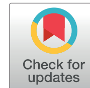

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# Imipramine Ameliorates Depressive Symptoms by Blocking Differential Alteration of Dendritic Spine Structure in Amygdala and Prefrontal Cortex of Chronic Stress-Induced Mice

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## Abstract

Previous studies have shown disrupted synaptic plasticity and neural activity in depression. Such alteration is strongly associated with disrupted synaptic structures. Chronic stress has been known to induce changes in dendritic structure in the basolateral amygdala (BLA) and medial prefrontal cortex (mPFC), but antidepressant effect on structure of these brain areas has been unclear. Here, the effects of imipramine on dendritic spine density and morphology in BLA and mPFC subregions of stressed mice were examined. Chronic restraint stress caused depressive-like behaviors such as enhanced social avoidance and despair level coincident with differential changes in dendritic spine structure. Chronic stress enhanced dendritic spine density in the lateral nucleus of BLA with no significant change in the basal nucleus of BLA, and altered the proportion of stubby or mushroom spines in both subregions. Conversely, in the apical and basal mPFC, chronic stress caused a significant reduction in spine density. The proportion of stubby or mushroom spines in these subregions overall reduced while the proportion of thin spines increased after repeated stress. Interestingly, most of these structural alterations by chronic stress were reversed by imipramine. In addition, structural changes caused by stress and blocking the changes by imipramine were corelated well with altered activation and expression of synaptic plasticity-promoting molecules such as phospho-CREB, phospho-CAMKII, and PSD-95. Collectively, our data suggest that imipramine modulates stress-induced changes in synaptic structure and synaptic plasticity-promoting molecules in a coordinated manner although structural and molecular alterations induced by stress are distinct in the BLA and mPFC.

**Key Words:** Depression, Dendritic spine, Basolateral amygdala, Medial prefrontal cortex, CaMKII, CREB

## INTRODUCTION

Synaptic plasticity is the ability of synapses to strengthen or weaken over time in response to environmental changes and underlies the modification of connectivity between neurons. Synaptic spine morphology and density are highly plastic in response to a variety of stimuli (Bourne and Harris, 2008; Kulkarni and Firestein, 2012). Importantly, the postsynaptic spine density of excitatory synapses is regarded as an important component of connectivity between neurons, ultimately modulating neuronal excitability (Kasai *et al*., 2003). Spine morphology also contributes to synaptic plasticity and function; thin and elongated spines are motile, transient, and form weaker synapse, whereas stubby or mushroom type spines form more stable and stronger synapse with larger postsynaptic densities (PSDs) that contain larger numbers of $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Peters and Kaiserman-Abramof, 1970; Matsuzaki *et al*., 2001; Tyler and Pozzo-Miller, 2003).

Depressive disorder is a devastating psychiatric illness that contributes to disability and morbidity that is becoming increasingly prevalent worldwide (Treadway and Zald, 2011). Increasing evidence implicates structural synapse changes and altered protein content in psychiatric pathophysiology. For example, the reduced gene expression of synaptic proteins including synapsin, microtubule-associated protein, and AMPA receptor subunits; dendritic atrophy; and pyramidal neuron retraction in the cornu ammonis 1 and dentate gyrus regions of the hippocampus have been observed in the depressed brain (Sapolsky, 2000; Duric *et al*., 2013).

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Stress is a precipitating factor for the development of mood disorders including depression. Multiple studies have demonstrated that repeated or chronic stress results in alteration of the structural and functional plasticity in several limbic and cortical areas, including the prefrontal cortex (PFC) and hippocampus (Sousa *et al*., 2000; Correll *et al*., 2005; Price and Drevets, 2010; Leuner *et al*., 2014). Chronic stress induces morphological changes in pyramidal neuron dendrites in the medial PFC (mPFC) including decreased length and branch numbers of apical dendrite (Chocyk *et al*., 2013; Luczynski *et al*., 2015) in addition to significant reduction of dendritic spine density and spine head diameter in layer V pyramidal neurons in the PFC (Li *et al*., 2011). Increased glutamate receptors expression in the mPFC were also simultaneously observed with impaired long-term potentiation (LTP) in mice subjected to maternal separation stress (Chocyk *et al*., 2013). The effects of chronic stress on morphological and molecular synaptic alterations in the PFC are similar to those reported for the hippocampus (Sousa *et al*., 2000; Li *et al*., 2011; Nishiyama and Yasuda, 2015), suggesting that chronic stress induces structural and functional plasticity deficits in the PFC and hippocampus.

