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GABAergic signaling within the Basolateral Amygdala Complex modulates resistance to the labilization/reconsolidation process

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ABSTRACT

It is well known that stress can affect mnemonic processes. In particular, stress before contextual fear conditioning induces a memory which exhibits resistance to being interfered with by Midazolam (MDZ) when applied after memory retrieval. Moreover, stress exposure strongly affects GABAergic transmission within the Basolateral Amygdala Complex (BLA), a brain structure critically involved in fear memory processing. The present study evaluated the involvement of GABAergic signaling within the BLA on the induction of resistance to memory reconsolidation interference. Results showed that MDZ administered intra-BLA before stress prevented the induction of resistance to the interfering effect of systemic administration of both MDZ and Propranolol on fear memory reconsolidation, when both applied after memory retrieval. The blockade of amygdala GABA-A receptors by the antagonist Bicuculline (BIC) before memory encoding induced resistance to interference by post-recall MDZ administration, similarly to that observed with stress exposure. Additionally, the systemic administration of D-cycloserine, a positive allosteric modulator of NMDA receptor, reverted the BIC-induced resistance to the MDZ interfering effect, in the same manner as that reported with stress-induced resistance.

In summary, these results suggest that the GABAergic signaling in the BLA at the moment of memory encoding is determinant for the induction of fear memory resistance to the onset of the labilization/reconsolidation process.

1. Introduction

There is a consensus that stress promotes the consolidation of emotionally arousing memories, including fear memory (Martijena & Molina, 2012; Roozendaal, McEwen, & Chattarji, 2009; Wolf, Atsak, de Quervain, Roozendaal, & Wingenfeld, 2016). In particular, a single stress exposure facilitates the emergence of persistent and robust fear memories when applied prior to the encoding process (Maldonado, Espejo, Martijena, & Molina, 2014; Maldonado, Martijena, & Molina, 2011; Rodriguez Manzanares, Isoardi, Carrer, & Molina, 2005). Moreover, a stressful experience before learning induces a fear memory that exhibits resistance to reconsolidation interference (Bustos, Giachero, Maldonado, & Molina, 2010) and also an apparent delay in extinction acquisition (Rodriguez Manzanares et al., 2005).

Our laboratory recently reported that fear memory reactivation in control animals increases the expression of GluN2B-NMDA receptor subunit and Zif-268 protein within the Basolateral Amygdala Complex (BLA), which could be implicated with the labilization and restabilization phases, respectively. However, these changes were absent in previously stressed animals, thus suggesting that the neurobiological changes associated to stress at the moment of memory encoding results in a memory trace unable to enter in the labilization/reconsolidation process (Espejo, Ortiz, Martijena, & Molina, 2016).

The BLA is critically involved in the formation of fear memories and in orchestrating an appropriate response to environmental threats. In addition, GABAergic signaling within this brain area plays a pivotal role in the emergence of fear memory (Wolff et al., 2014) and in the promoting influence of stress on fear memory consolidation (Martijena & Molina, 2012). Consistent with this view, stress attenuates GABAergic inhibitory control in the BLA, thereby facilitating excitatory transmission (Isoardi, Bertotto, Martijena, Molina, & Carrer, 2007). This coincides with the enhanced fear memory formation and the facilitated induction of long term potentiation (LTP) reported in the BLA (Rodriguez Manzanares et al., 2005). In support of these data, systemic or intra-BLA administration of Midazolam (MDZ), a positive modulator of GABA-A sites, prior to threatening stimulus prevents the promoting effects on fear memory (Giachero, Calfa, & Molina, 2013;

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Maldonado et al., 2011). Moreover, intra-BLA administration of bicuculline (BIC), a GABA-A receptor antagonist, induces a facilitating influence on the emergence of fear memory similar to that induced by the stressful experience (Giachero, Calfa, & Molina, 2015; Rodriguez Manzanares et al., 2005).

Taking all this evidence together, we hypothesize that the modulation of the GABAergic activity in the BLA before memory encoding is crucial for the induction of fear memory resistance to the posterior engagement of the labilization/reconsolidation process. In order to test this hypothesis, we evaluated the impact of intra-BLA MDZ infusion on the stress-induced memory resistance to the interfering effect of the systemic administration of MDZ and Propranolol (PROP), two drugs reported to disrupt fear memory reconsolidation. Therefore, we hypothesize that increasing GABAergic transmission in BLA before stress prevents the resistance to the disrupting effects of these drugs. Along the same line, we investigate the influence of intra-BLA BIC prior to memory encoding to test if this treatment affects the vulnerability to MDZ interfering effect. We predict that blockade of GABA-A sites in BLA simulates the resistance induced by stress to MDZ interfering effect on fear memory reconsolidation.

Both behavioral and molecular findings have shown that the use of systemic D-Cycloserine (DCS), a positive allosteric modulator of the NMDA receptor, prior to memory recall reverts the resistance to the interfering effect of MDZ in previously stressed animals (Bustos et al., 2010; Espejo et al., 2016). Similarly, intra-BLA infusion with DCS before reactivation also reverts memory resistance to the MDZ's interfering influence in stressed rats (Espejo et al., 2016). Accordingly with our assumption that BIC and stress may induce resistance by similar mechanisms, it could be expected that DCS reverts the BIC-induced resistance. For this, we evaluated the effect of pre-recall DCS injection in animals infused with BIC.

