animals in cxt-B did not reach significance ($p = 0.2$), the intragroup comparison among MDZ treated animals indicated that the reduction in the freezing levels in cxt-B was significant respect to the freezing level in cxt-A ($p < 0.003$) while freezing scores in SAL administered rats were not different in both contexts ($p = 0.75$). Altogether, the data corroborates that stress induces the facilitation of fear contextual generalization, and strengthens the notion that the attenuation of GABA-A inhibition in BLA induced by stress can be a critical factor for the manifestation of generalized fear memories.

The row data of the results reported here are accessible at the following link: https://data.mendeley.com/datasets/ws4yj7bw4t/1.

4. Discussion

The effect of previous stress on fear memory has been extensively studied since it can give insight to the understanding of the emergence of maladaptive memories that underlie anxiety and stress related disorders (Perusini et al., 2016; Martijena and Molina, 2012; Roozendaal et al., 2009). However, relatively little is known about the effects of stress on fear memory generalization, a critical symptom of anxiety disorders (Lissek, 2012). In the current work, we have shown that a single stress exposure before contextual fear conditioning promoted the generalization of this memory to a different context that was well discriminated in unstressed conditioned animals. Importantly, we have also found that the increase of fear in the non-conditioned context was dependent on an associative phenomenon rather than on a sensitized response due to previous stressors. The animals with stress experience that underwent a footshock deficit protocol, which did not generate the fear contextual memory, did not respond with fear in the unpaired context. This means that the increase of fear in the unpaired context is not an unspecific sensitized reaction due to prior stressors. Hence, an associative memory of the paired context was necessary to induce generalization. In fact, it has been suggested that animals generalize from one stimulus to another because the stimuli are judged to have, with some probability, the same consequence (Ghosh and Chattarji, 2015).

Our results extend previous findings which showed that chronic stress paradigms in mice (isolation and variable stress) favor the generalization of fear memories (Müller et al., 2014). Thus, stress prior to the fear conditioning could be changing the encoding of fear memory

such that the formation of this memory is qualitatively different from the acquisition without stress. In fact, it has been shown that stress increases fear learning (Maldonado et al., 2011; Giachero et al., 2013), retards the extinction of fear memory formation (Akirav et al., 2009) and renders this memory trace resistant to the labilization/reconsolidation process upon recall (Bustos et al., 2010; Espejo et al., 2016). Interestingly, those sequelae are also achieved by increasing the intensity of fear training (Wang et al., 2009) suggesting that an unrelated stressful experience presumably enhances the threatening/negative valence perceived during the conditioning processes. In agreement with this view, it has been long recognized that increasing the intensity and/or the number of shocks also favors the emergence of generalized memories (Baldi et al., 2004; Laxmi et al., 2003). This suggests that stress-induced neurobiological changes in those brain structures critically involved in fear learning could underlie the stress promoting influence on fear generalization. Accordingly, literature suggests that the basolateral amygdaloid complex (BLA) could be a critical locus (LeDoux, 2007; Roozendaal et al., 2009; Prager et al., 2016).

The BLA integrates sensory information from cortical and subcortical projections; it is centrally involved in emotional processing and generates the appropriate emotional reaction to environmental threats (LeDoux, 2007; Aggleton, 2000). It is well recognized that GABAergic interneurons within the BLA are responsible for controlling the activity of projecting glutamatergic cells through feedforward and feedback inhibition (Sah and Armentia, 2003; Dityatev and Bolshakov, 2005), providing a powerful inhibitory control of principal neurons in the BLA (Ehrlich et al., 2009; Paré and Collins, 2000). A large number of reports have emphasized that the GABAergic system in the amygdaloid complex is a key component in the modulation of emotional reactions to stressful stimuli (Prager et al., 2016). For instance, stress exposure results in a decreased chloride uptake mediated by GABA-A sites in this brain region (Martijena et al., 1997, 2002), as well as in a reduced benzodiazepine binding and the expression of α-1 GABA-A receptor mRNA in the BLA (Liu and Glowa, 2000). Accordingly, it has been revealed that a single restraint experience elicited BLA neuron hyperexcitability, which resulted from the reduction of recurrent GABAergic inhibition (Isoardi et al., 2007). Furthermore, stimulating GABA-A sites with MDZ attenuated both, the facilitating influence of stress on fear memory and synaptic excitability in the BLA (Rodriguez Manzanares et al., 2005). Thus, GABAergic neurotransmission in the BLA could serve as a dynamic gating mechanism, adjusting fear memory encoding according to the emotional state at the moment of the fear learning process (Martijena and Molina, 2012).