Although the amygdala is an essential region that governs stress responses and contributes to the development of depression, stress-induced structural plasticity related to a depressive phenotype has been less intensively investigated in this region. Several studies have reported that amygdala volume and activity were reduced in major depression (Frodl *et al*., 2002; Lange and Irle, 2004). Padival and colleagues described enhanced dendritic spine number in lateral (LAT) and basal (BA) nuclei of the BLA and increased tonic excitatory input in rats exposed to a relatively short-term restraint stress (Padival *et al*., 2013). However, no report regarding to the effects of antidepressant treatment on these stress-induced changes in subregions of amygdala was available. In addition, the effects of antidepressant on the signaling pathways underlying the changes in the parent dendrite and postsynaptic density were not reported.

Thus, the present study examined dendritic spine structural changes including spine number and morphology, and a functional plasticity-related signaling such as calcium/calmodulin-dependent kinase II/cyclic adenosine monophosphate responsive element binding protein (CAMKII/CREB) signaling in the BLA and mPFC of the chronic stress-induced depressed mouse model. We also examined whether the antidepressant imipramine could reverse alterations of dendritic spine structure and CAMKII/CREB cascade in these mice.

## MATERIALS AND METHODS

### Experimental mice

Seven week-old C57BL/6 male mice were obtained from Daehan Biolink, Inc (Eumsung, Chungbuk, Korea) and housed in clear plastic cages under specific pathogen-free conditions with a 12:12 h light-dark cycle (lights on at 6:00 and off at 18:00). Mice had free access to standard irradiated chow (Purina Mills, Seoul, Korea). Dankook University Animal Care and Use Committee granted approval for all experimental procedures involving animals.

### Experimental design

The mice were divided into three groups (control: CON, stress: RST, and stress with imipramine treatment: RST+Imi; 4-10 mice per group). To induce restraint stress, mice were individually placed into a well-ventilated 50 mL conical tube to prevent forward or backward movement. Stress was administered at set times from 10 a.m. to 12 p.m. for a duration of 2 h. This stress was repeated daily for 21 consecutive days. Control mice remained undisturbed in their home cages. All mice were removed from their housing environment and moved into non-housing cages in a separate room during restraint. For the group treated with both restraint and imipramine, daily intraperitoneal injections of imipramine (20 mg/kg) were administered 30 min before restraint stress (Fig. 1A). Control and restrained mice were treated with saline instead of imipramine.

### Behavioral assessment

For the tail suspension test (TST), mice were suspended in the air using an acrylic box 35 cm height×35 cm length×350 cm width, (Jeungdo Bio & Plant Co. LTD, Seoul, Korea) equipped with a hook; they were fixed to the hook with adhesive tape wrapped around the tail (at least 50 cm from the base). Each mouse was suspended by its tail for 6 min, and the immobility time was measured during the last 5 min. The social interaction (SI) test was based on Crawley's sociability method (Kaidanovich-Beilin *et al*., 2011). In brief, the apparatus was partitioned into three equal chambers (each 19 cm height×19 cm length×45 cm width) with dividing walls of clear Plexiglas that could be removed to allow free access to each chamber. The test mouse was first acclimatized by placing it in the closed-off center compartment for 5 min. An unfamiliar male mouse (C57BL/6, stranger) that had no prior contact with the test mouse was enclosed in a wire cup in either the left or the right chamber for 1 min, and then the test mouse was allowed to explore for 10 min by removing both the dividing walls. SI of the test mouse was quantified using the time spent (direct contact duration) with the novel conspecific (stranger). Social avoidance percentage was calculated with the following formula: (the duration of contact with empty cup/stranger cup)×100.

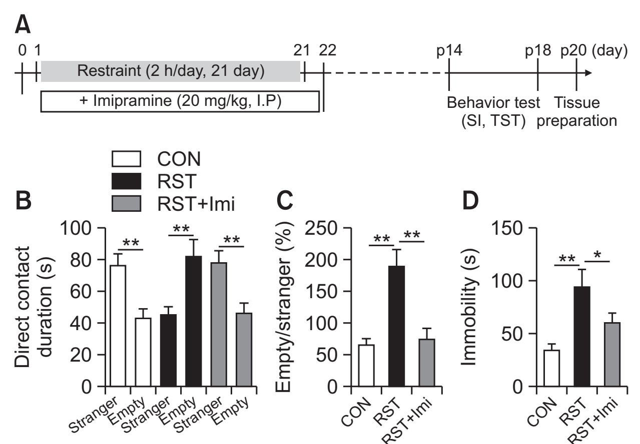


**Fig. 1.** Experimental design and depressive-like behavior tests. (A) Experimental design. (B) Quantitative analysis of direct contact duration for sociability. (C) Quantitative analysis of social avoidance. (D) Quantitative analysis of immobility time for despair. SI, Social interaction test; TST, tail suspension test. * and ** denote differences at $p<0.05$ and $p<0.01$, respectively.