2. Methods

2.1. Animals

Adult male Wistar rats (60 days old and weighing 280–320 g) from our breeding stock were housed in plastic cages (30 × 45 × 18 cm) with wood bedding in groups of 3–4 per cage, with food and water *ad libitum*. All animals were maintained in a 12 h light/dark cycle (with light from 7:00 am) and at a temperature of 21–22 °C. The protocols were approved by the Animal Care Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, which were consistent with the NIH Guide for the Care and Use of Laboratory animals. Efforts were made to minimize animal suffering and to reduce the number of animals used. All experiments were conducted between 9:00 and 14:00.

2.2. Stress

Animals were stressed by immobilization under intense light for 30 min in a experimental room (S group). Plastic restrainers were fitted close enough to the body to avoid any significant movements but to allow the normal breathing (Espejo et al., 2016). After the stress session, the rats were kept in the experimental room during 30 additional min and then 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. Drugs and administration

All drugs were dissolved in sterile saline (SAL, 0.9%, w/v). Midazolam (MDZ, Gobbi Novag S.A., Argentina) was used at concentrations of 3 mg/ml and 2 μg/μl for intraperitoneal (i.p.) and intra-BLA administration, respectively. Bicuculline methiodide (BIC, Fluka Biochemika-Sigma Aldrich, Switzerland) was dissolved at a concentration of 40 pmol/μl for intra-BLA administration (Giachero et al., 2015; Rodriguez Manzanares et al., 2005), and Propranolol (PROP, Sigma-Aldrich, USA) was systemically (i.p.) administered at a concentration of 10 mg/ml (Ortiz, Giachero, Espejo, Molina, & Martijena, 2015). D-Cycloserine (DCS, Sigma-Aldrich, USA) was used at a concentration of 15 mg/ml for i.p. injection (Bustos et al., 2010; Espejo et al., 2016), and the total volume of drug used in i.p. administration was 1.0 ml/kg, or an equivalent amount of SAL. For intra-BLA infusion, the total volume administered in all cases was 0.25 μl/side.

2.4. Contextual fear conditioning

- -Apparatus: The conditioning chamber was constructed of gray acrylic (20 × 23 × 20 cm) with a transparent lid, and 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 2.4 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.
- Fear Conditioning: Rats were individually placed in the conditioning chamber, and after 3 min of acclimatization (pre-shock period) received 3 unsignaled scrambled footshocks (0.65 mA, 3 s duration and 30 s intershock interval), with animals then being kept in the chamber for an additional 50 s (post-shock period). This protocol had also been used in a previous investigation (Espejo et al., 2016).
- Reactivation session: One day after training, rats were re-exposed to the training context for 5 min without shock delivery.
- Test sessions: One (Test 1) and eight (Test 2) days after the reactivation session, animals were reintroduced into the training context for 10 min without shock delivery.

The freezing responses of each rat were scored during the pre-shock and post-shock periods, as well as during the reactivation and testing sessions by the direct observation of an operator who was blind to the experimental condition. The total time spent freezing in each period was quantified using a stopwatch and expressed as a percentage of total time (Espejo et al., 2016). Freezing, a commonly used index of fear in rats, was defined as the total absence of body and head movement, except for those associated with breathing (Blanchard & Blanchard, 1969).

2.5. Surgery and intra-BLA infusion

The intra-BLA cannulae implantation, local infusion, and histological procedures were previously described by Giachero, Calfa, et al. (2013). The coordinates used relative to bregma were: anterior −3 mm; lateral ±5.0 mm; ventral −6.1 mm, this last coordinate was taken from the skull (Paxinos & Watson, 2009). Rats received a dose of penicillin-streptomycin after surgery to minimize the risk of infection. Moreover, each animal were closely observed after surgery to ensure the normal recovery.

A 7-day recovery period was allowed before starting the experiments. Rats were then bilaterally infused with their respective treatment at a flow rate of 0.25 μl/min, after which, the injectors were kept in place for an additional period of 60 s in order to allow drug diffusion. The injector protruded 2 mm beyond the guide cannulae in order to reach the BLA. 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. A total of ten animals were discarded for misplacement or tissular damage, and thus were replaced for new ones.

Representative image of the infusions site was obtained with a Fluoview FV1200 Confocal Laser Scanning Microscope (Olympus). Automatic stitching was made using the software FV10-ASW 4.0 and the automatic controller PRIOR ProScan III. The shadows were corrected with Adobe Photoshop.

2.6. Experimental design

Experiment 1: Animals were cannulated as described in Section 2. After the recovery period, all animals received an intra-BLA infusion of SAL and 10 min later (Rodriguez Manzanares et al., 2005) half of the rats were stressed (S group), with the remaining ones being handled as previously described (NS group). One day after stress, animals were fear conditioned and then reactivated after a further 24 h. Immediately after this reactivation, animals were randomly assigned to receive an injection of either SAL or MDZ and then were returned to their home cage, the retention tests were performed one and eight days later (Test 1 and Test 2, respectively). The resultant groups were: NS/SAL (n = 8), NS/MDZ (n = 8), S/SAL (n = 8), S/MDZ (n = 8).

Experiment 2a: BLA cannulated animals were all stressed (S) 10 min after being infused with either SAL or MDZ. One day later, rats were fear conditioned, and 24 h later, animals were reactivated and immediately after randomly administered with MDZ or SAL i.p., after that, were returned to the home cage. Fear memory retention tests were performed 1 and 8 days after the reminder session. The resultant groups were: SAL/SAL (n = 9), SAL/MDZ (n = 8), MDZ/SAL (n = 8), MDZ/MDZ (n = 8).

