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Stress influences the dynamics of hippocampal structural remodeling associated with fear memory extinction

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ABSTRACT

Fear extinction is defined as a decline in fear-conditioned responses following non-reinforced exposure to a fear conditioned stimulus, therefore the conditioned stimulus gains new predictive properties. Patients with anxiety related disorders (e.g.: PTSD) subjected to extinction-like exposure treatments often experience a relapse of symptoms. Stress is a risk factor for those psychiatric disorders and a critical modulator of fear learning that turns the memory resistant to the extinction process. Dendritic spines are the anatomical sites where neuronal activity reshapes brain networks during learning and memory processes. Thus, we planned to characterize the dynamics of synaptic remodeling before and after contextual fear extinction in the dorsal hippocampus (DH), and how this process is affected by a previous stress experience.

Animals with or without previous stress were contextually fear conditioned and one day later trained in an extinction paradigm. Rats were sacrificed one day after conditioning (pre-extinction) or one day after extinction for spine density analysis in the DH. We confirmed that stress exposure induced a deficit in extinction learning. Further, a higher density of dendritic spines, particularly mature ones, was observed in the DH of non-stressed conditioned animals at pre-extinction. Interestingly, after extinction, the spine levels returned to the control values. Conversely, stressed animals did not show such spines boost (pre-extinction) or any other change (post-extinction). In contrast, such standard dynamics of dendritic changes as well as the behavioral extinction was recovered when stressed animals received an intra-basolateral amygdala infusion of midazolam prior to stress.

Altogether, these findings suggest that stress hinders the normal dynamic of dendritic remodeling after fear extinction and this could be part of the neurobiological substrate that makes those memories resistant to be extinguished.

1. Introduction

Fear memories allow animals to anticipate and to avoid threats. However, alterations of the mechanisms implicated in these adaptive responses might lead to an excessive and an inappropriate state of fear and anxiety (e.g. PTSD, phobias) (Cohen, Janicki-Deverts et al., 2007).

Exposure therapy, based on fear extinction, is frequently used for treating traumatic memories in patients (Hermans, Craske et al., 2006; McNally, 2007; Hamm, 2009). The extinction process involves the encoding of a new contingency where the conditioned stimulus no longer predicts the event causing the manifestations of the primary exposure (Pavlov, 1927). However, most of the patients suffer the re-apparition of the symptoms. In this sense, it has been suggested that changes in the dynamics of memory extinction play an important role in anxiety and fear-related disorders. Given that stress is a prominent risk for such psychopathologies (Bremner, Krystal et al., 1995; Bremner & Brett, 1997; Bremner, Elzinga et al., 2008), here we examined the influence of stress on the hippocampal structural plasticity associated with the memory extinction.

It has previously been shown that different brain areas such as the hippocampus, the amygdala or the medial prefrontal cortex (mPFC), are critically involved in the formation of conditioned fear memories (Corcoran & Maren, 2001; Sierra-Mercado, Padilla-Coreano et al., 2011). Additionally, these brain areas play a pivotal role in stress

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responses, including plastic and behavioral manifestations as a consequence of the stress exposure (Watanabe, Gould et al., 1992; Sousa, Lukoyanov et al., 2000; Cerqueira, Taipa et al., 2007).

Of particular note regarding the hippocampus, various behavioral findings as well as neuroanatomical studies have demonstrated functional and anatomical differences along the septotemporal axis (Siegel & Tassoni, 1971; Swanson & Cowan, 1977). In this line, the dorsal region of the hippocampus (DH) has been suggested to play a selective role in contextual learning, while the ventral region (VH) might contribute to the modulation of fear and anxiety (Kjelstrup, Tuvnes et al., 2002; Calfa, Bussolino et al., 2007).

The majority of the excitatory contacts between neurons reside on dendritic spines (Yuste and Denk, 1995). Different experimental pieces of evidence have shown that the substrate for the storage of long-term memories, including contextual fear memories, rely on an appropriate structural plasticity in the form of changes in spine density (Kandel, 2001; Restivo, Vetere et al., 2009). Moreover, experimental observations have suggested that stress results in an over-activated neuronal fear circuit that can be correlated with the strengthening effect of stressful experiences on fear learning and fear memory formation (Rodriguez Manzanares, Isoardi et al., 2005). In line with this view, it was reported that stress exposure prior to fear conditioning reduces the emergence of memory extinction (Izquierdo, Wellman et al., 2006).

Interestingly, contextual fear memories are associated with increased DH dendritic spines density (Leuner, Falduto et al., 2003; Restivo, Vetere et al., 2009). Remarkably, little evidences relates the hippocampal structural plasticity to the formation of memory extinction; this could be important considering that the pharmacological inactivation of DH just before training (Sierra-Mercado, Padilla-Coreano et al., 2011) or extinction recall (Corcoran and Maren, 2004) induced a detrimental effect on the expression of fear memory extinction.

Then, the aim of our work is to evaluate how structural changes in DH might account for the impact of stressful experiences on the dynamics of memory extinction.

2. Material and methods

2.1. Animals

Male Wistar rats weighing 290–310 g from our breeding stock were housed in groups of 3–4 per cage with food and water ad libitum. All the animals were maintained in a 12 h light/dark cycle (lights on 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 Córdoba, 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 to the greatest extent. All the experiments were conducted between 9 a.m to 3 p.m. All animals were gently handled (2 min per animal) for five days before the beginning of the different experimental procedures.

2.2. Stress procedure

The animals were restrained for 60 min inside a plastic cylindrical container fitted close to the body, thus preventing animal movement except for the tail and the tip of the nose (Giachero, Calfa et al., 2013b). Control animals (no-stress) were gently handled without any stressful manipulation. After these manipulations, the animals were returned to their home cages.

2.3. BLA cannula implants and drug administration

Rats were anesthetized with a mixture of ketamine (55 mg/kg, i.p.; Ketajects) and xylazine (11 mg/kg i.p.; Xyla-Jects) and placed in a stereotaxic instrument (Stoelting, Wood Dale, IL) with the incisor bar set at -3.3 mm. Two stainless-steel guide cannulas (22 gauge; length 12 mm) aimed to the BLA were used following specific coordinates: anterior: −2.8 mm; lateral: ± 5.0 mm; ventral: −6.1 mm (Paxinos, 2007). The experiments started after a seven day recovery period (Giachero, Calfa et al., 2013b). Only animals with bilateral adequate injection sites were considered for statistical analysis.

Microinfusions were made using 30-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).

For intra-BLA drug administration, each rat was bilaterally infused with midazolam, a positive allosteric modulator of GABA-A receptor (Petersen, Braestrup et al., 1985) (MDZ; Gobbi Novag, Buenos Aires, Argentina) diluted in sterile isotonic saline (SAL, 0.9% w/v) and a dose of 1 μg/0.5 μl per side 10 min before stress exposure (Giachero, Bustos et al., 2013a; Giachero, Calfa et al., 2015).

2.4. Conditioning apparatus

The conditioning chamber (Cs) was made of a gray plastic wall (20 × 23 × 20 cm) with a clear lid. The floor consisted of 10 parallel stainless steel grid bars enclosed within a sound attenuating chamber. The grid floor was attached to a scrambled shocker (UgoBasile Biological Research Apparatus, Italy) to provide footshock. Illumination was supplied by a 2.5 W white light bulb, and the background noise was made by ventilation fans and the shock scrambler (55 dB).

2.5. Contextual fear conditioning

The fear conditioning protocol was similar to that previously described (Rodriguez Manzanares, Isoardi et al., 2005). On the day of the experiment, stressed (S) or non-stressed (NS) animals were randomly selected and transported from the housing room, individually placed in the conditioning chamber, and left undisturbed for a 3-min acclimation period (preshock period), following by 3 unsignaled scrambled footshocks (0.6 mA, 3 s duration and 30 s inter-shock interval; Cs-Us), with animals being kept in the chamber for an additional 2 min (post-shock period). For control purposes, the rats were placed in the conditioning chamber for the same period of time but did not receive the unsignaled footshock (Cs-noUs). At the end of this period, the rats were removed and subsequently placed in their home cages. In the experiments in which the animals were BLA implanted, the intensity of the footshock was 0.65 mA in order to induce levels of conditioning similar to those exhibited by animals without cannulae implantation since chronic cannulation tends to attenuate the expression of conditioned freezing (Fanselow, 1980; Lee, Milton et al., 2006). The chambers were cleaned with 10% aqueous ethanol solution before and after each session. The experimenters were unaware of the treatment condition.

2.6. Extinction training, retrieval and spontaneous recovery

Twenty-four hours after fear conditioning, the animals returned to the same context (CS) and were left undisturbed for 30 min. No shock was applied during the entire extinction training. After this procedure, the animals were transported to their home cages. On the next day, the animals were re-exposed to the conditioning chamber (CS) for 5 min for the extinction recall test and, later on, were returned to their home cages. In order to evaluate the spontaneous recovery of the original fear memory, 19 days after extinction recall, the animals were re-exposed to the CS for 5 min.