### Golgi staining

Golgi-Cox staining of brain tissue was conducted using a NovaUltra™ Golgi-Cox stain Kit (IHC World, Woodstock, MD, USA) according to the procedure suggested by the manufacturer. Briefly, 2 days after the last behavioral test, mice were anesthetized, decapitated, and the brain was rapidly removed. The fresh brain tissue was immediately immersed in a plastic jar filled with Golgi-Cox solution and stored in the dark at room temperature for 2 days. One day after immersion, the brain tissue was placed in fresh Golgi-Cox solution, and the impregnation was continued at room temperature in the dark for 14 days. When impregnation was finished, brain tissue was washed 2 times with phosphate-buffered saline (PBS) for 2 days, and then 100 μm thick sections were prepared using a vibratome (Leica, Wetzlar, Germany). The section was washed with distilled water (3 times, 5 min each) and stained with Post-Impregnation Solution for 10 min. Stained section was again washed with distilled water (3 times, 5 min each), mounted, and air-dried for 2 h. The slides were dehydrated in 70% and 95% ethanol for 5 min each, and then in 100% ethanol 2 times for 10 min each. The slides were then cleared with xylene 2 times for 10 min each and coverslipped with Permount. The slides were protected from light throughout the staining procedure and analysis.

### Analysis of spine density and morphology

Golgi-stained neurons were assessed with Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a DP71 camera (captured with a 60× objective, Olympus), and the spine numbers and morphology were analyzed using ImageJ software (National Institutes of Health, Image Engineering, Bethesda, MD, USA). We examined the most superficial pyramidal neurons on layer II/III of the mPFC and the BA and LAT of the BLA (defined based on a mouse brain atlas) (Horner and Arbuthnott, 1991). For each brain, 5-6 neurons that appeared to be completely filled were selected. Dendritic spines were defined as small protrusions ranging from 0.5-4 μm in length (Harris *et al*., 1992; Tyler and Pozzo-Miller, 2003); thus, all dendritic protrusions extending ≤4 μm in length from the parent dendrite were analyzed. Spine numbers and the morphologies of secondary and tertiary segments that were derived from the primary dendrites were evaluated at 10-μm steps from the first to fourth branch from the soma, where small soma with few dendrites and large soma with bipolar primary dendrite were excluded from this analysis. Spine density was expressed as the number of spines on a dendritic branch divided by the length of branch. Spine length was defined as the distance from the spine tip to the base. Distinct spine morphologies were classified as previously described (Peters and Kaiserman-Abramof, 1970; Harris *et al*., 1992) as stubby, mushroom, and thin. Stubby spines were defined as protrusion with a neck diameter similar to that of the head, all ≤1 μm in length. Mushroom spines were ≤2 μm in length with a neck diameter smaller than the head diameter. Thin spines were ≥2 μm in length with the elongated protrusion. The proportion of each type was quantified as ([spine number with each type on an individual branch/total spine number on an individual branch]×100).

### Immunohistochemistry

Anesthetized mice were perfused with 100 mM PBS (pH 7.4), followed by cold 4% paraformaldehyde in PBS. After perfusion, the brains were removed, they were fixed further for 18 h and transferred to 30% sucrose solution. Finally, 20-μm-thick sections were prepared using a vibratome (Leica). Free-floating sections were incubated with 0.3% hydrogen peroxide ($H_2O_2$), permeabilized with 0.3% Triton X-100, and nonspecific protein binding was blocked by incubation with 3% normal goat serum. Sections were incubated overnight at 4°C with anti-phospho(p)-CAMKII (Bioss Antibodies, Woburn, MA, USA), anti-PSD-95 (Abcam, Cambridge, UK) or anti-p-CREB antibody (Cell Signaling Technologies, Danvers, MA, USA), subsequently incubated with biotinylated secondary antibodies, and then visualized by the ABC method (ABC Elite kit, Vector Laboratories, Burlingame, CA, USA). The sections were mounted and assessed in digital images (captured at 100× magnification) using ImageJ software (National Institutes of Health, MD, USA).

### Statistical analysis

All values are reported as mean ± standard error of mean (SEM). Significant differences between groups were determined using *t*-tests and one-way analyses of variance (SPSS for Windows, version 18.0, Chicago, IL, USA). Values of $p<0.05$ were considered statistically significant.