Experiment 2b: BLA cannulated animals were infused, stressed and fear conditioned as described in Experiment 2a. One day after this conditioning, animals were reactivated and immediately after randomly administered with SAL or PROP i.p., and fear retention tests were performed 1 and 8 days after the reactivation session. The resultant groups were: SAL/SAL (n = 8), SAL/PROP (n = 8), MDZ/SAL (n = 10), MDZ/PROP (n = 9).

Experiment 3: BLA cannulated animals were administered with either BIC or SAL intra-BLA 15 min prior to fear conditioning (Giachero et al., 2015). One day later, rats were reactivated and then systemically administered with MDZ or SAL (in a random manner), being then returned to the home cage. Retention tests were performed 1 and 8 days after reactivation. The resultant groups were: SAL/SAL (n = 9), SAL/MDZ (n = 9), BIC/SAL (n = 7), BIC/MDZ (n = 10).

Experiment 4: BLA cannulated rats were intra-BLA infused with BIC or SAL and after 15 min subjected to fear conditioning as in Experiment 3. One day later, rats were randomly assigned to receive a systemic injection of DCS or SAL, and after 30 min were subjected to the reactivation session and immediately after administered with MDZ. After that, animals were returned to the home cage. Test 1 and Test 2 were performed as previously described. The resultant groups were: SAL/SAL (n = 7), SAL/DCS (n = 9), BIC/SAL (n = 9), BIC/DCS (n = 9).

2.7. Statistical analysis

The results were expressed as the means ± SEM of freezing percentage, and data were analyzed by the ANOVAs followed by Newman-Keuls post hoc test. Levene's test was used to evaluate variance homogeneity. For experiments with non-reactivated animals, Student's *t*-test was used (supplementary results). Each animal was considered as a unit of analysis. The significance level used for all statistical analyses was $p < 0.05$. Depending on the experiment, the factors analyzed were: Condition (NS vs S), Pretreatment (SAL vs MDZ; SAL vs BIC) and Treatment (SAL vs MDZ; SAL vs PROP; SAL vs DCS).

3. Results

3.1. Experiment 1. Prior stress induced resistance to the disrupting influence of MDZ on memory reconsolidation

This experiment was oriented at replicating our previous findings on stress-induced memory resistance in cannulated rats (Espejo et al., 2016). Additionally, we evaluated if intra-BLA infusion before memory encoding could affect fear conditioning or memory expression. The freezing response during the reactivation session is shown in Fig. 1C, with no differences in freezing expression being observed between the groups [$F(1,28) = 0.19$, $p = 0.66$]. In Test 1, NS/MDZ animals exhibited a decrease in freezing compared with the NS/SAL group. However, this decrease was not evident in S/MDZ animals. Moreover, this effect was maintained during Test 2. ANOVA revealed a significant Condition × Treatment interaction for both Test 1 [$F(1,28) = 19.37$, $p < 0.01$] and Test 2 [$F(1,28) = 18.09$, $p < 0.05$]. Post hoc comparisons confirmed that the freezing level during Test 1 and Test 2 in the NS/MDZ compared with the remaining groups, which did not differ from each other (Fig. 1D). Thus, confirming our previous findings (Bustos et al., 2010; Espejo et al., 2016), prior exposure to a stressful experience induces resistance to the interfering effect of MDZ on fear memory reconsolidation, in this case in cannulated animals. Moreover, prior intra-BLA infusion did not affect neither fear memory encoding nor fear expression.

3.2. Experiment 2. Intra-BLA administration of MDZ prior to stress prevented resistance to the interfering effect of MDZ and PROP on fear memory reconsolidation

The aim of this experiment was to evaluate the effect of MDZ administered intra-BLA prior to the threatening experience on the resistance to the reconsolidation-impairing effect of MDZ and PROP. As can be seen in Fig. 2B, MDZ and SAL administered animals displayed similar levels of freezing during reactivation [$(1, 29) = 1.02$, $p = 0.32$]. However, a decrease in freezing behavior was observed in the MDZ/MDZ group during both tests. In contrast, this effect was not observed in the SAL/MDZ group (Fig. 2C). ANOVA revealed a significant Pretreatment × Treatment interaction for Test 1 [$F(1,29) = 11.11$, $p < 0.01$] and Test 2 [$F(1,29) = 20.43$, $p < 0.01$], and post hoc comparisons confirmed a lower level of freezing for the MDZ/MDZ group during Test 1 and Test 2 compared with the remaining groups, which did not differ from each other (Experiment 2a).

It could be argued that the effect observed in the previous experiment may have been due the repeated administration of MDZ, thereby affecting the sensitivity of GABA-A receptors to MDZ's interfering effect. Therefore, we applied the same protocol as in the previous experiment, but using PROP as the disrupting agent after memory reactivation. Regardless of the pretreatment (SAL or MDZ) or the treatment (SAL or PROP), the four groups displayed similar levels of freezing during the reactivation session [$F(1,31) = 0.02$, $p = 0.89$] (Fig. 2E). Moreover, MDZ/PROP animals exhibited a decrease in their freezing behavior in both tests, whereas this reduction was not observed in SAL/PROP animals (Fig. 2F). ANOVA revealed a significant Pretreatment × Treatment interaction for Test 1 [$F(1,31) = 9.79$, $p < 0.01$] and Test 2 [$F(1,31) = 14.02$, $p < 0.01$], with the post hoc test indicating that the freezing levels displayed by MDZ/PROP animals were signifi-