The behavior of each rat was continuously videotaped in order to score freezing behavior during the different behavioral manipulations and to analyze *a posteriori*. The behavioral analysis was performed by an experienced researcher that was unaware of the condition of the animal (double blinded manner). Freezing, a commonly used index of fear in rats (Blanchard & Blanchard, 1969; Fanselow, 1980), was defined as the

total absence of body and head movement except for those associated with breathing.

2.7. Structural plasticity analysis

Dendritic spine visualization and analysis were performed as previously reported (Tyler & Pozzo-Miller, 2003; Calfa, Chapleau et al., 2012; Giachero, Calfa et al., 2013b, 2015). Briefly, animals were deeply anesthetized with chloral hydrate (400 mg/kg i.p.) before being perfused transcardially first by ice-cold PB (0.1 M, pH 7.4) and fixed using ice-cold 4% *para*-formaldehyde (dissolved in 0.1 M PB, pH 7.4). The brain was removed and post-fixed in the same fixative for 24 h at 4 °C, and then sectioned with a vibratome (200 μm thick) to isolate brain slices containing the DH which were collected in PBS 0.1%. Small droplets (< 10 μm) of a saturated solution of the lipophilic dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (DiI, InVitrogen; Carlsbad, CA) in fish oil (Pozzo-Miller, Inoue et al., 1999), was administrated in the *stratum radiatum* of CA1 DH. Thus, the DiI is randomly taken by the dendritic segment camming from different cells. Z-sections from labeled dendritic segments were collected using a Fluoview FV-300 laser-scanning confocal microscope (Olympus IX81 inverted microscope) with an oil immersion (NA 1.42) objective lens (PlanApo) from the *Centro de Microscopía Óptica y Confocal de Avanzada*, Córdoba, Argentina. The images were deconvolved using the "advanced maximum likelihood estimation algorithm" for Cell R software (Olympus Soft Imaging Solutions, Munchen, Germany), version 3.3, set with 15 iterations, and an overlay subvolume of 10 pixels. A theoretical point spread function was used.

The dendritic spine analysis was manually achieved using ImageJ software. Dendritic protrusions less than 3 μm length and contacting with the parent dendrite were considered for the analysis (Murphy & Segal, 1996; Chapleau, Calfa et al., 2009; Calfa, Chapleau et al., 2012).

From the z-section projection, the total number and also the number of each particular type of dendritic spine normalized to 10 μm of the dendritic segment length was counted and certainly, each spine was counted only once. A typical dendritic segment was about 25 μm long.

Spine types were classified considering the length (dimension from the base at the dendrite to the tip of its head, L), the diameter of the neck (measured as the maximum neck diameter, dn), and the diameter of the head (measured as the maximum head diameter, dh). Thus, individual spines were included in each category based on the specific ratios L/dn and dh/dn (Koh, Lindquist et al., 2002): type I or "stubby"-shaped dendritic spines, type II or "mushroom"- shaped dendritic spines, and type III or "thin"-shaped dendritic spines. Generally stubby spines present an L similar to the dn and the dh, and in general the magnitude is < 1 μm. Mushroom spines present a dh much larger than the dn in which the L is typically < 1 μm. Thin spines present an L longer than 1 μm that is much greater than the dn (Koh, Lindquist et al., 2002; Tyler & Pozzo-Miller, 2003). Examples of the dendritic spines types mentioned can be observed in Fig. 2Bi.

As previously reported (Tyler & Pozzo-Miller 2003; Chapleau, Calfa et al., 2009; Calfa, Chapleau et al., 2012; Giachero, Calfa et al., 2013b, 2015), we have included the "stubby"- and "mushroom"-shaped dendritic spines in the category of "mature" spines. This re-categorization is by virtue of the widespread Ca^{+2} transients in the parent dendrite and neighboring spines and due to the strength of the excitatory synapses formed on these spines (Harris, 1999; Segal & Andersen, 2000; Yuste, Majewska et al., 2000; Nimchinsky, Sabatini et al., 2002; Kasai, Matsuzaki et al., 2003).

2.8. Statistical analysis

All data collection was acquired in a blinded manner. Behavioral experiments were analyzed by a two-way ANOVA or repeated measures ANOVA for the percentage of time spent freezing followed by Bonferroni post-hoc test. Data were expressed as mean ± SD.

For the dendritic spines analysis, dendritic segments that belong to different slices from the same rat and from the same experimental group were considered for the statistical analysis. The distribution of the data does not rely on a normal distribution, and considering that mean values are rather insensitive to subtle changes, we used cumulative frequency plots to measure shifts in the total number of dendritic spines, mature dendritic spines, and thin dendritic spines per 10 μm of dendritic segment in the different experimental groups. Cumulative distribution probabilities were compared by Kolmogorov–Smirnov (KS) tests. Besides, we compared the total, mature and thin density of dendritic spines per 10 μm segment between rats from a same experimental group. Under this condition, no significant differences were observed intra-group ($P > 0.05$, KS test for all the comparisons).

Data were also expressed as median (quartile) and were compared by Kruskal–Wallis test or Mann Whitney *U* test as specified in each experiment. $P < 0.05$ was considered statistically significant.

3. Results

3.1. A single stress exposure affects the dynamics of fear memory extinction

In order to describe whether a single stress exposure affects the behavioral dynamics of the formation of the extinction of the fear memory, 40 animals were randomly subjected to a restraint stressful event (S) or just handled (non-stress; NS). Twenty-four hours later, animals were placed in a chamber for fear conditioning (CsUs) or remained in the chamber with no foot shock experience (CsNoUs). Thus, four experimental groups were evaluated: NS/CsNoUs: n = 9 rats; S/CsNoUs: n = 10 rats; NS/CsUs = n-11 rats; and S/CsUs: n = 10 rats.

On the following day, all the experimental animals were exposed for 30 min to the conditioning chamber for extinction training. An extinction recall test was performed the next day in a 5 min Cs exposure. Nineteen days later the animals were placed for 5 min in the same context (Cs) to evaluate spontaneous recovery (Fig. 1A).

For the analysis of fear memory extinction formation, we binned in a 5 min period the extinction training and compared the first 5 min (1–5 min) to the last 5 min (25–30 min) in order to observe the decay of fear behavior due to the extinction training. We also included the 5 min test of the extinction recall and the 5 min test of the spontaneous recovery test in the statistical analysis. A repeated measures ANOVA for the percentage of time spent freezing revealed a significant interaction between test trial x stress x conditioning $F(3, 108) = 13.982$, $p = 0.0001$). The relevant statistical information (Bonferroni post hoc test) revealed that the conditioned animals, independently of the stress exposure (NS/CsUs; S/CsUs), showed a higher level of freezing during the first 5 min exposure of the extinction training in comparison to the non-shocked animals ($p < 0.05$; Fig. 1Bi). Higher levels of freezing were still noticeable during the last 5 min of the extinction training only in the stressed-conditioned animals (S/CsUs) as compared to the rest of the experimental groups ($p < 0.05$; Fig. 1Bii). The analysis of the behavior during the extinction recall test revealed that the memory extinction was effectively formed in the conditioned non-stressed animals (Ns/CsUs) since the freezing response was similar to that shown by animals without prior conditioning ($p > 0.05$). However, higher levels of freezing were evident in S/CsUs animals in comparison to the rest of the experimental groups ($p < 0.05$; Fig. 1Biii), indicating that stress impeded the emergence of the memory extinction. As expected, the spontaneous recovery test showed that the NS/CsUs animals exhibited higher freezing levels in comparison to non-conditioned animals ($p < 0.05$). Moreover, no statistical differences were observed between NS/CsUs and S/CsUs groups during this test ($p > 0.05$; Fig. 1Biv).