## RESULTS

### Chronic restraint stress produced depressive-like behaviors and imipramine exerted an anti-depressive effect

We measured whether chronic restraint stress for 21 days (2 h/day) induced depressive-like behaviors and the effect of imipramine using the SI test for social preference and the TST for despair levels. Mice were subjected to 21 consecutive days of restraint, and depressive-like behaviors were evaluated 14 days after the last restraint stress exposure (Fig. 1A). In the SI test, the time spent in direct contact with the cup containing a stranger (direct contact duration) was significantly longer than that with the empty cup in control mice (Fig. 1B). In restrained mice, the direct contact duration was significantly shorter that that with the empty cup, and this was reversed by imipramine treatment (Fig. 1B). The ratio of the duration of contact with the empty cup relative to that with a stranger cup was markedly enhanced in restrained mice, and this increase was reversed by imipramine treatment (Fig. 1C). Consistent with the SI data, the TST analysis (Fig. 1D) showed that the immobile time of restrained mice was markedly increased compared with that of control mice, and enhanced immobility in restrained mice was reduced by imipramine treatment.

### Chronic restraint stress enhanced dendritic spine density in the LAT and altered the proportion of stubby or mushroom types in the BLA, and these alterations were reversed by imipramine

To elucidate the relationship between BLA spine structural plasticity and depressive-like behaviors we assessed dendritic spine density and morphology in the LAT and BA of the BLA following repeated restraint stress with or without imipramine treatment. The spine densities of the LAT in restrained mice were much denser than those measured in control mice, but this increase was reversed by imipramine treatment (Fig. 2B, 2C). The spine densities in the BA showed no significant dif-