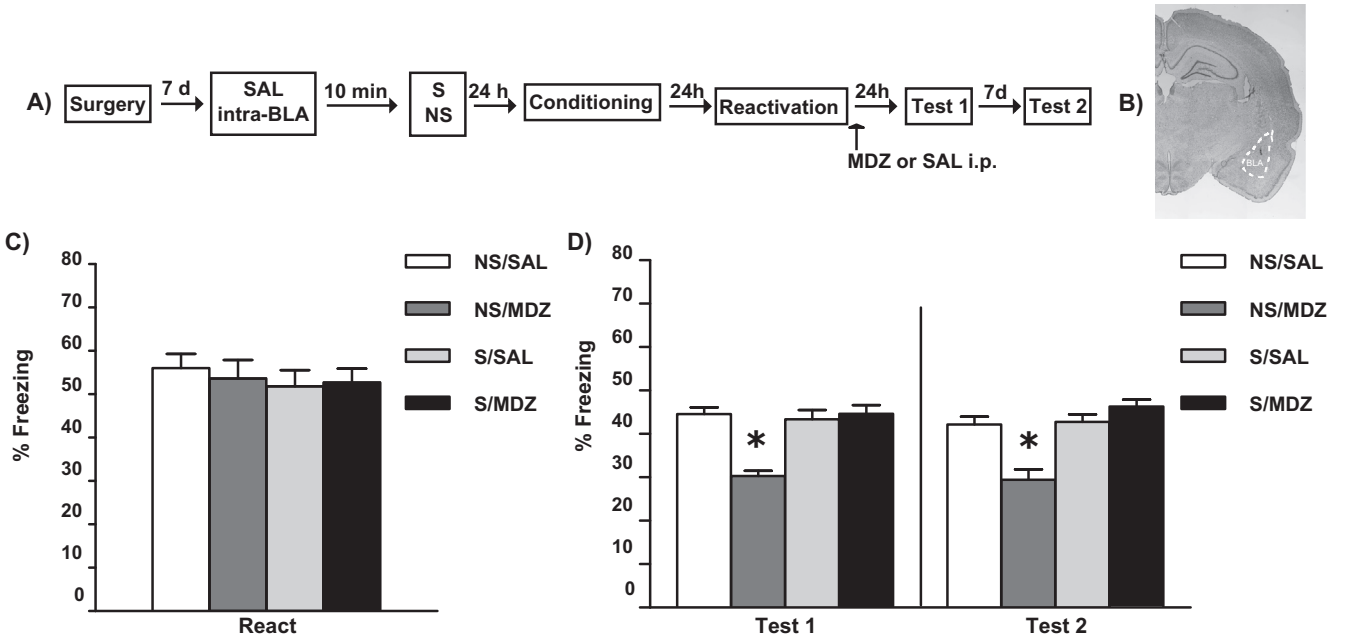

Fig. 1. Prior stress induced resistance to the disrupting influence of MDZ on memory reconsolidation. (A) Schematic representation of the experimental design. (B) Representative image of the infusion site in the BLA. (C) No differences between groups were observed in freezing during the reactivation session. (D) Stress prior to fear conditioning induced resistance to the interfering effect of MDZ on memory reconsolidation. NS/SAL (n = 8), NS/MDZ (n = 8), S/SAL (n = 8), S/MDZ (n = 8). Data are expressed as the mean ± SEM of the freezing percentage. (*) Significantly different compared with the remaining groups ($p < 0.01$).

cantly lower than those of the remaining groups, which did not differ from each other (Experiment 2b).

Taken together, this set of experiments suggests that the activation of the GABA-A receptor complex within the BLA by MDZ local infusion prior to the stressful experience prevented later on the resistance to the disrupting action of both drugs on fear memory reconsolidation.

To try to demonstrate that the interfering effect of both agents was dependent on the reactivation session, one group of cannulated animals was stressed after intra-BLA infusion with SAL or MDZ, and was fear conditioned one day later. After 24 h, animals were administered either with PROP or MDZ without re-exposure to the reminder (no reactivation), and were tested for fear behavior 24 h later. No differences were observed between animals administered with MDZ or SAL when MDZ [$t = -1.41$, $p = 0.18$] or PROP [$t = -0.31$, $p = 0.77$] was applied (Fig. S1).

3.3. Experiment 3. Intra-BLA infusion with BIC induced resistance to the interfering effect of MDZ on fear memory reconsolidation

The goal of this experiment was to study the effect of intra-BLA BIC infusion prior to fear conditioning on MDZ's disrupting action on memory reconsolidation. As shown in Fig. 3B, no difference was found in freezing scores between SAL or BIC administered animals during reactivation [$F(1,31) = 0.74$, $p = 0.39$]. However, the freezing behavior in SAL/MDZ group was lower during Test 1 and Test 2, whereas this reduction was not observed in BIC/MDZ treated animals (Fig. 3C). ANOVA revealed a significant Pretreatment × Treatment interaction for Test 1 [$F(1,31) = 18.2$, $p < 0.01$] and Test 2 [$F(1,31) = 13.41$, $p < 0.01$], and post hoc comparisons confirmed the significant reduction in freezing levels for the SAL/MDZ group compared with the remaining groups, which did not differ from each other.