These findings suggest that a single stress exposure unrelated to the cognitive task has a detrimental effect on the emergence of fear memory extinction. After 19 days of the extinction recall, a full fear behavior response was observed in those non-stressed animals that have exhibited extinguished freezing, indicating the occurrence of spontaneous

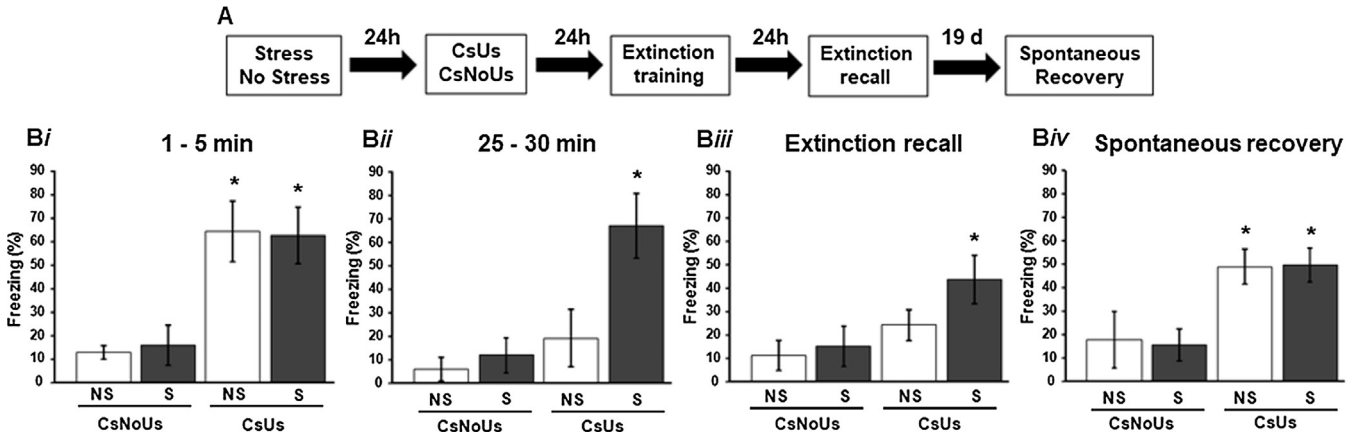

Fig. 1. A single stress exposure affects the dynamics of fear memory extinction. (A) Schematic representation of the experimental design. (B) Bar graph showing the freezing behavior response during the first (*i*) and *(ii)* the last 5 min of the extinction training. The next day animals underwent an extinction recall test (*iii*) and 19 days later the spontaneous recovery test (*iv*). Data are expressed as mean ± SD of the percentage of time spent freezing during the tests (N = 9–11 rats per group). *P < 0.05 compared with the rest of the experimental groups (repeated measures ANOVA, Bonferroni post hoc test).

recovery in these rats.

3.2. A single stress exposure prevents the formation-retraction of CA1 dorsal hippocampal dendritic spines associated to fear memory and to the extinction process

To explore whether fear memory extinction formation is associated with CA1 hippocampal structural plasticity and how it is influenced by stress exposure, 45 animals were randomly distributed to the experimental groups described in the first experiment, and sacrificed 24 h after conditioning (pre-extinction period) or 24 h after the end of the extinction training (post-extinction period) for dendritic spine analyses (Fig. 2A).

For those animals who received the extinction training (26 rats), repeated measures ANOVA for the percentage of time spent freezing during the first 5 min and the last 5 min of the extinction training revealed a significant effect of the triple interaction between stress x conditioning x period of test during extinction training as repeated measures ($F(1,22) = 5.42$, $p = 0.029$). As previously observed, a Bonferroni post hoc test revealed that the conditioned animals independently of the stress exposure (NS/CsUs and S/CsUs) presented a higher level of freezing behavior during the first 5 min Cs exposure ($p < 0.05$) in comparison to the non-conditioned animals. A high level of freezing was observed even during the last 5 min of extinction training only in the S/CsUs animals as compared to the rest of the experimental groups ($p < 0.05$).

3.2.1. Hippocampal dendritic spine analyses at the pre-extinction period

The animals were distributed into the four mentioned groups in which spine counts were performed on a total of 180 dendritic segments as follows: NS/CsNoUs: n = 48 segments, 1490 μm total dendritic length analyzed, 5 rats; NS/CsUs: n = 43 segments, 1216 μm, 5 rats; S/CsNoUs: n = 46 segments, 1517 μm, 4 rats; S/CsUs: n = 43 segments, 1277.3 μm, 5 rats). Fig. 2Bii shows representative examples of the different dendritic segments in the DH CA1 *stratum radiatum* for each particular experimental group.

The analysis of the cumulative probability distribution for the total density of dendritic spines in the DH showed a significant rightward shift toward a higher number in NS/CsUs animals in comparison to the rest of the experimental groups ($P < 0.05$ for each individual comparison, KS test; Fig. 2Ci). This shift also resulted in higher median (quartiles; total density/10 μm) in NS/CsUs: 15.44 [11.16–17.6] (Kruskal–Wallis test = 50.627; $p = 0.001$) in comparison to the rest of the experimental groups ($P < 0.001$; multiple comparison of mean ranks post hoc test) NS/CsNoUs: 10.31 [7.47–14.24], S/CsUS: 11.08 [7.33–14.96] and S/CsNoUs: 9.81 [6.94–15.33].

Such difference was detected essentially in mature dendritic spines since a significant rightward shift toward a higher number of this type of spines were detected in animals NS/CsUs in comparison to the rest of the experimental groups ($P < 0.05$ for each individual comparison, KS test; Fig. 2Cii). This shift also resulted in higher median (quartiles; total density/10 μm) in NS/CsUs: 11.66 [8.18–14.13] (Kruskal–Wallis test = 67.107) in comparison to NS/CsNoUs: 6.83 [3.89–9.94], S/CsUS: 6.63 [4.58–9.74] and S/CsNoUs: 6.93 [4.72–11.79] ($P < 0.001$; multiple comparison of mean ranks post hoc test).

The analysis of the cumulative probability distribution for the number of thin dendritic spines reflected no significant differences between groups ($P > 0.05$ for each individual comparison, KS test; Fig. 2Ciii). The median of thin dendritic spines (quartiles; thin spines density/10 μm) are as follow: NS/CsUs: 3.47 [1.63–5.89], NS/CsNoUs: 3.77 [2.04–6.01], S/CsUs: 3.33 [1.36–5.35] and S/CsNoUs: 2.87 [1.03–5.03], in where no significant changes were detected (Kruskal–Wallis test = 67.107; $p > 0.05$; multiple comparison of mean ranks post hoc test).

3.2.2. Hippocampal dendritic spine analyses at the post-extinction period

The animals were distributed into the four mentioned groups and spine counts were performed on a total of 173 dendritic segments as follows: NS/CsNoUs: n = 31 segments, 820 μm total dendritic length analyzed, 6 rats; NS/CsUs: n = 48 segments, 1330 μm, 8 rats; S/CsNoUs: n = 44 segments, 1468 μm, 6 rats; S/CsUs: n = 50 segments, 1593 μm, 6 rats). Fig. 2Biii shows representative examples of the different dendritic segments in the CA1*stratum radiatum* DH for each particular experimental group.

The analysis of the cumulative probability distribution for the total number of dendritic spines reflected no significant differences between groups ($P > 0.05$ for each individual comparison, KS test, Fig. 2Di). The median (quartiles; total density/10 μm) are as follows: NS/CsUs: 12.16 [9.05–17.39], NS/CsNoUs: 11.21 [9.28–15.94], S/CsUs: 11.17 [8.71–14.51] and S/CsNoUs: 10.73 [7.41–14.42]. No significant changes were detected between the different experimental groups (Kruskal–Wallis test = 6.90; $p > 0.05$; multiple comparisons of mean ranks post hoc test).

Similarly, the analysis of the cumulative probability distribution for the number of mature dendritic spines reflected no significant differences between groups ($P > 0.05$ for each individual comparison, KS test; Fig. 2Dii). The median (quartiles; mature spines density/10 μm) are as follows: NS/CsUs: 7.93 [6.09–11.73], NS/CsNoUs: 7.89 [5.73–10.19], S/CsUs: 7.21 [4.72–11.22] and S/CsNoUs: 7.03 [4.25–9.2]. No statistical differences were detected between the