The present results strongly suggest that this mechanism is also involved in the stress-induced promoting effect on the generalization of fear memory. Bicuculline, a GABA-A antagonist administered in BLA before fear conditioning, emulated the promoting effect of stress on fear generalization whereas the administration of MDZ intra-BLA prior to the stress session decreased the generalization index. However, the interpretation of those experiments should be taken cautiously. The regulation of the firing rate by GABAergic interneurons controls the flow of information from the BLA, and evidence indicates that local inhibitory circuits in the amygdala mediate its functioning (Prager et al., 2016). Hence, the effects observed after the administration of midazolam or bicuculline are probably having a broader impact in efferents from the amygdala and also the modulatory afferents that it receive, including dopaminergic, serotoninergic, cholinergic and adrenergic neuromodulator systems which make synapses with interneurons and principal neurons (Prager et al., 2016).

At the cellular level, the increase in the excitability of the BLA neurons by stress could alter the selectivity of the amygdala neurons to respond to the specific context (Ghosh and Chattarji, 2015). For instance, recordings in the lateral amygdala following discrimination training to two discrete cues identified separate populations of neurons that signaled either generalized or cue-specific associations. Increasing generalized fear by increasing the foot shock intensity enhanced the excitability of LA neurons. Furthermore, the same LA neurons that were cue specific before the behavioral shift to generalized fear lost their specificity afterwards, thereby tilting the balance of activity towards a greater proportion of generalizing neurons (Ghosh and Chattarji, 2015). How does stress increase the excitability of basolateral amygdala neurons? There is a plethora of neurotransmitters, neuromodulators and hormones that are released during stress that will reach amygdala neurons (Joëls and Baram, 2009). From those, one of the best understood is the glucocorticoid system. Stress activates the hypothalamus–pituitary–adrenal axis which leads to the release of glucocorticoid hormones (mainly cortisol in humans and corticosterone in rodents) from the adrenal cortex. These hormones can access the brain easily and, once there, bind to mineralocorticoid receptors and glucocorticoid receptors to exert both, rapid non-genomic and slow genomic actions on physiology and behavior (de Quervain et al., 2017). Interestingly, it has been demonstrated that corticosterone application on brain slices enhanced their intrinsic excitability and decreased the impact of GABA-A inhibitory postsynaptic potentials (Duvarci and Paré, 2007). A recent work reported that glucocorticoid- induced reduction of spontaneous inhibitory GABA currents was mediated by non-genomic mechanisms which induced the endocannabinoid suppression of presynaptic GABA release (Di et al., 2016). These could be potential mechanisms that underlie the reduction of gabaergic inhibition observed after stress (Rodriguez Manzanares et al., 2005; Isoardi et al., 2007; Liu et al., 2014).

Altogether, these data go along with the neuroimaging studies in humans which show that stress related disorders like PTSD and panic disorder are associated with an enhanced activity of the amygdala (Shin and Liberzon, 2010). Interestingly, generalization protocols applied to this clinical population (Lissek et al., 2014; Kaczkurkin et al., 2017) and non-clinical population with higher state anxiety (Dibbets and Evers, 2017) or trait anxiety (Dibbets et al., 2015) show overgeneralization of cues. Overgeneralization refers to excessive fear responding towards stimuli that are rather dissimilar to the Cs + and it is considered maladaptive (Lissek, 2012). In this context, it is important to mention that the generalization chamber used in this work is quite dissimilar to the training chamber suggesting that our protocol could be suitable for studying maladaptive generalization.