In order to try to demonstrate that the reactivation session is a prerequisite to be able to observe the interfering effect of MDZ, cannulated animals were locally administered in the BLA with either BIC or SAL and underwent the same conditioning protocol as previously described. Then, one day later, both groups were administered with MDZ without being reactivated and further tested after 24 h. No difference was observed between the BIC or SAL groups in freezing expression groups during this test [$t = 1.13$, $p = 0.28$] (Fig. S2).

These results showed that, in a similar way to stress, intra-BLA administration of BIC prior to memory encoding induced a memory trace that after reactivation exhibited resistance to MDZ's interfering effect, suggesting that the blockade of GABA-A sites within BLA at the moment of memory encoding limited the posterior occurrence of the labilization/reconsolidation process.

3.4. Experiment 4. Systemic administration with DCS restored the BIC-induced resistance to the interfering effect of MDZ on memory reconsolidation

Given that DCS promotes destabilization of resistant memory in stressed animals (Bustos et al., 2010; Espejo et al., 2016), we explore whether DCS can also facilitates destabilization of other resistant memories at retrieval, as that induced by previous intra-BLA BIC (a GABA-A receptor blocker). Therefore, we evaluate the influence of DCS administration on the resistance generated by local administration of BIC in the BLA. As illustrated in Fig. 4B, regardless of the pretreatment (SAL or BIC) or the treatment (SAL or DCS), all groups displayed similar levels of freezing during reactivation [$F(1,30) = 0.3$, $p = 0.58$]. In Test 1, BIC/SAL/MDZ animals exhibited a robust freezing whereas the SAL/SAL/MDZ, SAL/DCS/MDZ and BIC/DCS/MDZ groups showed a lower freezing behavior, with these effects being maintained during Test 2 (Fig. 4C). ANOVA revealed a significant Pretreatment × Treatment interaction for Test 1 [$F(1,30) = 20.83$, $p < 0.01$] and Test 2 [$F(1,30) = 16.97$, $p < 0.01$], and the post hoc test confirmed that the freezing level of the BIC/SAL/MDZ group was significantly higher than that of the remaining groups, which did not differ from each other.

These results suggest that DCS administration prior to memory recall restored the susceptibility to the MDZ disruptive effect on fear memory reconsolidation in BIC pretreated rats.