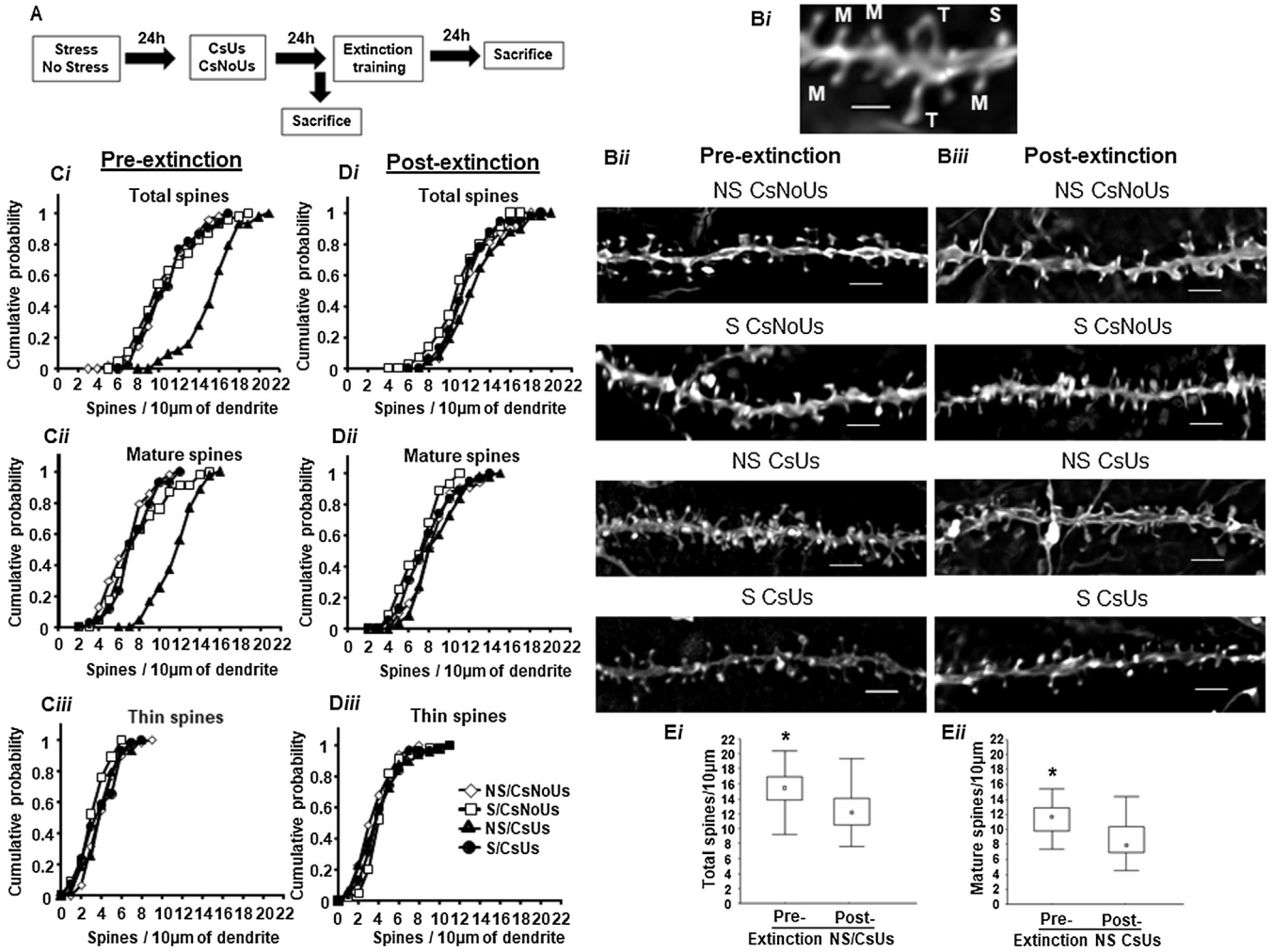

Fig. 2. A single stress exposure prevents the formation-retraction of CA1 dorsal hippocampal dendritic spines associated to fear memory and to the extinction process. (A) Schematic representation of the experimental design. (B) (*i*) Representative example showing the morphology of the different types of dendritic spines observed: (S) stubby-shaped dendritic spines, (M) mushroom- shaped dendritic spines and (T) thin-shaped dendritic spines. Bar: 1 µm. (*ii, iii*) Representative examples of apical dendritic segments of CA1 dorsal hippocampal pyramidal neurons (*stratum radiatum*) which were selected for quantitative analysis of dendritic spines from animals of each experimental group (N = 5 rats per group). Bar scale: 2 µm. (C and D) Cumulative frequency of total (*i*), mature (*ii*), and thin (*iii*) dendritic spine density on apical dendrites of hippocampal CA1 pyramidal cells at pre-extinction (C) and post extinction period (D). $P < 0.05$, Kolmogorov–Smirnov test (NS/CsUs compared with the rest of the experimental groups). E) Graphs showing the median (dot) interquartile (bar) and low and upper values (error bars) of total (*i*) and mature (*ii*) density of dendritic spines for conditioned non-stressed animals at pre extinction training as compared to the post extinction training period; $^{*}P < 0.05$, Mann Whitney *U* test.

experimental groups (Kruskal–Wallis test = 3.397; $p > 0.05$; multiple comparisons of mean ranks post hoc test).

With respect to the analysis of thin dendritic spines, the cumulative probability distribution reflected a significant left shift in NS/CsNoUs but just in comparison to S/CsNoUs ($P < 0.05$; KS test; Fig. 2Diii. Such difference is evident in the median (quartiles; thin spines density/ 10 µm) of the different experimental groups: NS/CsUs: 3.43 [1.44–7.02], NS/CsNoUs: 3.14 [1.61–5.91], S/CsUs: 3.69 [1.77–6.5] and S/CsNoUs: 3.85 [2.29–5.87] (Kruskal–Wallis test = 13.433; $p < 0.05$; multiple comparison of mean ranks post hoc test).

This different pattern of structural plasticity observed for each particular period of time, before or after extinction training, was notably evident when the comparison was performed between them. Thus, a significant difference was observed in the total density of dendritic spines for conditioned non-stressed animals at pre extinction training as compared to the post-extinction training period (Mann Whitney *U* test = 468; $p = 0.0007$; Fig. 2Ei). Furthermore, the density of mature dendritic spines in NS/CsUs animals at pre-extinction period presented a higher number in comparison to the post-extinction period (Mann Whitney *U* test = 362; $p < 0.0001$; Fig. 2Eii). No changes were observed with respect to thin dendritic spines ($p > 0.05$). However, no significant differences were detected in stressed animals independently of the fear conditioned ($p > 0.05$).

These results suggest a critical dynamic of CA1-DH dendritic spine remodeling due contextual fear conditioning, an effect that vanished after the formation of the memory extinction.

3.3. The recall of the original memory did not induce additional hippocampal structural plasticity

We then assessed the effect of a simple exposure to the same context for 5 min on DH structural remodeling that is after a period of time that does not allow the extinction of the freezing response. This experiment controls that the extinction process is the responsible for the reduction of the density of DH dendritic spines and not the mere exposure to the Cs. Seventeen animals were randomly selected for stress exposure or just handled. One day after this manipulation, all the animals were exposed to the fear conditioning protocol or to the control procedure. After 24 h, the animals were re-exposed to the conditioned chamber for the 5 min retrieval experience. One day after, the animals were

