RAPID COMMUNICATION

Hippocampal Dendritic Spines Remodeling and Fear Memory Are Modulated by GABAergic Signaling Within the Basolateral Amygdala Complex

Marcelo Giachero, Gaston D. Calfa,* and Victor A. Molina*

ABSTRACT: GABAergic signaling in the basolateral amygdala complex (BLA) plays a crucial role on the modulation of the stress influence on fear memory. Moreover, accumulating evidence suggests that the dorsal hippocampus (DH) is a downstream target of BLA neurons in contextual fear. Given that hippocampal structural plasticity is proposed to provide a substrate for the storage of long-term memories, the main aim of this study is to evaluate the modulation of GABA neurotransmission in the BLA on spine density in the DH following stress on contextual fear learning. The present findings show that prior stressful experience promoted contextual fear memory and enhanced spine density in the DH. Intra-BLA infusion of midazolam, a positive modulator of GABAa sites, prevented the facilitating influence of stress on both fear retention and hippocampal dendritic spine remodeling. Similarly to the stress-induced effects, the blockade of GABAa sites within the BLA ameliorated fear memory emergence and induced structural remodeling in the DH. These findings suggest that GABAergic transmission in BLA modulates the structural changes in DH associated to the influence of stress on fear memory. © 2015 Wiley Periodicals, Inc.

KEY WORDS: dendritic spines; fear memory; stress; dorsal hippocampus; basolateral amygdale

INTRODUCTION

There is growing consensus that stress affects several distinct cognitive processes (Roozendaal et al., 2009) and it is widely accepted that threatening experiences promote emotionally arousing memories, including fear memory (Cordero et al., 2003). Particularly, a stressful experience such as a restraint event has the specific characteristic to facilitate the emergence of a robust and persistent contextual fear memory following a single training trial, though this one by itself is incapable to yield a fully fear response at testing in unstressed rats (Maldonado et al., 2011, 2014; Giachero et al., 2013a,b).

A number of studies have shown that stress exposure affects the inhibitory GABAergic system within the basolateral amygdala complex (BLA) (Martijena et al., 2002; Roozendaal et al., 2009; Hadad-Ophir et al., 2014). In addition, the GABAergic neurotransmission in this brain area plays a major role on the influence of emotional arousing stimuli on fear memory formation (Rodriguez Manzanares et al., 2005). In fact, an acute restraint session elicits BLA hyperexcitability, consequential of the attenuation of recurrent GABA inhibition (Isoardi et al., 2007). What is more, the influence of stress on associative fear memory coincides with the stress-dependent facilitation of long-term potentiation (LTP) in BLA (Rodriguez Manzanares et al., 2005; Suvrathan et al., 2014). A similar facilitating effect on the emergence of fear memory is induced following the intra-BLA administration of bicuculline (BIC), a GABAa receptor antagonist (Rodriguez Manzanares et al., 2005). In line with these findings, systemic or intra-BLA midazolam (MDZ) infusion, a positive modulator of GABAa sites, prevents the facilitating influence of stress on fear memory formation (Rodriguez Manzanares et al., 2005; Maldonado et al., 2011; Giachero et al., 2013a) and on LTP generation in the BLA (Rodriguez Manzanares et al., 2005). Collectively, these findings support the view that the GABAergic neurotransmission in BLA plays a major role on the modulation of stress on fear memory.

The BLA is reciprocally connected with the hippocampus (Pikkarainen et al., 1999; Pitkanen et al., 2000; Petrovich et al., 2001). Moreover, there is molecular and physiological evidence of a functional interaction between both brain areas (Seidenbecher et al., 2003; McIntyre et al., 2005; Huff et al., 2006).

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Besides, both areas are part of a brain circuitry critically involved in the formation of contextual fear memory (Zelikowsky et al., 2014), where the hippocampus, particularly its dorsal part (dorsal hippocampus, DH), plays a pivotal role in the contextual representation following contextual fear conditioning (Kim and Fanselow, 1992). In turn, the BLA is critically involved in the attachment of the emotional significance to the incoming stimuli that is transmitted to the DH to form a contextual representation with relevant emotional valence (Richter-Levin and Akirav, 2003). Furthermore, some authors propose that the BLA modulates the storage memory process in the hippocampus, among other brain regions (McGaugh, 2004). Similarly, the hippocampus has been proposed to be a downstream target of BLA neurons in contextual fear (Kim et al., 1993; Maren and Fanselow, 1997).

It is well established that dendritic spines express the structural platform for excitatory synaptic contacts between neurons (Yuste and Denk, 1995). Besides, hippocampal structural plasticity, in the form of changes in spine density following learning, provides a substrate for the storage of long-term memories, including contextual fear memories (Kandel, 2001; Restivo et al., 2009; Giachero et al., 2013b) although to date, no studies report whether stress-induced changes on the GABAergic transmission in BLA affect the structural rearrangement in DH associated with fear memory.

To address this issue, this study examines the influence of stress-induced changes on the GABAergic transmission within the BLA on the hippocampal spine density associated with long-term contextual fear memory.

RESULTS

CA1 Hippocampal Structural Rearrangement is Associated With Contextual Fear Memory Following a Weak Fear Conditioning Procedure in Stressed Animals

To investigate whether a single stress exposure before a weak fear-conditioning protocol induces structural plasticity in DH, a total of 42 animals were randomly subjected to a single restraint stress session (S) or just handled (nonstress, NS). The next day, the animals were placed in the conditioning chamber (Cs) and received a single unsignaled footshock (Us) (Cs-Us, week training procedure) or remained in the chamber without the footshock (Cs-noUs) experience. The freezing behavior in response to a subsequent Cs exposure was evaluated 1 and 6 days later. In another set of experiments, the animals were sacrificed 1 day after conditioning for dendritic spine analysis (Fig. 1A; see detailed methods). Thus, the experimental groups were as follows: NS/Cs-noUs; NS/Cs-Us; S/Cs-noUs; and S/Cs-Us.

During conditioning, at the preshock period, animals displayed a low level of freezing with no significant difference between the experimental groups (two-way ANOVA analysis, $P > 0.05$). During the postshock period, animals that received the shock, regardless of the restraint exposure, presented a higher amount of freezing in comparison to nonshocked animals (two-way ANOVA analysis, $P < 0.05$).