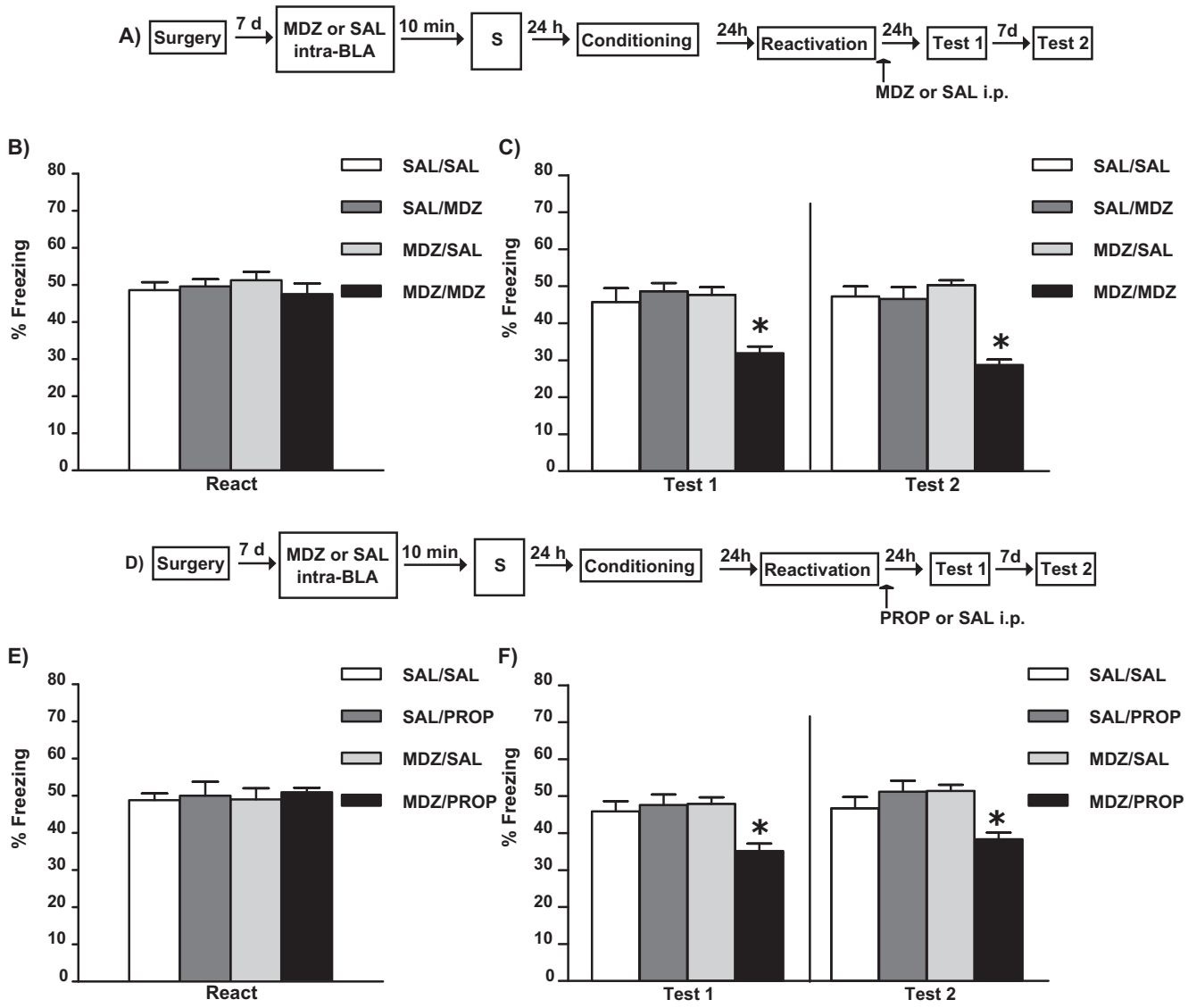

Fig. 2. Intra-BLA administration of MDZ prior to stress prevented the resistance to the interfering effect of MDZ and PROP on fear memory reconsolidation. (A) Schematic representation of the experimental design, Experiment 2a. (B) No differences between groups were observed in freezing during the reactivation session. (C) Intra-BLA administration of MDZ before stress prevented the stress-induced resistance to the interfering effect of MDZ on memory reconsolidation. SAL/SAL (n = 9), SAL/MDZ (n = 8), MDZ/SAL (n = 8), MDZ/MDZ (n = 8). (D) Schematic representation of the experimental design, Experiment 2b. (E) No differences between groups were observed in freezing during the reactivation session. (F) Intra-BLA administration of MDZ before stress prevented the stress-induced resistance to the interfering effect of PROP on memory reconsolidation. SAL/SAL (n = 8), SAL/PROP (n = 8), MDZ/SAL (n = 10), MDZ/PROP (n = 9). Data are expressed as the mean ± SEM of the freezing percentage. (*) Significantly different compared with the remaining groups ($p < 0.01$).

4. Discussion

As expected, and confirming previous evidence (Bustos et al., 2010; Espejo et al., 2016), exposure to a single stressful experience prior to contextual fear conditioning induced a memory trace that upon reactivation exhibits a reduced vulnerability to the disrupting effect of MDZ on fear memory reconsolidation (Fig. 1C), whereas the same treatment decreased freezing behavior during testing in unstressed animals.

"Memory destabilization" is conventionally accepted to be when memory expression is affected after experimental manipulations following reactivation (Schwabe, Nader, & Pruessner, 2014). Therefore, our findings are indicative that stress prior to fear conditioning limits the onset of retrieval-induced instability, suggesting that the neurobiological changes caused by stress in BLA, at the moment of memory formation, can impair later on the emergence of the memory labilization/reconsolidation process following recall. Moreover, this view is further supported by molecular evidence, since prior stress restricted the onset of molecular events closely associated to the labilization/reconsolidation process (Espejo et al., 2016). Additionally, it has been reported that stress-induced resistance is observed even in a 7-day old memory (Bustos et al., 2010), a period in which the majority of the stress effects have vanished. This suggests that the stress-induced resistance is due to changes in the encoding process rather than to a direct effect of stress upon memory reactivation.

Here, we have shown that stimulating the GABA-A sites within the BLA by MDZ infusion prior to stress prevented resistance to the disrupting effect of MDZ on memory reconsolidation. Moreover, MDZ administered intra-BLA prior to stress also allowed the disruptive effect of the β-adrenergic antagonist PROP, another well known reconsolidation-interfering agent (Ortiz et al., 2015; Przybyslawski, Roullet, & Sara, 1999; Soeter & Kindt, 2011). These results indicate that the restored interference in the MDZ/MDZ