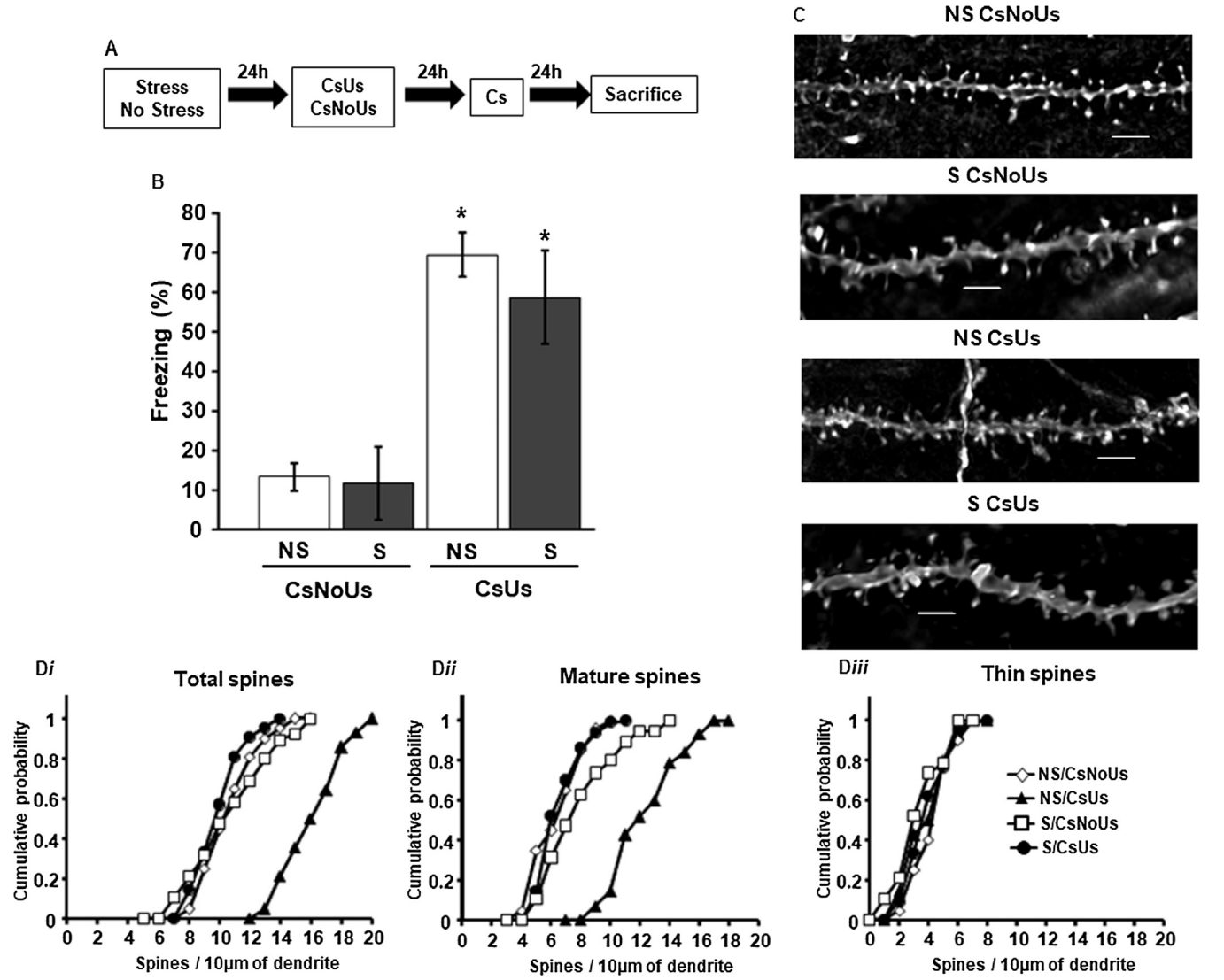

Fig. 3. The recall of the original memory did not induce additional hippocampal structural plasticity. (A) Schematic representation of the experimental design. (B) Bar graph showing the freezing behavior response during the 5 min recall, one day after fear conditioning. Data are expressed as mean ± SD of the percentage of time spent freezing, $^{*}P < 0.05$ compared with the rest of the experimental groups (ANOVA, Bonferroni post hoc test). (C) Representative examples of apical dendritic segments of CA1 dorsal hippocampal pyramidal neurons (stratum radiatum) which were selected for quantitative analysis of dendritic spines from animals of each experimental group (n = 3–5 rats per group). Bar scale: 2 µm. (D) Cumulative frequency of total (*i*), mature (*ii*), and thin (*iii*) dendritic spine density on apical dendrites of hippocampal CA1 pyramidal cells one day after recall of fear memory ($P < 0.05$, Kolmogorov–Smirnov test, NS/CsUs compared with the rest of the experimental groups).

sacrificed for structural plasticity analysis (Fig. 3A).

For the time spent freezing during the 5 min test, a two-way ANOVA analysis showed a significant effect of the conditioning ($F(1,13) = 161.42$; $p < 0.0001$). A Bonferroni post hoc test revealed that the conditioned animals independently of the stress exposure (NS/CsUs or S/CsUs) exhibited higher levels of freezing response in comparison to the non-conditioned animals ($p < 0.05$) (Fig. 3B).

For CA1 DH structural plasticity 24 h after the 5 min retrieval, the spine counts were performed on a total of 74 dendritic segments as follows: NS/CsNoUs: n = 20 segments, 624 µm total dendritic length analyzed, 5 rats; NS/CsUs: n = 14 segments, 550 µm, 3 rats; S/CsNoUs: n = 19 segments, 582 µm, 5 rats; S/CsUs: n = 21 segments, 611 µm, 4 rats). Fig. 3C shows representative examples of the different dendritic segments for each particular experimental group. The analysis of the cumulative probability distribution for the total density of dendritic spines reflected a significant rightward shift toward a higher number in animals NS/CsUs in comparison to the rest of the experimental groups [$P < 0.05$ for each individual comparison, KS test; Fig. 3Di]. This shift also resulted in a higher median (quartiles; total density/10 µm) in NS/CsUs: 15.91 [13.43–18.73] with respect to the rest of the groups: NS/CsNoUs: 10.34 [8.39–13.27], S/CsUs: 9.77 [7.36–11.84] and S/CsNoUs: 10.74 [6.44–15.11] (Kruskal–Wallis test $H(3–74) = 30.89$; $P < 0.001$; multiple comparison of mean ranks post hoc test, $P < 0.001$).

A similar rightward shift toward higher density in NS/CsUs animals compared to the rest of the experimental groups was observed for mature dendritic spines ($P < 0.05$ for each individual comparison, KS test; Fig. 3Dii). In a similar manner, a higher median (quartiles; mature dendritic spine density/10 µm) in NS/CsUs: 11.76 [9.22–15.53] was observed in comparison to the rest of the groups NS/CsNoUs: 6.88 [4.48–7.96], S/CsUs: 5.89 [4.86–7.50] and S/CsNoUs: 7.51 [4.79–11.79] (Kruskal–Wallis test $H(3–74) = 32.79$; $P < 0.001$; multiple comparison of mean ranks post hoc test, $P < 0.001$).

Such significant differences were not reflected in the analysis of density of thin dendritic spines ($P > 0.05$ for each individual comparison, KS test; Fig. 3Diii). The median (quartiles; thin dendritic spine

density/10 μm) are as follows: NS/CsUs: 4.03 [1.94–5.33], NS/CsNoUs: 4.24 [2.82–5.92], S/CsUs: 3.88 [2.24–5.52] and S/CsNoUs: 2.95 [0.95–5.51] (Kruskal–Wallis test H(3–74) = 4.60; P = 0.203).

Thus, a brief recall experience of the original memory did not generate any further significant change on dendritic spine rearrangement in CA1 DH 24 h after memory recall as compared to the elevation already observed just before memory recall (pre-extinction period).

3.4. MDZ intra basolateral amygdala (BLA) prevented the deleterious effects of stress on memory extinction and on hippocampal structural changes

In order to prevent the deleterious effects of a single stress exposure on fear memory extinction formation and on dendritic spine remodeling, a total of 52 animals were subjected to intra-BLA MDZ or SAL infusion 15 min prior to stress or control manipulation. One day later, all animals were fear conditioned (CsUs). Thus, four experimental groups were evaluated: SAL/NS/CsUs (n = 10): animals that received intra-BLA SAL and then subjected to handling and one day later fear conditioned; SAL/S/CsUs (n = 13): animals that received intra-BLA SAL prior to stress exposure and 24 h later subjected to fear conditioning; MDZ/NS/CsUs (n = 13): animals that received intra-BLA MDZ prior to handling and 24 h later fear conditioned; and MDZ/S/CsUs (n = 16): animals that received intra-BLA MDZ prior to stress and 24 h later fear conditioned. The next day, a subset of animals for each experimental group was sacrificed for structural plasticity analysis or was exposed for 30 min to the Cs for extinction training. The recall of the memory extinction was performed one day later (Fig. 4A).

For the analysis of the fear memory extinction, we performed the same analysis as previously described: we binned in a 5 min period the extinction training and the first 5 min were compared to the last 5 min in order to observe the fear behavior decay due to the extinction training. We also included in the statistical analysis the 5 min recall extinction test: SAL/NS/CsUs (n = 6), SAL/S/CsUS (n = 8), MDZ/NS/CsUs (n = 10) and MDZ/S/CsUS (n = 13).

A repeated measure ANOVA for the percentage of time spent freezing revealed a significant effect of test trial x stress x drug administration F(2, 66) = 3.709, p = 0.0297). Thus, the relevant statistical information (Bonferroni post hoc test) revealed that independently of the stress and MDZ administration, the animals displayed a higher freezing response during the first 5 min of the extinction training, an indicative of the classically conditioned response (Fig. 4Bi). Interestingly, stressed animals that were MDZ BLA treated presented a reduced freezing expression during the last 5 min extinction training, with no statistical differences in comparison to non-stressed rats either SAL or MDZ administered (P > 0.05). On the contrary, the stressed animals SAL/S/CsUs, continued with a higher fear expression in comparison to the rest of the experimental groups (p < 0.05; Fig. 4Bii). A similar behavior was observed during the recall of the memory extinction, where MDZ BLA treated animals sustained the reduced freezing response independently of the stress condition (p < 0.05; Fig. 4Biii).

With the aim of evaluating the structural plasticity under this manipulation, the rest of the subset of the experimental animals (n = 15 rats) were distributed into the four mentioned groups and sacrificed 24 h after conditioning (pre-extinction time). The dendritic spine counts were performed on a total of 109 dendritic segments as follows: SAL/NS/CsUs: n = 27 segments, 775 μm total dendritic length analyzed, 4 rats; MDZ/NS/CsUs: n = 24 segments, 986 μm, 3 rats; SAL/S/CsUs: n = 35 segments, 986 μm, 5 rats; MDZ/S/CsUs: n = 23 segments, 713 μm, 3 rats). Fig. 4C shows representative examples of the different dendritic segments in the CA1 DH *stratum radiatum* for each particular experimental group.

The analysis of the cumulative probability distribution for the total density of dendritic spines reflected a significant leftward shift toward a reduced number in animals SAL/S in comparison to SAL/NS, MDZ/NS and MDZ/S (P < 0.05, Kolmogorov– Smirnov (KS) test; Fig. 4Di). A no significant change was observed between MDZ/NS, SAL/NS and MDZ/S (p > 0.05). This shift resulted in a higher median (quartiles; total density/10 μm) in SAL/NS: 19 [16.84–21.59], MDZ/NS: 17.57 [15.73–19.89] and MDZ/S: 18 [15.17–20.17] with respect to the SAL/S group: 12.49 [7.51–17.71] (Kruskal–Wallis test H(3–108) = 53.24; P < 0.001; multiple comparison of mean ranks post hoc test, P < 0.001).