During testing (test 1 and test 2), the fear memory in animals subjected to the weak fear training was only evident in animals previously exposed to a stress experience (Fig. 1B). A repeated measures ANOVA for the percentage of time spent freezing revealed a significant effect of stress ($F(1,26) = 48.562$, $P = 0.00001$), conditioning ($F(1,26) = 47.183$, $P = 0.00001$), and stress $\times$ conditioning ($F(1,26) = 36.023$, $P = 0.00001$). This significant statistical information revealed that the freezing behavior responses observed at both, test 1 and test 2, were significantly elevated in stressed and conditioned animals ($P < 0.05$, Bonferroni post hoc test). In addition, nonstressed conditioned animals exhibited a similar freezing response in comparison to control animals (NS/Cs-noUs and S/Cs-noUs) at test 1 and test 2 ($P > 0.05$, Bonferroni post hoc test).

For CA1 hippocampal structural plasticity (Fig. 1A), the animals were distributed into the four mentioned groups in which spine counts were performed on a total of 101 dendritic segments as follows: NS/Cs-noUs: $n = 20$ segments, 730.48 μm total dendritic length analyzed, three rats; NS/Cs-Us: $n = 24$ segments, 831.2 μm, three rats; S/Cs-noUs: $n = 23$ segments, 881.09 μm, three rats; S/Cs-Us: $n = 24$ segments, 836.92 μm, three rats). Figure 1C shows representative examples of the different dendritic segments in the DH *stratum radiatum* CA1 for each particular experimental group.

The results from the dendritic spine analysis showed a higher density of dendritic spines in CA1 hippocampal area only in the stressed animals that had been subjected to the weak training procedure. The analysis of the cumulative probability distributions for the total density of dendritic spines reflected a significant rightward shift toward higher numbers in animals S/Cs-Us in comparison to the rest of the experimental groups [$P < 0.05$ for each individual comparison, Kolmogorov–Smirnov (KS) test; Fig. 1D]. This shift also resulted in a higher median (quartiles; total density/10 μm) in S/Cs-Us 16.17 (15.78–16.71) with respect to the rest of the groups: NS/Cs-noUs: 12.36 (11.89–12.87), NS/Cs-Us: 12.33 (11.62–12.91), and S/Cs-noUs: 12.34 (11.93–13.18) (Kruskal–Wallis test = 50.627; $P < 0.001$; multiple comparison of mean ranks post hoc test, $P < 0.001$). A similar rightward shift toward a higher density in S/Cs-Us animals compared with the rest of the experimental groups was observed for mature dendritic spines ($P < 0.05$ for each individual comparison, KS test; Fig. 1E). In a similar manner, a higher median (quartiles; mature dendritic spine density/10 μm) in S/Cs-Us: 13.04 (11.68–13.78) was observed in comparison to the rest of the experimental groups: NS/Cs-noUs: 9.97 (8.59–10.59), NS/Cs-Us: 9.60 (9.01–10.57), and S/Cs-noUs: 10.18 (8.70–10.69) (Kruskal–Wallis test = 33.61; $P < 0.001$; multiple comparison of mean ranks post hoc, $P < 0.001$). The higher density of total dendritic spines was also evident for thin dendritic spines, in which a rightward shift to greater density was also observed in