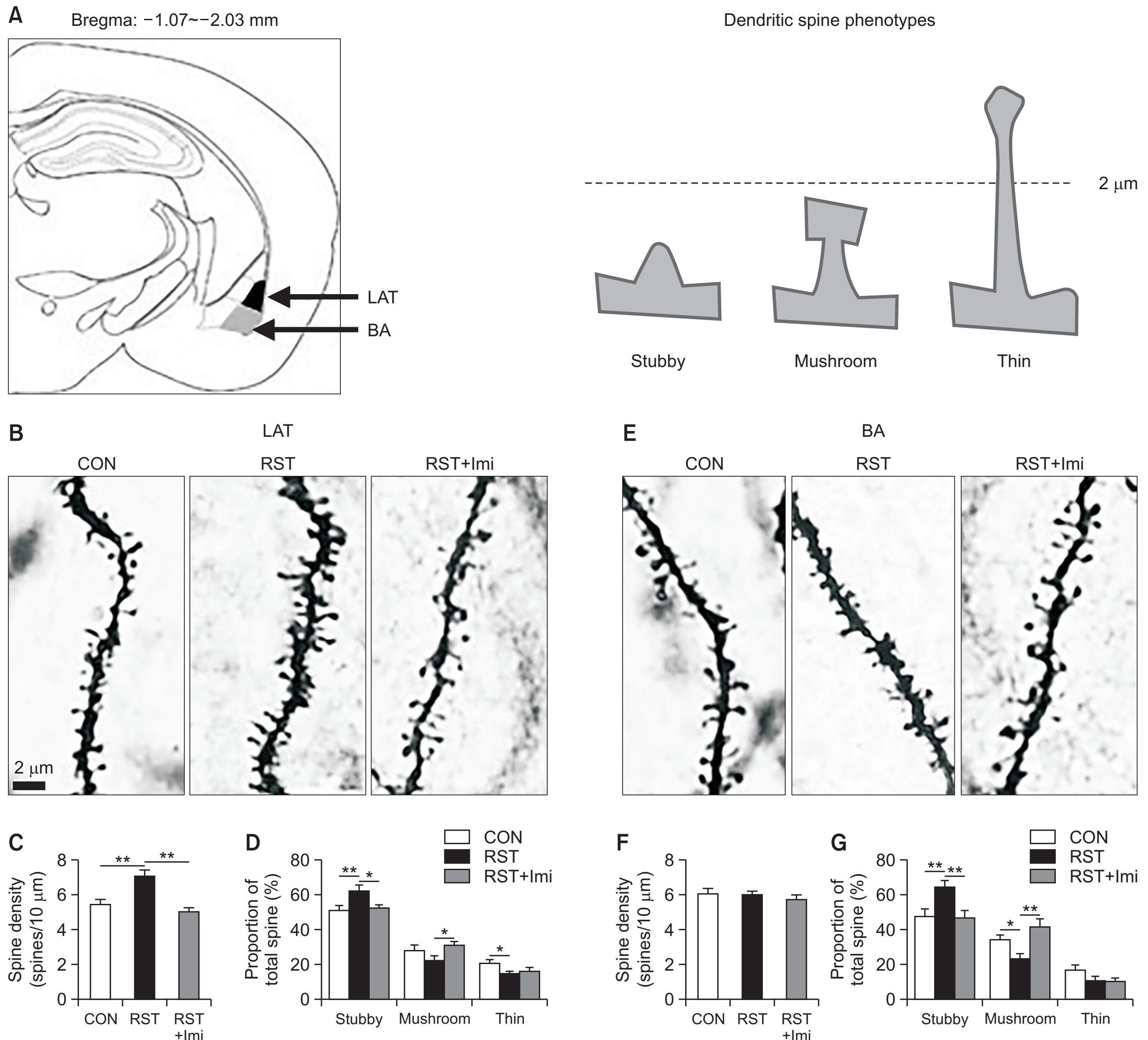


**Fig. 2.** Chronic restraint stress affected dendritic spine structure in the basolateral amygdala (BLA). (A) The LAT and BA region of the BLA from mouse atlas and spine phenotypes. Photomicrographs showing dendritic spine structure (B), quantitative analysis of spine density (C), and the proportion of different spine subtype (D) in the LAT region. Photomicrographs showing dendritic spine structure (E), quantitative analysis of spine density (F), and the proportion of spine subtype (G) in the BA region. * and ** denote differences at $p<0.05$ and $p<0.01$, respectively.

ference among the groups (Fig. 2E, 2F).

Then we observed spine morphology alterations following repeated stress. In the LAT the proportion of stubby spines increased in response to repeated stress but this increase was blocked by imipramine (Fig. 2D). The proportion of mushroom spines decreased by restraint stress and reversed significantly by imipramine (Fig. 2D). Similarly, the proportion of thin spines reduced by repeated stress and this reduction was reversed by imipramine but not significantly (Fig. 2D). In the BA, the proportion of stubby spines in restrained mice was significantly enhanced compared with that of control mice, and this increase was reversed by imipramine (Fig. 2G). In contrast, the proportion of mushroom spines in restrained mice decreased relative to that of control mice, which was reversed by imipramine (Fig. 2G). The proportion in thin spine morphology was not significantly different among groups (Fig. 2G).

## Chronic restraint stress reduced dendritic spine density and the proportion of stubby and mushroom spines in the mPFC, and these alterations were reversed by imipramine

Repeated stress-induced alteration of dendritic spine in the BLA prompted us to assess that of the mPFC as it is also known to be implicated in depression. The dendritic spine