Similarly, a leftward shift toward a reduced density of mature dendritic spines was observed in SAL/S in comparison to MDZ/NS, MDZ/S and SAL/NS animals (P < 0.05 for the different comparisons to SAL/S/CsUs, KS test; Fig. 4Dii). This shift resulted in a higher median (quartiles; mature dendritic spines/10 μm) in SAL/NS: 13.92 [11.27–17.04], MDZ/NS: 12.61 [10.25–13.85] and MDZ/S: 11.77 [9.73–13.94] with respect to the SAL/S group: 7.38 [5.25–12.94] (Kruskal–Wallis test H(3–108) = 46.88; P < 0.001; multiple comparison of mean ranks post hoc test, P < 0.001).

In the same way, those changes were also evident for the analysis of thin dendritic spines, where SAL/S presented a lesser density compared to MDZ/NS, MDZ/S and SAL/NS animals (P < 0.05 for the different comparisons to SAL/S, KS test; Fig. 4Diii). This shift resulted in a higher median (quartiles; thin dendritic spines/10 μm) in SAL/NS: 5.59 [3.87–7.45], MDZ/NS: 5.49 [3.11–7.47] and MDZ/S: 5.98 [3.41–9.22] with respect to the SAL/S group: 3.91 [1.89–5.49] (Kruskal–Wallis test H(3–108) = 24.55; P < 0.001; multiple comparison of mean ranks post hoc test, P < 0.001).

Overall, MDZ intra BLA prior to stress exposure prevented the stress-induced deleterious effects on the formation/expression of the fear memory extinction alongside its influence on the dynamic of hippocampal structural remodeling.

4. Discussion

The main finding that emerges from the present study is that the formation of the fear memory extinction in non-stressed animals, is accompanied by a particular structural plasticity dynamics at CA1 DH. There was a boost of dendritic spines density, particularly mature ones, after fear encoding (pre-extinction), that was dampened to control values after the extinction training. Therefore, the decay in the number of total and mature dendritic spines in unstressed conditioned rats is presumably associated with the formation of the memory extinction. However, no changes were evident between pre- and post-extinction in conditioned stressed rats in comparison to unstressed conditioned animals. Thus, such dynamic pattern is absent in stressed conditions, an effect presumably related to the stress-induced detrimental effect on the extinction behavioral performance (Izquierdo, Wellman et al., 2006; Akirav, Segev et al., 2009; Wilber, Walker et al., 2011).

Based on the previous viewpoint, the memory extinction would be the responsible for the decrease of dendritic spines density. In fact, dendritic spines remodeling of CA1 DH following a brief recall experience (5 min), that does not induce extinction, did not generate any extra significant dendritic spine rearrangement to what has already been observed just before memory recall (pre-extinction period) or, any reduction or change in the morphology of the dendritic spines. Therefore, these results further support the view that the dynamic of hippocampal structural plasticity due to the extinction training is potentially associated with the emergence of the memory extinction.

As many researchers have demonstrated, and previously stated, dendritic spines are the locus for the excitatory contact between neurons (Yuste & Denk, 1995; Peters & Palay, 1996). As such, the reshaping of dendritic spines, conducing to a proper structural plasticity in particular brain regions might be the substrate for the storage of long-term memories (Kandel, 2001; Restivo, Vetere et al., 2009). In this way, it was pointed out that structural changes are necessary for the stabilization and persistence of those memories (Kandel, 2001; Restivo, Vetere et al., 2009). Based on the above consideration, we have made two fundamental inquiries; first, we conjecture that fear memory

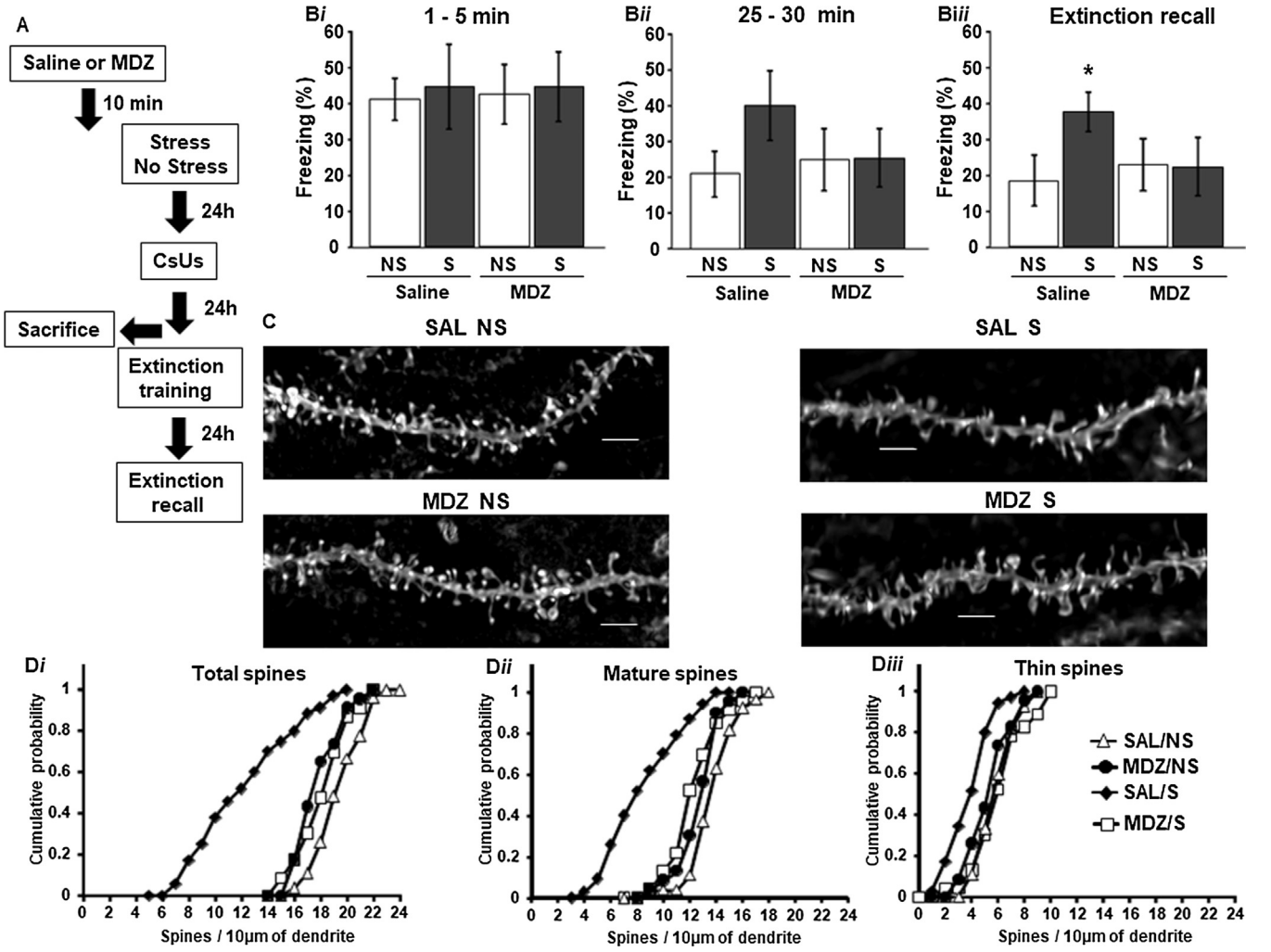

Fig. 4. Midazolam intra basolateral amygdala complex prevented the deleterious effects of stress on memory extinction and on hippocampal structural changes. (A) Schematic representation of the experimental design. (B) Bar graphs showing the freezing behavior response during the first (*i*) and the last (*ii*) 5 min of extinction training, and during the extinction recall (iii). Data are expressed as mean ± SD of the percentage of time spent freezing, $^{*}P < 0.05$ compared with the rest of the experimental groups (ANOVA, Bonferroni post hoc test). (C) Representative examples of apical dendritic segments of CA1 dorsal hippocampal pyramidal neurons (*stratum radiatum*) which were selected for quantitative analysis of dendritic spines from animals of each experimental group (n = 3–5 rats per group). Bar scale: 2 µm. (D) Cumulative frequencies of total (*i*), mature (*ii*), and thin (*iii*) dendritic spine density on apical dendrites of hippocampal CA1 pyramidal cells one day after extinction training ($P < 0.05$, Kolmogorov–Smirnov test, SAL/S compared with the rest of the experimental groups).

extinction formation requires dynamics changes in CA1 DH structural remodeling; secondly, we were wondering whether the stress exposure, that hinders fear memory extinction formation, might also prevent the dynamic of such DH structural remodeling.

A higher density of dendritic spines, particularly mature ones was only observed in unstressed conditioned animals at pre-extinction. This finding suggests a critical activity of CA1 DH necessary for the formation and the expression of the fear memory as previously shown (Leuner, Alduto et al., 2003; Giachero, Calfa et al., 2013b, 2015). Interestingly, the values for the total and particularly mature dendritic spines reached control values after extinction. In a similar way, Vetere and colleagues (Vetere, Restivo et al., 2011) have reported a higher number of dendritic spines associated to remote contextual fear memory in the anterior cingulate cortex (aCC), a critical area for memory system consolidation (Frankland, Bontempi et al., 2004), returning to basal levels after extinction. Concurrently, Heinrichs and colleagues (Heinrichs, Leite-Morris et al., 2013) demonstrated a subtle but significant spine density and dendritic branch intersection increase in BLA neurons of fear conditioned animals that were reversed after extinction training. Thus, these observations open the possibility to consider that the reshaping of the dendritic spines at DH represents a critical sign for memory extinction formation.