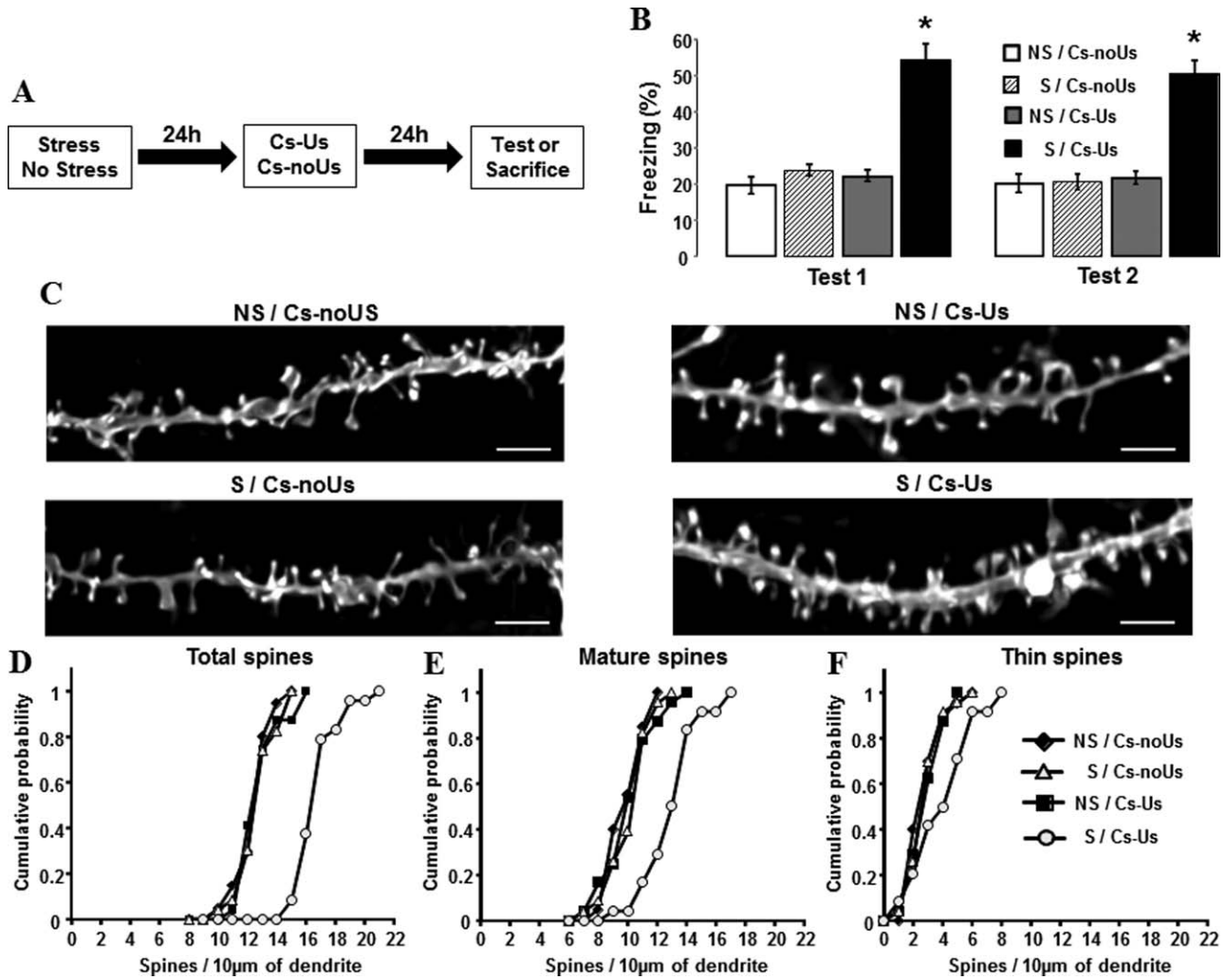

FIGURE 1. A single stress session before the weak fear conditioning protocol induces CA1 hippocampal dendritic spine remodeling and enhances fear memory. (A) Schematic representation of the experimental design. (B) Bar graph showing the freezing behavior response during the test 1 and the test 2 sessions. Data are expressed as mean ± SEM of the percentage of time spent freezing during the tests ($n = 7$–8 rats per group). *$P < 0.05$ compared with the rest of the experimental groups (repeated measures 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 ($n = 3$ rats per group). Bar scale: 2 µm. (D–F) Cumulative frequency of total (D), mature (E), and thin (F) dendritic spine density on apical dendrites of hippocampal CA1 pyramidal cells ($P < 0.05$, Kolmogorov–Smirnov test).

S/Cs-Us animals compared with the rest of the experimental groups ($P < 0.05$ for each individual comparison, KS test; Fig. 1F). However, differences between median (quartiles; thin dendritic spine density/10 µm) were not statistically significant [S/Cs-Us: 3.97 (2.18–5.12), NS/Cs-noUs: 2.52 (1.69–3.40), NS/Cs-Us: 2.51 (1.94–3.33), and S/Cs-noUs: 2.41 (1.99–3.20)] (Kruskal–Wallis test = 6.99; $P = 0.0721$).

Intra-BLA MDZ Administration Prevents the Effects of Stress on Both Fear Memory and Structural Plasticity in CA1 Hippocampal Area

To prevent the stress influence on fear memory and on the concomitant structural rearrangement in CA1 hippocampal area, a total of 40 animals were bilaterally cannulated in BLA (Fig. 2B), and randomly infused with MDZ or SAL 10 min before stress exposure (S) or control manipulation (NS). One day later, all animals were placed in the conditioning chamber (Cs) and subjected to a single unsignaled footshock (Us). The freezing behavior to a subsequent Cs exposure was evaluated 1 and 6 days later. An additional group of rats was sacrificed 24 h after conditioning for dendritic spine analysis (Fig. 2A). Thus, the experimental groups were as follows: SAL/S; SAL/NS; MDZ/S; and MDZ/NS (see detailed methods).

During conditioning, at the preshock period animals displayed a low level of freezing with no significant difference between the experimental groups (two-way ANOVA analysis,