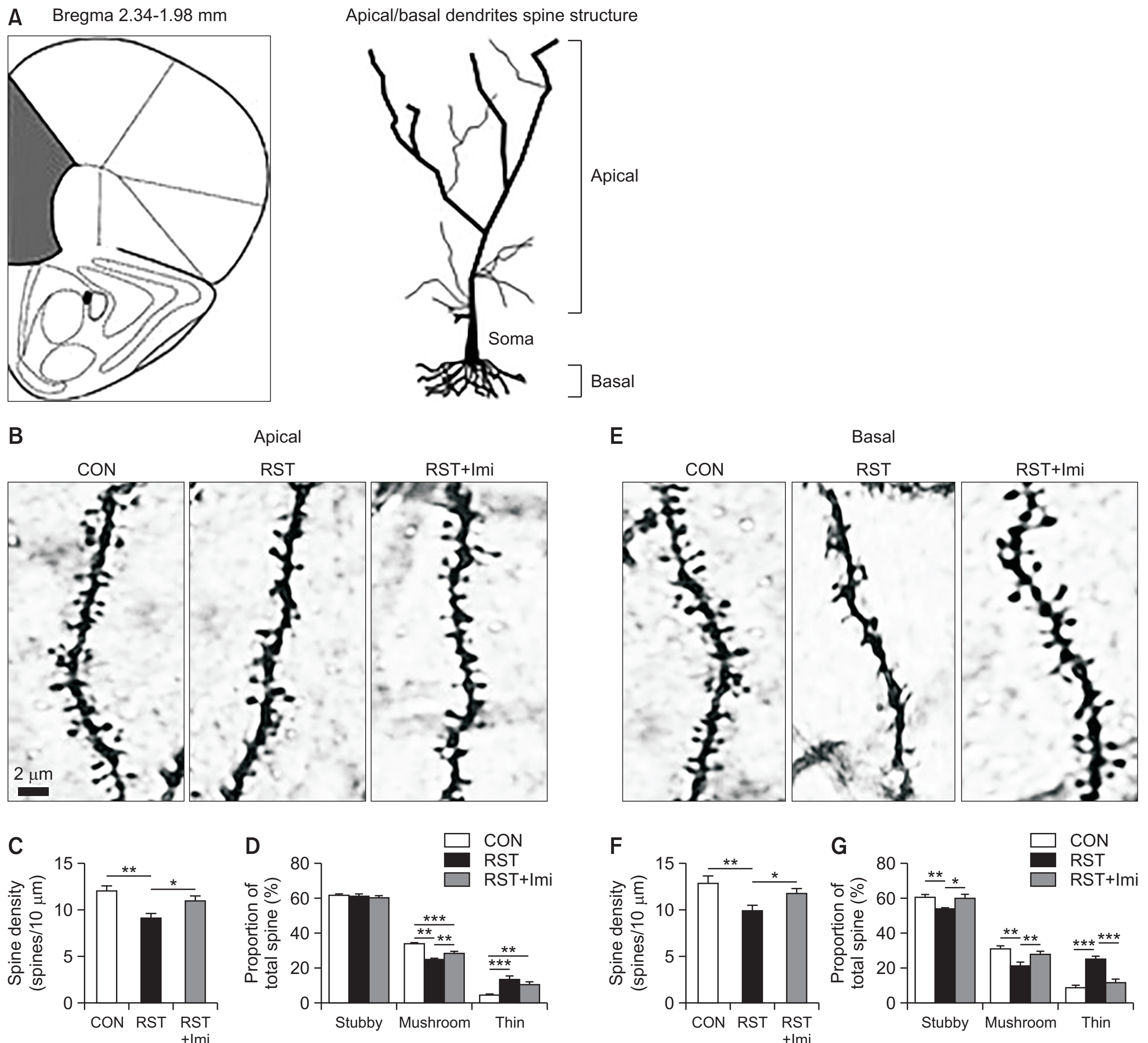


**Fig. 3.** Chronic restraint stress affected dendritic spine structure in the medial prefrontal cortex (mPFC). (A) The mPFC region analyzed and a representative image from the apical and basal dendrite spine structure. Photomicrographs showing dendritic spine structure (B), quantitative analysis of spine density (C), and the proportion of different spine subtype (D) in apical layer. Photomicrographs showing dendritic spine structure (E), quantitative analysis of spine density (F), and the proportion of different spine subtype (G) in the basal layer. *, ** and *** denote differences at $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

number on both the apical (Fig. 3B, 3C) and basal dendrites (Fig. 3E, 3F) in the mPFC in restrained mice was significantly reduced relative to that of control mice, and this reduction was reversed by imipramine.

Analysis of spine morphology on the apical layer showed that the proportion of stubby spines was similar among groups (Fig. 3D). The proportion of mushroom in restrained mice was reduced and this reduction was recovered by imipramine treatment (Fig. 3D). In contrast, the proportion of thin spines increased significantly by restraint stress but this increase was slightly but not significantly blocked by imipramine (Fig. 3D). On the basal layer, the alterations in the proportion of spine types were similar to those on the apical layer. The proportion of stubby spines was slightly reduced by stress and this reduction was reversed by imipramine (Fig. 3G). A similar result was observed in case of mushroom spines. In contrast, the proportion of thin spines was increased in stressed mice but this increase was blocked by imipramine (Fig. 3G).

### Chronic restraint stress enhanced the pCREB and pCAMKII immunoreactivities in the BLA, which were reversed by imipramine

Given that chronic stress produced structural changes in dendritic spines in the BLA and mPFC, we assessed the rela-

tionship between structural plasticity and molecular changes by examining the levels of pCAMKII and pCREB, which are the most characterized signaling proteins involved in synaptic plasticity. In the BLA, the immunoreactivities of pCREB in the LAT and BA of restrained mice were significantly higher compared to control mice (Fig. 4A), and this increase was reduced by imipramine in both the LAT and BA (Fig. 4B). The changes in pCAMKII immunoreactivity were more prominent and similar to that of pCREB on LAT and BA (Fig. 4C, 4D).

### Chronic restraint stress downregulated the pCREB and pCAMKII immunoreactivities in the mPFC which were reversed by imipramine

In comparison with BLA, pCAMKII/CREB levels in the mPFC were opposite to those observed in the BLA. The immunoreactivities of pCREB were markedly reduced by chronic stress, and this reduction was blocked by imipramine (Fig. 5A, 5B). The expression pattern of pCAMKII was similar to that of pCREB in the mPFC (Fig. 5C, 5D).

### Chronic restraint stress oppositely regulated PSD-95 expression in the BLA and mPFC

We determined the expression levels of PSD-95 in the BLA (LAT and BA) and mPFC. In the BLA, the immunoreactivities of PSD-95 of restrained mice were significantly higher than that of control mice in the LAT and BA (Fig. 6A, 6C). In contrast with the BLA, PSD-95 decreased in the mPFC following chronic stress (Fig. 6B, 6C). The decreased PSD-95 expression in response to chronic stress was reversed by imipramine treatment in both the LAT and BA of the BLA and mPFC (Fig. 6C).