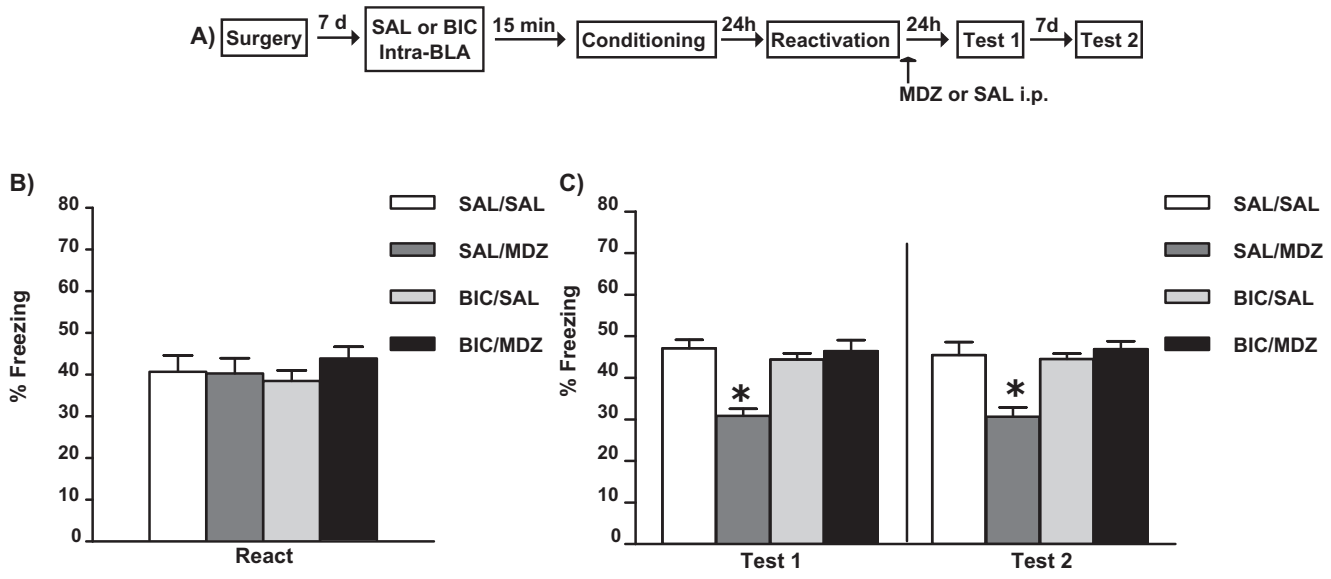

Fig. 3. Intra-BLA infusion with BIC induced resistance to the interfering effect of MDZ on fear memory reconsolidation. (A) Schematic representation of the experimental design. (B) No differences between groups were observed in freezing during the reactivation session. (C) Intra-BLA administration of BIC before fear conditioning induced resistance to the interfering effect of MDZ on memory reconsolidation. SAL/SAL (n = 9), SAL/MDZ (n = 9), BIC/SAL (n = 7), BIC/MDZ (n = 10). Data are expressed as the mean ± SEM of the freezing percentage. (*) Significantly different compared with the remaining groups ($p < 0.01$).

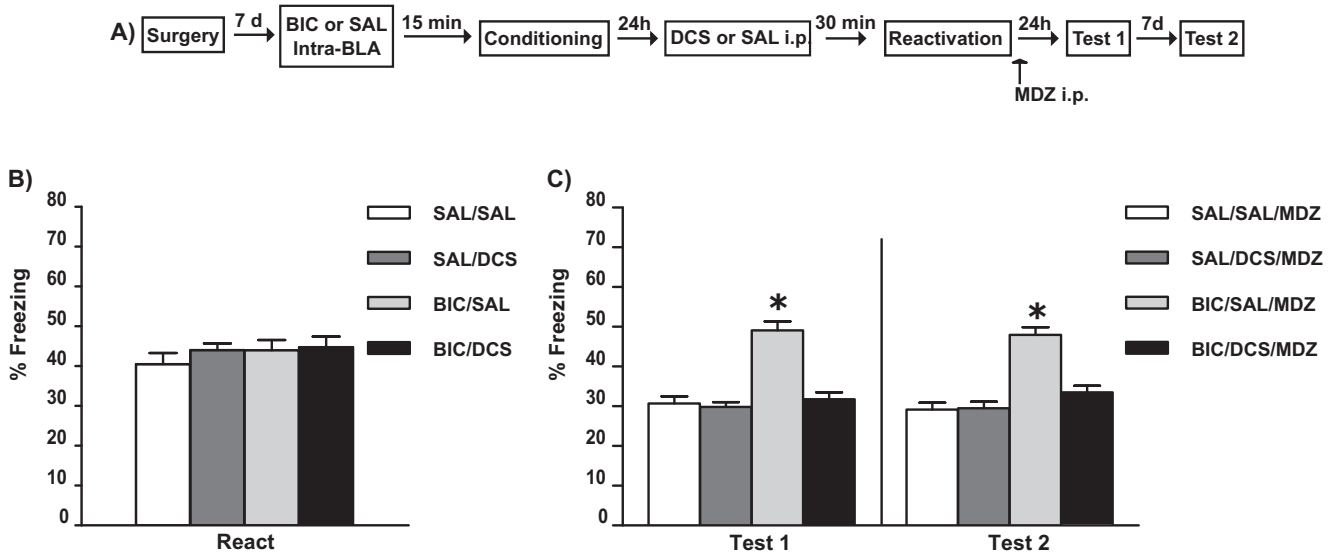

Fig. 4. Systemic administration with DCS restored the BIC-induced resistance to the interfering effect of MDZ on memory reconsolidation. (A) Schematic representation of the experimental design. (B) No differences between groups were observed in freezing during the reactivation session. (C) Systemic administration of DCS before memory reactivation reverted the BIC-induced resistance to the interfering effect of MDZ on memory reconsolidation. SAL/SAL (n = 7), SAL/DCS (n = 9), BIC/SAL (n = 9), BIC/DCS (n = 9). Data are expressed as the mean ± SEM of the freezing percentage. (*) Significantly different compared with the remaining groups ($p < 0.01$).

group is not attributable to the repeated administration of the benzodiazepine ligand. Our findings also demonstrated a long-lasting reduction of freezing following MDZ or PROP in unstressed animals, since this effect observed in Test 1 is maintained at least one week later (Test 2). Additionally, the decrease in freezing behavior following MDZ or PROP was not noticeable when both agents were applied without memory reactivation, demonstrating that the interfering effect of both drugs is dependent on reactivation-induced destabilization, as previously reported (Ortiz et al., 2015). Collectively, the above findings suggest that GABAergic signaling in BLA, at the moment of memory encoding, plays a crucial role on the influence of stress in fear memory resistance to the reconsolidation process.

In agreement with this notion, the current study demonstrated that the blockade of GABA-A sites within the BLA with BIC prior to memory formation also resulted in a resistant memory. The observed effects were dependent on memory reactivation, since there no were differences in freezing scores between non-reactivated groups. Moreover, given that the half life of BIC at physiological conditions is about 45 min (Olsen, Ban, Miller, & Johnston, 1975), we suggest that the BIC-induced resistance is not due to a direct influence on memory reactivation, but it is a consequence of reducing GABAergic transmission in BLA at the time of memory encoding.