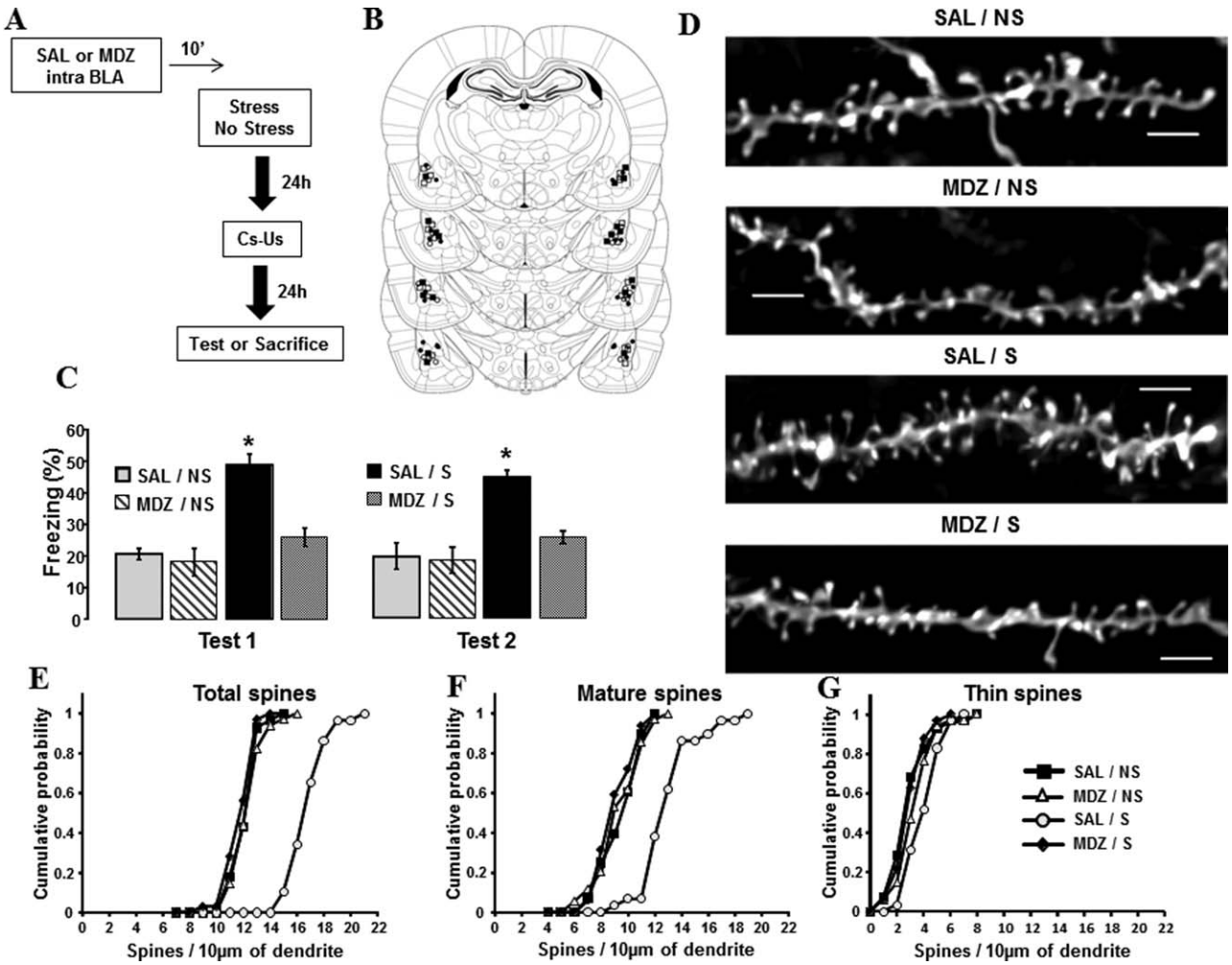

FIGURE 2. MDZ intra-BLA before stress prevents both CA1 hippocampal dendritic spines boost and fear memory. (A) Schematic representation of the experimental design. (B) Schematic drawings of coronal sections showing the location of the cannula placement in BLA (adapted from Paxinos and Watson, 2007): ■ SAL/NS; □ MDZ/NS; ● SAL/S; and ○ MDZ/S. (C) Bar graph showing the freezing behavior response during the test 1 and test 2. Data are expressed as mean ± SEM of the percentage of freezing spent during the test ($n = 7$–8 rats per group). *$P < 0.05$ compared with the rest of the experimental groups (repeated measures ANOVA, Bonferroni post hoc test). (D) Representative examples of apical dendritic segments of CA1 pyramidal neurons (*stratum radiatum*) which were selected for quantitative analysis of dendritic spines ($n = 3$ rats per group). Scale bar: 2 µm. (E–G) Cumulative frequency of total (E), mature (F), and thin (G) spine density on apical dendrites of hippocampal CA1 pyramidal cells ($P < 0.05$, Kolmogorov–Smirnov test).

$P > 0.05$). During the postshock period, the animals presented a higher amount of freezing regardless of the SAL or MDZ treatment (two-way ANOVA analysis, $P > 0.05$).

During testing (test 1 and test 2) the higher freezing observed in SAL stressed rats was prevented by intra-BLA MDZ before stress (Fig. 2C). A repeated measures ANOVA for the percentage of time spent freezing revealed a significant effect of the treatment ($F(1,24) = 18.773$, $P = 0.00023$), the stress ($F(1,24) = 42.541$, $P < 0.001$), and treatment × stress ($F(1,24) = 12.797$, $P = 0.00152$). Thus, the statistical analysis shows a higher freezing behavior in SAL stressed animals in comparison to the rest of the experimental groups ($P < 0.05$, Bonferroni post hoc test; Fig. 2C).

For spine density analysis, a set of the animals were randomly distributed into the four groups, where spine counts were performed on a total of 123 dendritic segments as follows: SAL/NS: $n = 28$ segments, 853.11 µm total dendritic length analyzed, three rats; SAL/S: 29 segments, 928.61 µm, three rats; MDZ/NS: 34 segments, 1085.91 µm, three rats; MDZ/S: 32 segments, 972.89 µm, three rats. Figure 2D shows representative examples of the different dendritic segments in the *stratum radiatum* CA1 hippocampal area for each particular experimental group.

The results from the dendritic spine analysis show that the activation of GABAa sites by MDZ in BLA before stress exposure prevented the remodeling in the CA1 hippocampal area of

fear-trained animals. The analysis of the cumulative probability distributions for the total density of dendritic spines revealed a significant rightward shift toward higher number of total spines in SAL/S animals when compared with the rest of the experimental groups ($P < 0.05$ for each individual comparison, KS test; Fig. 2E). Interestingly, MDZ/S animals presented a cumulative probability distribution that is comparable to SAL- or MDZ-treated nonstressed animals ($P > 0.05$ for each individual comparison, KS test; Fig. 2E). This shift was also evident when comparing the median (quartiles; mature spine density/10 μm) of SAL/S: 16.39 (15.58–17.14) with respect to the other experimental groups: SAL/NS: 12.18 (11.41–12.73); MDZ/NS: 12.19 (11.57–12.76); and MDZ/S: 11.49 (10.88–12.36) (Kruskal–Wallis test = 67.412; $P = 0.00001$; multiple comparison of mean ranks post hoc, $P < 0.001$). Similar to total dendritic spines, MDZ intra-BLA prevented the increased number of mature dendritic spines induced by both stress exposure and fear training. The cumulative probability distributions for the density of mature dendritic spines reflected a significant rightward shift toward a higher number in SAL/S animals in comparison with the rest of the experimental groups ($P < 0.05$ for each individual comparison, KS test; Fig. 2F). MDZ-treated animals presented a cumulative probability distributions comparable to the nonstressed animals either SAL or MDZ infused ($P > 0.05$ for each particular comparison, KS test; Fig. 2F). This shift was also evident when compared the median (quartiles; mature spine density/10 μm) of SAL/S: 12.65 (11.73–13.2) vs. SAL/NS: 9.28 (8.10–10.37), MDZ/NS: 8.86 (8.02–10.49); and MDZ/S: 8.61 (7.66–10.12) (Kruskal–Wallis test = 53.81; $P = 0.00001$; multiple comparison of mean ranks post hoc, $P < 0.001$). The analysis of thin dendritic spines resulted in a rightward shift toward higher density of thin spines in SAL/S animals compared with SAL/NS ($P = 0.0132$, KS test; Fig. 2G). Similarly, a rightward shift was observed in SAL/S in comparison with MDZ/S ($P = 0.0051$, KS test; Fig. 2E). No significant differences were detected for the rest of the experimental groups' comparisons ($P > 0.05$ for each individual comparison, KS test). The same significant effect was also evident for median (quartiles; thin spine density/10 μm) analysis: SAL/NS: 2.68 (1.85–3.25), SAL/S: 3.77 (2.8–4.67); MDZ/NS: 3.07 (2.19–3.25); MDZ/S: 2.7 (2.2–3.51) (Kruskal–Wallis test = 14.50; $P = 0.0023$; multiple comparison of mean ranks post hoc, $P < 0.001$).