Besides the role of the amygdala reported here, stress effects in other areas could also modulate the encoding of fear memory in a generalizing direction. For instance, blocking the activity of ventral hippocampus or the anterior cingulate cortex at remote times, when memories are generalized, returned the precision of the memory so that rats only freezed in the training context (Cullen et al., 2015). Conversely, the blockade of the activity of other brain areas (e.g. prefrontal cortex, nucleus reuniens) leads to contextual fear generalization at recent time points (Xu and Südhof, 2013). Interestingly, these effects occur when the blocking was performed before the encoding but not at post training intervals (Xu and Südhof, 2013). Notably, those brain areas are well- known targets of chronic and acute stress (Christoffel et al., 2011; Wilson et al., 2015; Bender et al., 2016).

Another interesting finding in the present work was that the facilitatory effect of stress on fear generalization was abolished when animals retrieved the paired context before the generalizing context. This result is in agreement with other studies (Huckleberry et al., 2016; Winocur et al., 2009) and presumably suggests that the retrieval in the original context makes the memory more precise. One plausible mechanism for this improvement is through reconsolidation triggered by the retrieval in the original context (Forcato et al., 2014). However, using a similar stress protocol to the one used in this work has shown that prior stress renders the memory resistant to the reconsolidation process (Bustos et al., 2010; Espejo et al., 2016). This implies that memory reconsolidation is not always necessary for making fear memory more precise.

Another interpretation could be that rather than reflecting enhanced

specificity of the contextual fear memory representation, the results may reflect decreased contextual fear due to extinction. If the exposure to context-A before testing in context-B indeed resulted in extinction of fear, there would be less to generalize and thus less basis for a difference between the stress and no-stress groups during the context-B test. However, fear memories formed after stress are resistant to extinction (Hoffman et al., 2014; Akirav et al., 2009) and the time exposure usually required to extinguish the fear memory is quite longer than the time used here for memory evaluation (de la Fuente et al., 2011), making this interpretation unlikely. Alternatively, the exposure in context-A before context-B could serve to improve the context-A representation making it easier to discriminate a different context. In favor of this interpretation, Biedenkapp and Rudy (2007) showed that pre-exposure of the training context (but not a different context) before the contextual fear conditioning prevented the generalization induced by the passing of time (in this protocol 15 days after fear conditioning).

From another point of view, it has been proposed that there are dissociable neuronal processes to identify the threat, on the one hand; and ambiguity-based uncertainty evaluation, on the other hand (Onat and Büchel, 2015). Following this model, even though animals are able to discriminate both contexts, stress could increase the ambiguity/uncertainty system which also controls the fear response, driving an enhancement in fear response in the unpaired context. This uncertainty could be reduced when the animals recall the memory in the original context prior to the unpaired context and hence, preventing the fear generalization. Further experiments are necessary to elucidate the mechanism implicated in this potential enhanced precision.

In conclusion, our data indicate that stress prior to fear conditioning promotes the generalization of fear memory to a safe context, which is similar to what happened in anxiety and stress related disorders, suggesting that this model can have a translational potential to understand the neurobiology of maladaptive generalization. Furthermore, a reduction in the GABAergic inhibitory control within the BLA, during memory encoding, seems to be critically involved in the generalization of contextual fear memories, making the gabaergic system an important target for disorders where the generalization of fear is a core symptom.

Ethical statement

The protocols used in the present study were approved by the Animal Care Committee of the “Facultad de Ciencias Químicas”, National University of Cordoba, which is consistent with the NIH Guide for the Care and Use of Laboratory animals. The number of animals used, as well as their suffering, was minimized at the minimum possible.

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