In accordance with different authors, dendritic spines are highly plastic since they respond to synaptic activity (Yuste & Denk, 1995; Fischer, Kaech et al., 1998; Maletic-Savatic, Malinow et al., 1999). Therefore, the induction of the long-term synaptic plasticity (LTP) causes enlargement of hippocampal spine head with a critical intracellular increase of calcium levels in mature dendritic spines (Matsuzaki, Honkura et al., 2004). Interestingly, low-frequency stimulation known for its long-term synaptic depression (LTD) causes some spines to shrink or even disappear (Zhou, Homma et al., 2004). In this way, it was observed that LTD and the synaptic depotentiation by the regulation of the endocytosis of GluA2-containing AMPARs in LA play a critical role in tone-cued fear memory extinction (Kim, Lee et al., 2007). Based on these findings and on the current results, it is plausible to hypothesize that a reduced dendritic spine density and CA1 DH-LTD underlies contextual fear memory extinction. Additional experiments need to be performed in order to clarify this issue.

Consistently with a wide number of researchers (Ammassari-Teule, 2016), the present findings show that animals that were previously stressed exhibited a behavioral impairment in fear memory extinction formation. We hypothesized that stress impacts on the structural

plasticity and thus, underlies the behavioral effects observed. In contrast to unstressed conditioned rats, stressed conditioned animals did not show the dendritic spines boost following fear encoding. The brain areas involved in memory extinction formation, such as hippocampus, mPFC, amygdala (Sierra-Mercado, Padilla-Coreano et al., 2011) are also involved in the stress response (McEwen, 2007). Therefore, it can be supposed that stress itself would change the number of hippocampal dendritic spines. In this line of reasoning, chronic stress exposure -21 d/6h immobilization stress (Watanabe, Gould et al., 1992; Magarinos, McEwen et al., 1996; Chen, Dube et al., 2008) or 10 d/2h immobilization stress (Vyas, Mitra et al., 2002)- presented contrasting effects depending on the brain area. Whereas chronic stress protocols induced a reduction of hippocampal dendritic spines and dendritic arborization particularly at CA3 hippocampal sub-area on BLA pyramidal cells -presumed glutamatergic-, presented a higher number of dendritic branch points. In a similar way, acute stress exposures induce different structural plasticity changes. Accordingly, 30 min restraint stress exposure with the addition of tail shocks induced a higher number of dendritic spines at CA1 hippocampal sub-area (Shors, Chua et al., 2001); a single force swimming exposition generated a reduced dendritic branch number and length at principal IL-mPFC neurons, with no changes in the PL-mPFC neurons (Izquierdo, Wellman et al., 2006). In our laboratory, 30 min (Giachero, Calfa et al., 2013b, 2015) or 60 min (present work) restraint stress did not induce significant changes in the number or the morphology of the dendritic spines at CA1 DH when animals where sacrificed 48 h after this environmental challenge. As many other researchers did, we conjectured that this difference resides on the consequence of the magnitude of the stress applied (Shors, Chua et al., 2001), the requirement of different brain areas or sub-areas for information processing (Watanabe, Gould et al., 1992; Izquierdo, Wellman et al., 2006) and/or the time after stress exposure.

In this work, the emotional relevant experience did not change by itself the hippocampal structural plasticity; however, it has a detrimental action on dendritic spine remodeling of conditioned animals. Although it has been suggested that the formation of fear is closely associated with dendritic spine remodeling in DH, the current results show that conditioned stressed rats exhibited fear memory without spine remodeling. A possible explanation could be that other molecular changes in DH might be supporting the cognitive process. Additionally, the changes in spine density could be occurring in other brain areas involved in the formation of fear memory. For instance, an impaired hippocampal spatial learning, LTP generation and a reduced dendritic branching was observed in the hippocampus after animals were exposed to stress. On the contrary, a similar stress exposure facilitated the consolidation of emotionally arousing memories, LTP generation and dendritic branching in BLA (Vyas, Mitra et al., 2002; Vyas, Jadhav et al., 2006; Roozendaal, McEwen et al., 2009). In addition, in previous experiments from our lab (Bender, Otamendi et al., 2018), we have observed that conditioned animals with prior stress showed fear generalization (higher freezing in a different, not conditioned, context). This finding reinforces the idea that the role of DH was altered since it is well known that contextual fear generalization is correlated with a lack of hippocampal activity/involvement (Jasnow, Lynch et al., 2017).

It has been suggested that stress exposure leads to a reduction in the GABAergic inhibitory control on glutamatergic pyramidal projection neurons in BLA (Isoardi, Bertotto et al., 2007). Thus, this reduced GABAergic signaling would result in an unmasked activation of pyramidal neurons, and consequently, an enhanced excitability of BLA neurons (Rodriguez Manzanares, Isoardi et al., 2005). Consistent with this view, we have previously reported that stimulating GABA-A sites within the BLA by MDZ infusion prior to restraint prevented the stress-induced influence on fear memory (Rodriguez Manzanares, Isoardi et al., 2005; Giachero, Calfa et al., 2013b) and fear generalization (Bender, Otamendi et al., 2018).

In line with this evidence, the present findings show that intra-BLA MDZ administration prior to restraint prevented the stress-induced deleterious influence on memory extinction, and enhanced dendritic spines to a level similar to those observed in unstressed rats at pre-extinction training. Consistent with previous findings, this result highlights the critical role of the GABAergic transmission in BLA for the stress influence on fear memory and on the structural plasticity associated to different phases of fear memory, in our particular case, to fear memory extinction. Besides, these results support the idea that the behavioral and spine remodeling manifestations are critically affected by the emotional state, demonstrating a potential critical association of a dynamic structural plasticity in DH with the emergence of the memory extinction.

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References

Akirav, I., Segev, A., et al. (2009). D-cycloserine into the BLA reverses the impairing effects of exposure to stress on the extinction of contextual fear, but not conditioned taste aversion. *Learning Memory, 16*(11), 682–686.
Ammassari-Teule, M. (2016). Is structural remodeling in regions governing memory an univocal correlate of memory? *Neurobiology of Learning and Memory, 136*, 28–33.
Bender, C. L., Otamendi, A., et al. (2018). Prior stress promotes the generalization of contextual fear memories: Involvement of the gabaergic signaling within the basolateral amygdala complex. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 83*, 18–26.
Blanchard, R. J., & Blanchard, D. C. (1969). Crouching as an index of fear. *J Comp Physiol Psychol, 67*(3), 370–375.
Bremner, J. D., & Brett, E. (1997). Trauma-related dissociative states and long-term psychopathology in posttraumatic stress disorder. *Journal of Traumatic Stress, 10*(1), 37–49.
Bremner, J. D., Elzinga, B., et al. (2008). Structural and functional plasticity of the human brain in posttraumatic stress disorder. *Progress in Brain Research, 167*, 171–186.
Bremner, J. D., Krystal, J. H., et al. (1995). Functional neuroanatomical correlates of the effects of stress on memory. *Journal of Traumatic Stress, 8*(4), 527–553.
Calfa, G., Bussolino, D., et al. (2007). Involvement of the lateral septum and the ventral Hippocampus in the emotional sequelae induced by social defeat: Role of glucocorticoid receptors. *Behavioural Brain Research, 181*(1), 23–34.
Calfa, G., Chapleau, C. A., et al. (2012). HDAC activity is required for BDNF to increase quantal neurotransmitter release and dendritic spine density in CA1 pyramidal neurons. *Hippocampus, 22*(7), 1493–1500.
Cerqueira, J. J., Taipa, R., et al. (2007). Specific configuration of dendritic degeneration in pyramidal neurons of the medial prefrontal cortex induced by differing corticosteroid regimens. *Cerebral Cortex, 17*(9), 1998–2006.
Chapleau, C. A., Calfa, G. D., et al. (2009). Dendritic spine pathologies in hippocampal pyramidal neurons from Rett syndrome brain and after expression of Rett-associated MECP2 mutations. *Neurobiology of Diseases, 35*(2), 219–233.
Chen, Y., Dube, C. M., et al. (2008). Rapid loss of dendritic spines after stress involves derangement of spine dynamics by corticotropin-releasing hormone. *Journal of Neuroscience, 28*(11), 2903–2911.
Cohen, S., Janicki-Deverts, D., et al. (2007). Psychological stress and disease. *JAMA, 298*(14), 1685–1687.
Corcoran, K. A., & Maren, S. (2001). Hippocampal inactivation disrupts contextual retrieval of fear memory after extinction. *Journal of Neuroscience, 21*(5), 1720–1726.
Corcoran, K. A., & Maren, S. (2004). Factors regulating the effects of hippocampal inactivation on renewal of conditional fear after extinction. *Learning Memory, 11*(5), 598–603.
Fanselow, M. S. (1980). Conditioned and unconditional components of post-shock freezing. *Pavlov Journal of Biological Science, 15*(4), 177–182.
Fischer, M., Kaech, S., et al. (1998). Rapid actin-based plasticity in dendritic spines. *Neuron, 20*(5), 847–854.
Frankland, P. W., Bontempi, B., et al. (2004). The involvement of the anterior cingulate cortex in remote contextual fear memory. *Science, 304*(5672), 881–883.
Giachero, M., Bustos, S. G., et al. (2013a). A BDNF sensitive mechanism is involved in the fear memory resulting from the interaction between stress and the retrieval of an established trace. *Learning Memory, 20*(5), 245–255.
Giachero, M., Calfa, G. D., et al. (2013b). Hippocampal structural plasticity accompanies the resulting contextual fear memory following stress and fear conditioning. *Learning Memory, 20*(11), 611–616.