GABAa Sites' Blockade in BLA Prior to the Weak Fear Conditioning Induced Hippocampal Rearrangement Associated With Long-Term Fear Memory

We next evaluated whether GABAa sites' blockade by BIC intra-BLA induces a facilitating effect on both fear memory and hippocampal structural changes, presumably associated with long-term fear memory.

A total of 40 animals were bilaterally cannulated in BLA (Fig. 3B), and randomly BIC or SAL infused. Ten minutes later, half of the animals was subjected to conditioning (Cs-Us) and the other half was exposed to the training chamber (Cs) without footshock (Cs-noUs). Thus, the experimental groups were as follows: SAL/Cs-noUs; SAL/Cs-Us; BIC/Cs-noUs; and BIC/Cs-Us (see detailed methods). One and 6 days later, a group of animals was re-exposed to the context for behavioral testing. The rest of the animals were sacrificed for dendritic spine analysis 1 day after conditioning (Fig. 3A).

During conditioning, at the preshock period animals displayed a low level of freezing with no significant difference between the experimental groups (two-way ANOVA analysis, $P > 0.05$). During the postshock period, animals that received the shock, regardless of the BIC or SAL treatment, presented a higher amount of freezing in comparison to nonshocked animals (two-way ANOVA analysis, $P < 0.05$).

During testing (test 1 and test 2), the freezing behavior exhibited by conditioned intra-BLA BIC animals was higher than the freezing behavior displayed by the rest of the experimental groups (Fig. 3C). A repeated ANOVA analysis revealed a significant effect of the treatment ($F(1, 24) = 29.100$, $P = 0.00002$), the conditioning ($F(1, 24) = 47.699$, $P = 0.000001$), and the treatment × conditioning interaction ($F(1, 24) = 39.351$, $P = 0.000001$). Thus, the statistical analysis shows that the GABAa receptor blockade before conditioning facilitates fear memory formation, effect that resembles the influence of the stress exposure.

We next evaluated the dendritic spine density in the CA1 hippocampal area resulting from this manipulation (Fig. 3A). Spine counts were performed on a total of 113 dendritic segments as follows: SAL/Cs-noUs: $n = 26$ segments, 843.85 μm total dendritic length analyzed, three rats; SAL/Cs-Us 26 segments, 808.55 μm, three rats; BIC/Cs-noUs 30 segments, 948.35 μm, three rats; BIC/Cs-Us 31 segments, 1100.67 μm, three rats. Figure 3D shows representative examples of the different dendritic segments in the *stratum radiatum* CA1 hippocampal area for each particular experimental group.

The results from the dendritic spine analysis show that intra-BLA BIC combined with fear training promoted a higher number of total dendritic spines, particularly mature ones, in CA1 hippocampal area. The analysis of the cumulative probability distributions for the total density of dendritic spines reflected a significant rightward shift toward a higher number of total spines in animals Bic/Cs-Us than in the rest of the experimental groups ($P < 0.05$ for each individual comparison, KS test; Fig. 3E). This shift also resulted in a higher median (quartiles; total density/10 μm) in BIC/Cs-Us [17.22 (16.35–18.18)] with respect to the rest of the experimental groups: SAL/Cs-noUs: 12.07 (11.48–12.68); BIC/Cs-noUs: 12.04 (10.52–16.52); and SAL/Cs-Us: 11.93 (11.50–12.55) (Kruskal–Wallis test = 53.07142; $P < 0.001$; multiple comparison of mean ranks post hoc, $P < 0.001$). For mature dendritic spines, a similar rightward shift toward a higher density in BIC/Cs-Us animals than in the rest of the experimental groups was detected ($P < 0.05$ for each individual comparison, KS test; Fig. 3F). Besides, SAL/Cs-Us animals presented a rightward shift when compared with SAL/Cs-noUs animals ($P = 0.0128$, KS test). This result was evident in the analysis of the median (quartiles; mature spine density/10 μm) in which BIC/Cs-Us [12.19 (10.78–13.60)] exhibited a higher density than in the rest of the groups: SAL/Cs-noUs: 7.89 (6.78–9.30);