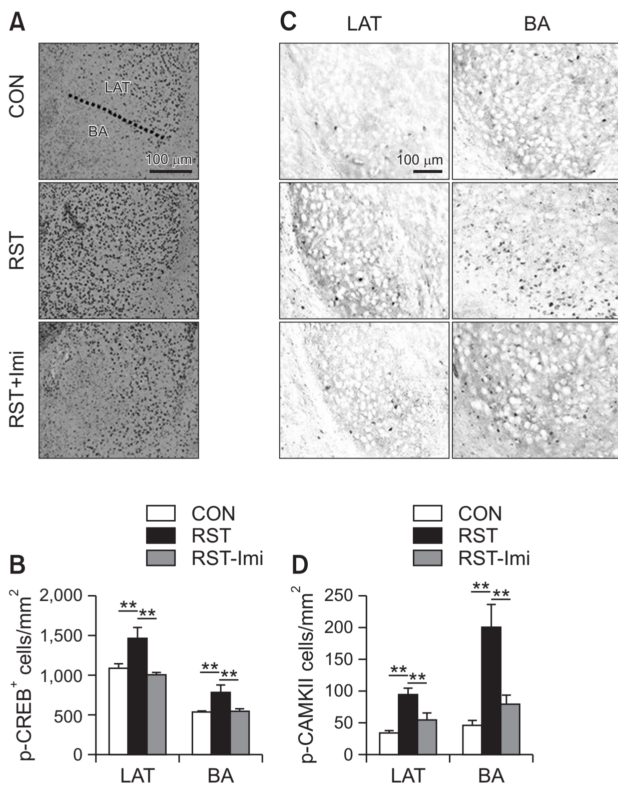


**Fig. 4.** Chronic restraint stress affected the p-CREB and p-CAMKII levels in the BLA. Photomicrographs showing anti-pCREB-positive immunoreactivity in BLA (A), and quantitative analysis of immunoreactivity (B; Y-axis is the normalized level). Photomicrographs showing anti-pCAMKII-positive immunoreactivity (C) in the BLA, and quantitative analysis (D) of immunoreactivity (Y-axis is the normalized level). The data are presented as the mean ± SEM (n=4 animals/group). ** denotes a difference at $p$<0.01.

## DISCUSSION

Our results demonstrate that 21 consecutive days of restraint stress induced depressive-like behaviors and altered the structural plasticity of dendritic spines in the BLA and mPFC, concomitant with changes in pCAMKII/CREB and PSD-95 expression. These behavioral, structural, and molec-