Giachero, M., Calfa, G. D., et al. (2015). Hippocampal dendritic spines remodeling and fear memory are modulated by GABAergic signaling within the basolateral amygdala complex. *Hippocampus, 25*(5), 545–555.
Hamm, A. O. (2009). Specific phobias. *Psychiatric Clinics of North America, 32*(3), 577–591.
Harris, K. M. (1999). Structure, development, and plasticity of dendritic spines. *Current Opinion in Neurobiology, 9*(3), 343–348.
Heinrichs, S. C., Leite-Morris, K. A., et al. (2013). Dendritic structural plasticity in the basolateral amygdala after fear conditioning and its extinction in mice. *Behavioural Brain Research, 248*, 80–84.
Hermans, D., Craske, M. G., et al. (2006). Extinction in human fear conditioning. *Biological Psychiatry, 60*(4), 361–368.
Isoardi, N. A., Bertotto, M. E., et al. (2007). Lack of feedback inhibition on rat basolateral amygdala following stress or withdrawal from sedative-hypnotic drugs. *European Journal of Neuroscience, 26*(4), 1036–1044.
Izquierdo, A., Wellman, C. L., et al. (2006). Brief uncontrollable stress causes dendritic retraction in infralimbic cortex and resistance to fear extinction in mice. *Journal of Neuroscience, 26*(21), 5733–5738.
Jasnow, A. M., Lynch, J. F., 3rd, et al. (2017). Perspectives on fear generalization and its implications for emotional disorders. *Journal of Neuroscience Research, 95*(3), 821–835.
Kandel, E. R. (2001). The molecular biology of memory storage: A dialogue between genes and synapses. *Science, 294*(5544), 1030–1038.
Kasai, H., Matsuzaki, M., et al. (2003). Structure-stability-function relationships of dendritic spines. *Trends in Neurosciences, 26*(7), 360–368.
Kim, J., Lee, S., et al. (2007). Amygdala depotentiation and fear extinction. *Proceedings of the National Academy of Sciences of the United States of America, 104*(52), 20955–20960.
Kjelstrup, K. G., Tuvnes, F. A., et al. (2002). Reduced fear expression after lesions of the ventral hippocampus. *Proceedings of the National Academy of Sciences of the United States of America, 99*(16), 10825–10830.
Koh, I. Y., Lindquist, W. B., et al. (2002). An image analysis algorithm for dendritic spines. *Neural Computation, 14*(6), 1283–1310.
Lee, J. L., Milton, A. L., et al. (2006). Reconsolidation and extinction of conditioned fear: Inhibition and potentiation. *Journal of Neuroscience, 26*(39), 10051–10056.
Leuner, B., Falduto, J., et al. (2003). Associative memory formation increases the observation of dendritic spines in the hippocampus. *Journal of Neuroscience, 23*(2), 659–665.
Magarinos, A. M., McEwen, B. S., et al. (1996). Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shrews. *Journal of Neuroscience, 16*(10), 3534–3540.
Maletic-Savatic, M., Malinow, R., et al. (1999). Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science, 283*(5409), 1923–1927.
Matsuzaki, M., Honkura, N., et al. (2004). Structural basis of long-term potentiation in single dendritic spines. *Nature, 429*(6993), 761–766.
McEwen, B. S. (2007). Physiology and neurobiology of stress and adaptation: Central role of the brain. *Physiological Reviews, 87*(3), 873–904.
McNally, R. J. (2007). Mechanisms of exposure therapy: How neuroscience can improve psychological treatments for anxiety disorders. *Clinical Psychology Review, 27*(6), 750–759.
Murphy, D. D., & Segal, M. (1996). Regulation of dendritic spine density in cultured rat hippocampal neurons by steroid hormones. *Journal of Neuroscience, 16*(13), 4059–4068.
Nimchinsky, E. A., Sabatini, B. L., et al. (2002). Structure and function of dendritic spines. *Annual Review of Physiology, 64*, 313–353.
Pavlov, I. P. (1927). *Conditioned reflexes.* London: United Kingdom, Oxford University Press.
Paxinos, G., & Watson, C. (2007). *The rat brain in stereotaxic coordinates.* San Diego: Academic Press.
Peters, A., & Palay, S. L. (1996). The morphology of synapses. *Journal of Neurocytology, 25*(12), 687–700.
Petersen, E. N., Braestrup, C., et al. (1985). Evidence that the anticonflict effect of midazolam in amygdala is mediated by the specific benzodiazepine receptors. *Neuroscience Letters, 53*(3), 285–288.
Pozzo-Miller, L. D., Inoue, T., et al. (1999). Estradiol increases spine density and NMDA-dependent Ca2+ transients in spines of CA1 pyramidal neurons from hippocampal slices. *Journal of Neurophysiology, 81*(3), 1404–1411.
Restivo, L., Vetere, G., et al. (2009). The formation of recent and remote memory is associated with time-dependent formation of dendritic spines in the hippocampus and anterior cingulate cortex. *Journal of Neuroscience, 29*(25), 8206–8214.
Rodriguez Manzanares, P. A., Isoardi, N. A., et al. (2005). Previous stress facilitates fear memory, attenuates GABAergic inhibition, and increases synaptic plasticity in the rat basolateral amygdala. *Journal of Neuroscience, 25*(38), 8725–8734.
Roozendaal, B., McEwen, B. S., et al. (2009). Stress, memory and the amygdala. *Nature Reviews Neuroscience, 10*(6), 423–433.
Segal, M., & Andersen, P. (2000). Dendritic spines shaped by synaptic activity. *Current Opinion in Neurobiology, 10*(5), 582–586.
Shors, T. J., Chua, C., et al. (2001). Sex differences and opposite effects of stress on dendritic spine density in the male versus female hippocampus. *Journal of Neuroscience, 21*(16), 6292–6297.
Siegel, A., & Tassoni, J. P. (1971). Differential efferent projections from the ventral and dorsal hippocampus of the cat. *Brain, Behavior and Evolution, 4*(3), 185–200.
Sierra-Mercado, D., Padilla-Coreano, N., et al. (2011). Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. *Neuropsychopharmacology, 36*(2), 529–538.
Sousa, N., Lukoyanov, N. V., et al. (2000). Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement. *Neuroscience, 97*(2), 253–266.
Swanson, L. W., & Cowan, W. M. (1977). An autoradiographic study of the organization of the efferent connections of the hippocampal formation in the rat. *The Journal of Comparative Neurology, 172*(1), 49–84.
Tyler, W. J., & Pozzo-Miller, L. (2003). Miniature synaptic transmission and BDNF modulate dendritic spine growth and form in rat CA1 neurones. *Journal of Physiology, 553*(Pt 2), 497–509.
Vetere, G., Restivo, L., et al. (2011). Extinction partially reverts structural changes associated with remote fear memory. *Learning Memory, 18*(9), 554–557.
Vyas, A., Jadhav, S., et al. (2006). Prolonged behavioral stress enhances synaptic connectivity in the basolateral amygdala. *Neuroscience, 143*(2), 387–393.
Vyas, A., Mitra, R., et al. (2002). Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *Journal of Neuroscience, 22*(15), 6810–6818.
Watanabe, Y., Gould, E., et al. (1992). Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. *Brain Research, 588*(2), 341–345.
Wilber, A. A., Walker, A. G., et al. (2011). Chronic stress alters neural activity in medial prefrontal cortex during retrieval of extinction. *Neuroscience, 174*, 115–131.
Yuste, R., & Denk, W. (1995). Dendritic spines as basic functional units of neuronal integration. *Nature, 375*(6533), 682–684.
Yuste, R., Majewska, A., et al. (2000). From form to function: Calcium compartmentalization in dendritic spines. *Nature Neuroscience, 3*(7), 653–659.
Zhou, Q., Homma, K. J., et al. (2004). Shrinkage of dendritic spines associated with long-term depression of hippocampal synapses. *Neuron, 44*(5), 749–757.