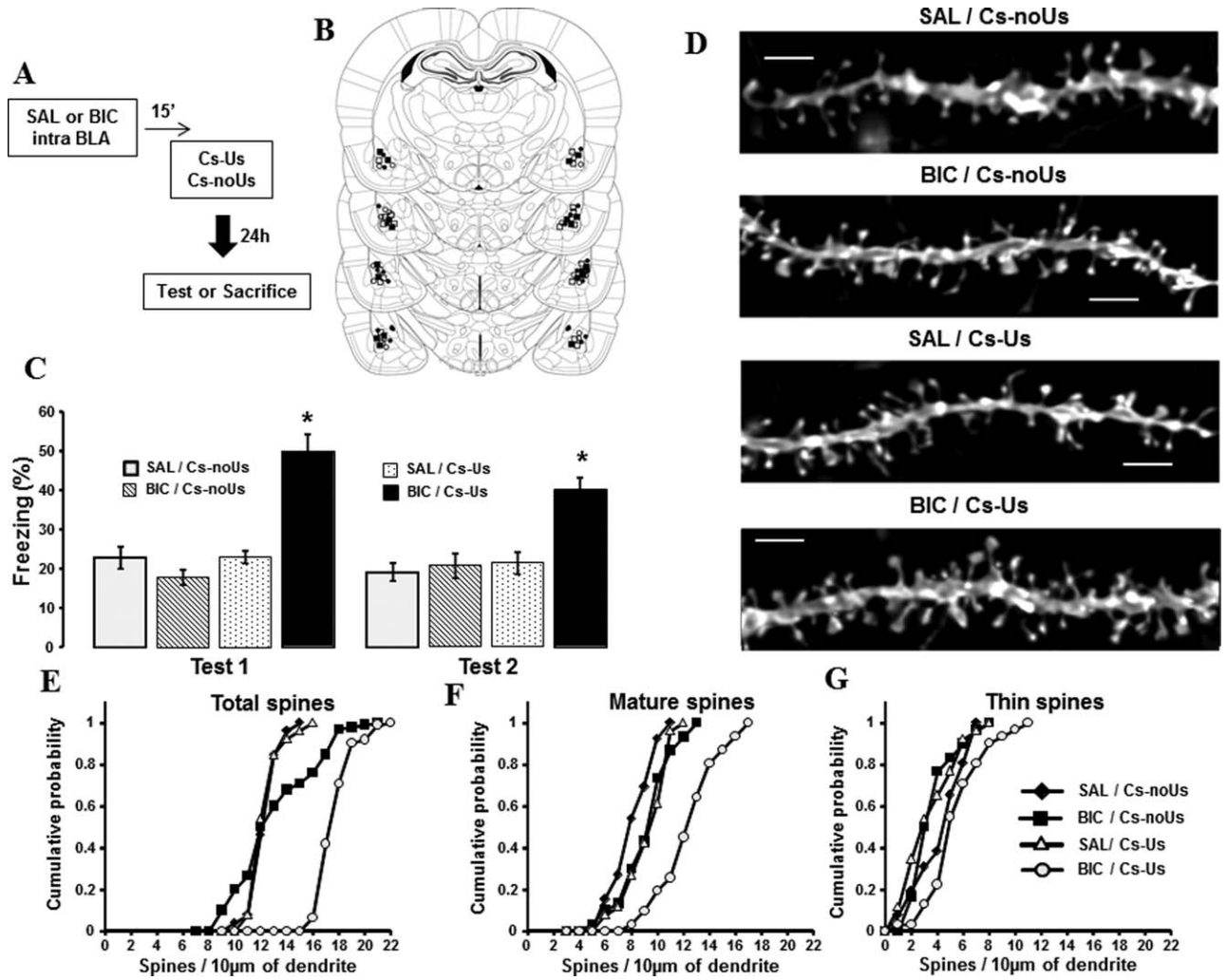

FIGURE 3. The blockade of GABAa sites in BLA previous to the weak fear conditioning protocol facilitates both CA1 hippocampal dendritic spines remodeling and fear memory. (A) Schematic representation of the experimental design. (B) Schematic drawings of coronal sections showing the location of the cannula placement in BLA (adapted from Paxinos and Watson, 2007): ■ SAL/Cs-noUs; □ BIC/Cs-noUs; ● SAL/Cs-Us; and ○ BIC/Cs-Us. (C) Bar graph showing the freezing response during the test 1 and the test 2. Data are expressed as mean ± SEM of the percentage of freezing spent during the test ($n = 7$–8 rats per group). *$P < 0.05$ compared with the rest of the experimental groups (repeated measures ANOVA, Bonferroni post hoc test). (D) Representative examples of apical dendritic segments of CA1 pyramidal neurons which were selected for quantitative analysis of dendritic spines ($n = 3$ rats per group). Scale bar: 2 µm. (E–G) Cumulative frequency of total (E), mature (F), and thin (G) spine density on apical dendrites of hippocampal CA1pyramidal cells ($P < 0.05$, Kolmogorov–Smirnov test).

BIC/Cs-noUs: 9.26 (7.93–10.12); and SAL/Cs-Us: 9.77 (7.86–10.04) (Kruskal–Wallis test = 46.9124; $P < 0.001$; multiple comparison of mean ranks post hoc, $P < 0.001$). The multiple comparisons of mean ranks for all groups revealed a nonsignificant difference between SAL/Cs-Us and SAL/Cs-noUs. The analysis of the cumulative probability distributions for thin dendritic spines showed a significant rightward shift toward a higher number of total thin spines in BIC/Cs-Us than in SAL/Cs-Us and BIC/Cs-noUs ($P < 0.05$ for both comparison, KS test; Fig. 3G). Besides, BIC/Cs-noUs presented a cumulative probability distribution with a left shift toward lesser number of spines than SAL/Cs-noUs ($P < 0.05$, KS test; Fig. 3F). Those changes were partially reflected in the median (quartiles; thin spine density/10 µm) comparisons: BIC/Cs-Us: 4.91 (4.03–6.52); SAL/Cs-noUs: 4.28 (2.29–5.89); BIC/Cs-noUs: 2.95 (2.20–3.85); and SAL/Cs-Us: 2.61 (1.56–4.55) (Kruskal–Wallis test = 17.7193; $P = 0.0005$). The multiple comparison of mean ranks for all groups revealed a significant difference between Bic/Cs-Us animals and the rest of the experimental groups ($P < 0.001$).

DISCUSSION

Previously stressed rats exposed to a single context-shock experience exhibited a robust freezing behavior during test 1

and test 2, indicating that a stress experience promotes the formation of a long-lasting fear memory. Such memory has been reported to be crucially dependent on context-shock association rather than to an unspecific generalization or to a sensitized response to the shock due to the prior restraint experience (Maldonado et al., 2011, 2014). Stress, by reducing the inhibitory GABAergic control on BLA-projecting neurons (Isoardi et al., 2007), facilitates LTP generation in BLA and enhances fear learning (Rodriguez Manzanares et al., 2005). What is more, stress-induced facilitation on fear memory using a weak training protocol was significantly attenuated by intra-BLA MDZ pretreatment, a result that strengthens this view. It is noteworthy that intra-BLA MDZ prevents the induction of LTP in BLA neurons (Rodriguez Manzanares et al., 2005). Accordingly, we demonstrated that the selective blockade of GABAa receptors within BLA before the context-shock association facilitated fear memory. In fact, such facilitation is similar to the one observed in stressed animals. Hence, our findings complement and extend previous studies supporting a critical role of the GABAergic signaling within the BLA on the influence of stress-induced contextual fear memory.