It has been suggested that stress exposure leads to a reduction in the GABAergic inhibitory control on glutamatergic pyramidal

projection neurons in BLA (Isoardi et al., 2007). Thus, this diminished inhibitory input would result in an unmasked activation of pyramidal neurons, and consequently, an enhanced excitability of BLA neurons (Rodriguez Manzanares et al., 2005). This would give support to the widely reported stress promoting influence on the emergence of associative fear memory, which coincides with a reduced inhibitory GABAergic control and facilitated generation of LTP in BLA neurons (Rodriguez Manzanares et al., 2005). Thus, the decrease in GABAergic transmission caused by either stress or the pharmacological blockade of the GABA-A sites in the BLA at the time of memory encoding is likely to play a crucial influence on the induction of fear memory resistance to the occurrence of the labilization/reconsolidation process following recall.

In addition, the present findings as well as previously reported results sustain the widespread view that BLA is a primary locus in mediating not only fear memory formation and reconsolidation, but also having a crucial influence on the vulnerability to the occurrence of destabilization upon reactivation. In agreement, previous studies reported that intra-BLA administration of BIC mimics the behavioral influence of stress on fear memory formation and on the increased hippocampal dendritic spines associated with fear memory (Giachero et al., 2015; Rodriguez Manzanares et al., 2005). Moreover, it is important to remark that the above-mentioned evidence concurs with the hypothesis that transient disinhibition in projection neurons is a mechanism that contributes to memory encoding and expression (Letzkus, Wolff, & Luthi, 2015; Wolff et al., 2014).

It is known that robust fear memories are less vulnerable to reconsolidation disruption. For instance, increasing footshock trials during acquisition induced a memory trace that is less vulnerable to interference after recall (Suzuki et al., 2004; Wang, de Oliveira, & Nader, 2009). Therefore, it is reasonable to suggest that increasing the fear status by prior stress or by increasing footshock intensity, promotes the resistance to the interfering effect of drugs on fear memory reconsolidation.

It is well established that previous stress promotes fear memory formation (Martijena & Molina, 2012). A similar stressor to that used in the present study potentiates fear expression during recall and testing when it interacts with a weak training protocol (Giachero, Bustos, Calfa, & Molina, 2013; Giachero, Calfa, et al., 2013; Giachero et al., 2015; Maldonado et al., 2011, 2014). Moreover, the current results showed no difference between NS and S or between BIC and SAL animals in freezing during reactivation. However, we cannot exclude that this treatments facilitate fear expression; since such effect may be masked due to the fact that it is close to a ceiling effect (our cannulated rats express a maximum of 50–55% of freezing even with higher intensity footshocks). In addition, previous findings showed that a similar stressor attenuated extinction, and as previously noted, resistance to extinction could reflect the strength of the learning process (Rodriguez Manzanares et al., 2005). Collectively, the present data, as well as previous findings, support the notion that experiencing a stress episode that is unrelated to the cognitive task results in a more robust fear memory trace.

It is important to emphasize that activation of NMDA sites before reactivation is a prerequisite for the labilization/reconsolidation process after retrieval (Tronson & Taylor, 2007). Hence, the stimulation of these receptors, for instance by DCS (a partial agonist of the NMDA receptors (Rouaud & Billard, 2003)), should restore vulnerability to MDZ's disruptive action after retrieval in resistant memories, as that exhibited by intra-BLA BIC treated rats. Consistent with this, the present findings revealed that systemic DCS prior to reactivation restored MDZ disrupting effect on memory reconsolidation in BIC pretreated animals. Furthermore, DCS administration did not affect freezing during reactivation in either BIC or SAL administered animals, which is in agreement with previous data on stressed and non-stressed animals (Bustos et al., 2010). Moreover, the fact that the stress-induced and the BIC-induced resistances can be reverted by DCS also suggests similar mechanisms for each of those interventions that underlie the induction of resistance to the onset of the labilization/reconsolidation process.

Interestingly, it has been reported that stressed rats treated systemically or intra-BLA with DCS prior to reactivation did not show the usual resistance to the reconsolidation-interfering agent (Espejo et al., 2016). Furthermore, it has been suggested that GluN2B subunits of NMDA sites are required for memory destabilization, since intra-BLA administration of a selective antagonist of this NMDA subtype prevents the instability induced by fear memory reactivation (Ben Mamou, Gamache, & Nader, 2006; Milton et al., 2013). In another study, reactivation elevated BLA GluN2B expression in unstressed rats, whereas, this increase was not detected in resistant memories, as shown by stressed animals (Espejo et al., 2016). Additionally, the role of NMDA activation on the induction of lability has been observed in different types of resistant memories (Gazarini, Stern, Piornedo, Takahashi, & Bertoglio, 2014; Ortiz et al., 2015). Therefore, taken together, all this evidence supports the idea that the activation of NMDA receptors is critical in the reversal of the resistance generated by either stress or the pharmacological blockade of GABA-A receptors in the BLA.

5. Conclusion

Suppression of GABAergic input in the BLA should alter the excitation–inhibition balance in the BLA and increase BLA principal neuron excitability. Encoding the information under this circumstance may induce neurobiological changes in the consolidation process that limit the future vulnerability for retrieval-induced destabilization and reconsolidation of fear memory. Moreover, if the labilization/reconsolidation process is an expression of the dynamic nature of memory, as previously suggested (Nader, 2015), then GABAergic signaling in BLA at the moment of memory encoding may define memory flexibility at retrieval. Thus, elucidating the neural mechanism and the anatomical locus of the stress influence on memory processing, and particularly, on the dynamic properties of the fear memory trace may provide new insights into the prevention and treatment of pathologies associated with stress-related memories.

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Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nlm.2017.06.004.

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