Consistent with our current behavioral findings, prior stress exposure in fear-trained rats enhanced the total density of dendritic spines in DH. Importantly, dendritic spines are the structural loci for the majority of the excitatory synapses on the mammalian CNS (Yuste, 2011) and their particular morphology influences its functional properties (Matsuzaki et al., 2001; Yuste and Bonhoeffer, 2001). In this line, spine enlargement has been associated with LTP (Matsuzaki et al., 2004) while spine shrinkage and posterior retraction has been observed in response to low-frequency stimulation (Zhou et al., 2004). Our results showed that both mature and thin dendritic spines presented a higher density in fear-trained stressed animals. It is important to indicate that neither the fear-conditioning protocol used in this work nor the stress exposure per se was able to induce an increment in the number of dendritic spines. Another study using a stronger stressor in rats (30-min restraint stress with the addition of tail shocks; Shors et al., 2001) reported an increment in the number of dendritic spines at CA1 DH. This difference in the stress protocol could induce a vast synaptic rearrangement with the observable consequences such as the increment in dendritic spines. The measurement of the stress effect by itself in this work was performed approximately 48 h after the end of the stressful experience (see Fig. 1 for a schematic detail of the experiment). Moreover, a shorter delay (24 h) (Giachero et al., 2013b) after a similar single stress session did not change the number or the morphology of dendritic spines in CA1 DH.

In addition to the absence of an effect following stress per se in synaptic remodeling, the weak fear-conditioning protocol was not able to induce an increment in the number of dendritic spines. This is consistent with a previous study where it was found that hippocampal CA1 structural remodeling accompanies the facilitated fear memory following the combination of a weak fear conditioning training and a later threatening experience (Giachero et al., 2013b). These results suggested that both the contextual fear memory and the remodeling of dendritic spines in DH are interrelated.

We do not discard any physiological change at the synapse level that can also support the expression of the fear memory after the animals have been exposed to the stressful experience, but a new set of electrophysiological experiments are needed to demonstrate such expression.

Thus, the present results highlight the fact that the formation, enlargement, and even dendritic spine maturation are relevant for the synaptic rearrangement by the ongoing synaptic activity, which is assumed to contribute to the expression of the fear memory.

Our findings are supported by previous studies that have demonstrated that the emergence of a long-term contextual fear memory is associated with dendritic spines rearrangement in the hippocampus (Leuner et al., 2003; Restivo et al., 2009; Giachero et al., 2013b). Importantly, MDZ intra-BLA prevented the effect of stress exposure on the hippocampal structural synaptic remodeling associated with fear retention. In addition, the local infusion of the selective antagonist of GABAa sites, BIC, in BLA before the training experience induced hippocampal dendritic spine remodeling comparable to the one promoted by stress. In this line, it is interesting to remark that the cumulative distribution of total spines in BIC-treated animals without further conditioning seems to present a widespread distribution (Fig. 3E) in comparison to the other experimental groups and to the stress application alone. However, applying a nonhierarchical clustering method (*k*-means) in these results, it was possible to observe a nonclustering division ($P > 0.05$), meaning that one group of data was obtained. However, we cannot fully discard any particular synaptic rearrangement unobservable under these conditions that can be induced by the hyperactivity generated by the BIC intra-BLA administration.

Collectively, our findings suggest that the hippocampal structural remodeling associated with stress-induced memory formation is, at least in part, under the modulation of the GABAergic neurotransmission in BLA.

The functional interaction between the amygdala complex and the DH on the dynamics involved in establishing a persistent fear memory trace has been extensively studied (McGaugh, 2004; Hermans et al., 2014). Synchronization of theta activity in the amygdalo-hippocampal network has been reported following conditioned fear (Seidenbecher et al., 2003). Besides, single or chronic stress exposures affect the synchronized oscillatory activity between the CA1 and CA3 hippocampal area and the lateral amygdala (LA). Granger causality spectra revealed a strong directional influence from the LA to CA1 that persisted throughout and even 10 days after chronic stress (Ghosh et al., 2013), suggesting a growing dominance of amygdalar activity over the hippocampus during and after chronic stress. In addition, the hippocampal formation integrates contextual stimuli with information about the unconditioned stimulus coming from the BLA via the entorhinal cortex (Sparta et al., 2014). Furthermore, BLA regulates the expression of immediate-early gene in DH, related to memory

formation after contextual fear conditioning, because the inactivation of BLA prevents the increase of hippocampal Arc protein and c-fos mRNA (McIntyre et al., 2005; Huff et al., 2006). Interestingly, it has been suggested that Arc mRNA is enriched near the synapses that were stimulated (Steward et al., 1998; Bramham et al., 2008). Finally, Arc protein enhancement was reported to occur in dendritic spines and critically involved in LTP (Plath et al., 2006).

In conclusion, at the level of neuronal network, our findings extend the notion that the structural changes in DH provide a substrate for memory retention. In addition, although the modulatory role of BLA is not relevant in the retention of neutral context, it has been suggested to be crucial in the formation of emotionally arousing memories (Zelikowsky et al., 2014). What is more, this study highlights the pivotal role of the BLA GABAergic mechanism on the influence of stress on hippocampal structural plasticity and fear memory.

DETAILED METHODS

Animals

Adult male Wistar rats (60 days old, weighing between 280 and 320 g) from a colony established at the Departamento de Farmacología-IFEC, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, were used. All animals were housed in standards laboratory Plexiglas cages in groups of three per cage. Animals were maintained on standard conditions (12 h light/dark cycle; lights on at 0700–1900 h); room temperature 21–23°C; food and water ad libitum. All the experimental protocols used in this work were approved by the Animal Care Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, which are consistent with the standards outlined in the NIH Guide for the Care and Use of Laboratory Animals. The number of animals used, as well as their suffering, was kept at the minimum possible needed to accomplish the goals of this study.

Stressor

Animals were transferred in their own home cages to an experimental room, and located for 30 min inside a plastic cylindrical restrainer fitted close to the body, preventing animal movement except for the tail and the tip of the nose. At the end of the stress session, animals were returned to the colony room. No other subjects were present in the experimental room during stress exposure (Giachero et al., 2013b). Fear conditioning was performed 1 day after stress exposure. Control unstressed animals were transferred to the experimental room, gently handled, and then returned to the colony room.

Conditioning Apparatus

The conditioning chamber (Cs) was made of gray plastic wall ($20 \times 23 \times 20$ cm^3) 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 available by ventilation fans and the shock scrambler (55 dB). The chambers were cleaned with 10% aqueous ethanol solution before and after each session. Experiments were always performed between 1400 and 1600 h with the experimenters unaware of the treatment condition.

Contextual Fear Conditioning

As previously described (Maldonado et al., 2011; Giachero et al., 2013b), on the day of the experiment, stressed or nonstressed 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: a single unsignaled footshock (0.3 mA; 3 s duration) (Cs-Us) or the rats remained in the conditioning chamber for the same period of time but did not receive the unsignaled footshock (Cs-noUs). The animals remained in the chamber for an additional 50 s (postshock period). At the end of this period, rats were removed and subsequently placed in their home cages. This manipulation elicits a minimal level of freezing at testing. In the experiments in which animals were BLA implanted, the intensity of the footshock was 0.5 mA in order to induce levels of conditioning similar to those exhibited by animals without cannulae implantation because chronic cannulation tends to attenuate the expression of conditioned freezing (Fanselow, 1980; Lee et al., 2006).

Test Sessions

Rats were re-exposed to the Cs without shocks for 3 min, 1 day (test 1), and 6 days after training (test 2). Freezing behavior was assessed as a measure of fear memory during test 1 and during test 2. One week before experiments rats were handled daily for ~60 s each. The behavior of each rat was continuously videotaped in order to score freezing behavior during the preshock and postshock period, and during the entire 3-min test sessions. The total time spent freezing in each period was quantified (in seconds) using a stopwatch and expressed as the percentage of total time. Freezing, a commonly used index of fear in rats (Blanchard and Blanchard, 1969; Fanselow, 1980), was defined as the total absence of body and head movement except those associated with breathing.

Surgery and Intracranial Infusions

Under aseptic conditions, 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 and Watson, 2007). These coordinates were established from pilot studies in our laboratory (Giachero et al., 2013a,b). The guide cannulas were secured in place using acrylic cement and two stainless-steel screws were anchored to the skull. Stainless "dummy cannulas" protruding 0.5 mm beyond the tips were placed inside the guide cannulas to prevent occlusion. After surgery, animals received a subcutaneous injection of a penicillin/streptomycin suspension to reduce the risk of infections. Animals were allowed to recover from surgery for 5–7 days before the experimental procedures. Microinfusions were made using 33-gauge infusion cannulas that extended 2 mm beyond the guide cannulas implanted in the BLA. The infusion cannulas were connected via polyethylene tubing (PE 10, Becton Dickinson, MD) to a 10 μl microsyringe (Hamilton, Reno, NV) mounted on a microinfusion pump (Cole-Parmer® 74900-Series).

Drugs and Drug Administration

MDZ (GobbiNovag, Buenos Aires, Argentina) was diluted in sterile isotonic saline (SAL, 0.9% w/v) and a dose of 1 μg/0.5 μl per side was used for intra-BLA administration 10 min before stress exposure.

BIC was dissolved and diluted in sterile isotonic saline (SAL, 0.9% w/v) and bilaterally infused at a dose of 10 pmol/0.5 μl per side 15 min before conditioning. The BIC dose selected is well below doses that have been found previously to induce convulsion and brain seizure activity (Turski et al., 1985; Sanders and Shekhar, 1991; Dickinson-Anson and McGaugh, 1997). Besides, this dose was previously reported to facilitate fear memory (Rodriguez Manzanares et al., 2005).

Structural Plasticity Analysis

Dendritic spine visualization and analysis was performed as previously reported by other researchers and by our laboratory (Tyler and Pozzo-Miller, 2003; Calfa et al., 2012; Giachero et al., 2013b). Concisely, under deeply anesthesia (chloral hydrate, 400 mg/kg i.p.), animals were transcardially perfused first by ice-cold PB (0.1 M, pH 7.4) and then fixed using ice-cold 4% *para*-formaldehyde (PFA) (in 0.1 M PB, pH 7.4). After brain removed and postfixed (4% PFA, 24 h, 4°C), coronal sections (200 μm thick) containing the DH were obtained with a vibratome and collected in 0.1% PBS. The CA1 DH was stained with 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 et al., 1999) by microinjection with a patch pipette and positive pressure application (Giachero et al., 2013b). Using a Fluoview FV-300 laser-scanning confocal microscope (Olympus IX81 inverted microscope) with a 60× oil immersion (NA 1.42) objective lens (PlanApo) from the Centro de Microscopía Óptica y Confocal de Avanzada, Córdoba, Argentina, z-sections from labeled dendritic segments were collected. 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 achieved manually using ImageJ software. Dendritic protrusions less than 3 μm length and contacting with the parent dendrite were considered for the analysis (Murphy and Segal, 1996; Chapleau et al., 2009; Calfa et al., 2012). Special consideration was taken to select a single dendritic segment, presumably from different neurons but from CA1 *stratum radiatum*, in lights of the high density of labeled dendrites. Thus, 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 that each spine was counted only once.

Spine types were classified as previously (Koh et al., 2002; Tyler and Pozzo-Miller, 2003; Boda et al., 2004): type I or "stubby"-shaped dendritic spines, type II or "mushroom"-shaped dendritic spines, and type III or "thin"-shaped dendritic spines. Different measurements were taken for each dendritic protrusion in order to classify them, in brief: 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, *d*n), and the diameter of the head (measured as the maximum head diameter, *d*h) (Koh et al., 2002). Thus, individual spines were included in each category based on the specific ratios *L*/*d*n and *d*h/*d*n (Koh et al., 2002; Tyler and Pozzo-Miller, 2003; Calfa et al., 2012; Giachero et al., 2013b).

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

Statistical Analysis

Behavioral experiments were analyzed by a two-way ANOVA or by a repeated-measure ANOVA, depending on the experimental design, followed by Bonferroni post hoc test. Data were expressed as mean ± SEM. For the dendritic spine 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 use cumulative frequency plots to measure shifts in the total number of dendritic spines, mature and thin dendritic spines per 10 μm of dendritic segment in the different experimental groups. Cumulative distribution probabilities were compared by KS test. Under this considerations, the total density of dendritic spines as well as mature and thin dendritic spines, no

significant differences were observed between rats from each particular experimental group comparing the results from the different dendritic segments ($P > 0.05$, KS test for all the comparisons).

Data were also expressed as median (quartile) and compared by Kruskal–Wallis test and multiple comparison of mean rank was used as post hoc. $P < 0.05$ was considered statistically significant. We performed a compromise power analyses to determine the statistical power given the number of observations, sample means, and SD, using G*Power (Faul et al., 2007). All data collection were achieved in a blinded manner.

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