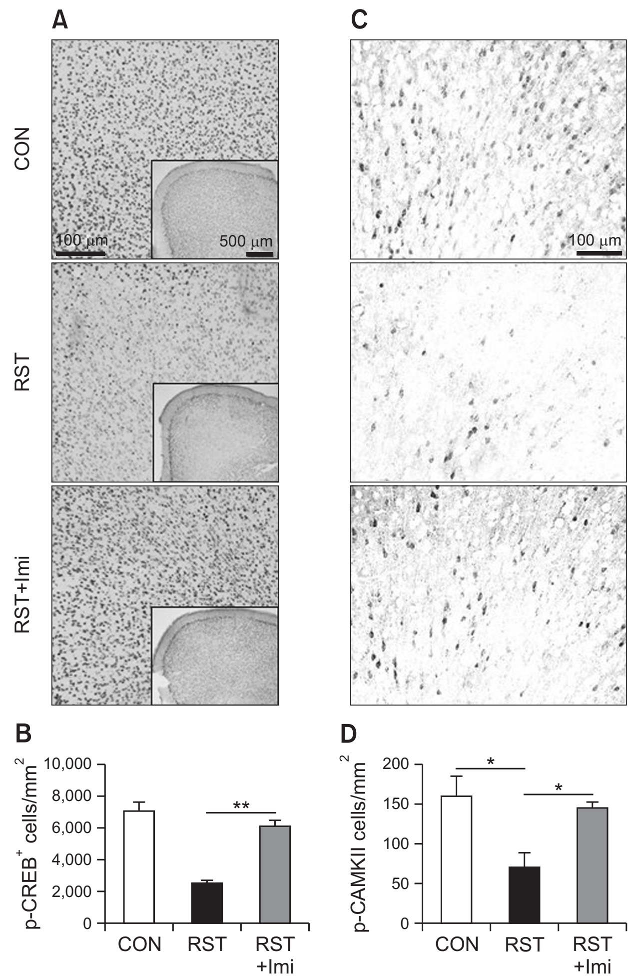


**Fig. 5.** Chronic restraint stress affected p-CREB and p-CAMKII levels in the mPFC. Photomicrographs showing anti-pCREB-positive immunoreactivity in the mPFC (A), and quantitative analysis of immunoreactivity (B; Y-axis is the normalized level). Photomicrographs showing anti-pCAMKII-positive immunoreactivity in the mPFC (C) and quantitative analysis of immunoreactivity (D; Y-axis is the normalized level). The data are presented as the mean ± SEM (n=4 animals/group). * and ** denote differences at $p$<0.05 and $p$<0.01, respectively.

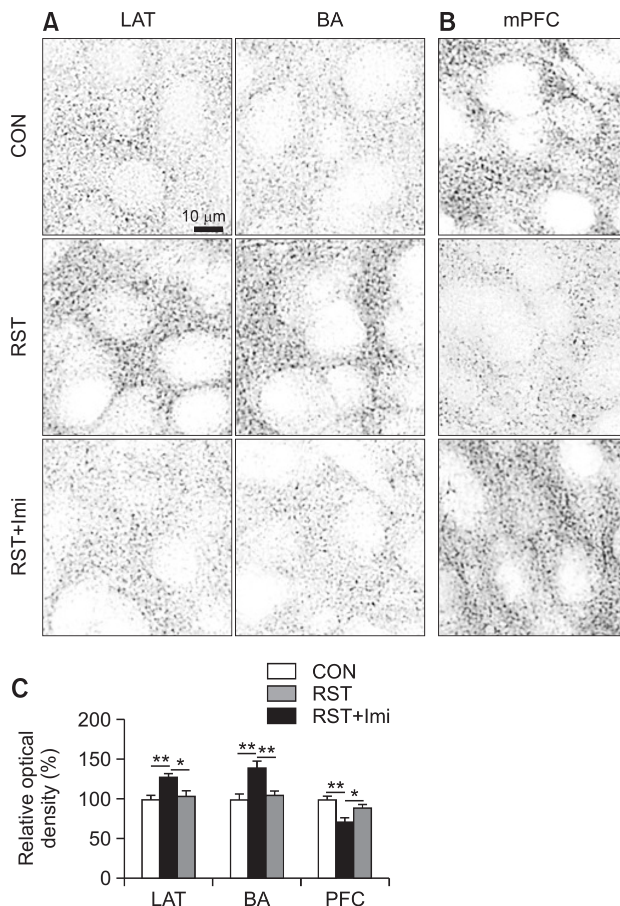


**Fig. 6.** Chronic repeated restraint stress affected PSD-95 expression levels in the BLA and mPFC. Photomicrographs showing anti-PSD-95-positive immunoreactivity in the BLA (A) and mPFC (B). Quantitative analysis of immunoreactivity (C; Y-axis is the normalized level). The data are presented as the mean ± SEM. * and ** denote differences at $p<0.05$ and $p<0.01$, respectively.

ular changes following chronic stress were region specific and reversed by an antidepressant imipramine.

Clinical and animal studies have shown that chronic stress causes psychiatric illnesses such as anxiety and depression. In the present study repeated restraint stress for 21 days enhanced social avoidance and immobility as measured by the SI test and TST, respectively (Fig. 1) and reversed by imipramine treatment in agreement with our previous studies and others (Pham *et al*., 2003; Leem *et al*., 2014). Thus, the chronic restraint stress paradigm used in the present study is valid for investigating behavioral, cellular, and molecular events of a depressive phenotype.

A large body of evidence has demonstrated that synaptic plasticity is impaired in psychiatric disorders such as depression. Chronic or repeated stress altered structural and functional plasticity in some limbic structures such as the PFC, hippocampus, and amygdala (Duman and Monteggia, 2006; Li *et al*., 2011; Quan *et al*., 2011; Padival *et al*., 2013; Luczynski *et al*., 2015; Yi *et al*., 2017). Accumulating evidence shows that in both patients with major depression and animal model of chronic stress, the PFC is hypoactive and apical dendrite atrophy is observed, and deficits in excitatory responsiveness to inputs occur; conversely, the amygdala becomes hyperactive and exhibits hypertrophy and hyperexcitability (Rosenkranz *et al*., 2010). One conceivable mechanism underlying neural activity-dependent behavioral performance is structural plasticity of dendritic spines, which is a key component in determining synaptic properties such as synaptic efficacy and excitatory neurotransmission (Tavosanis, 2012). The functional significance of dendritic spine structure is based on the observation that spine density affects connectivity between neurons and influences neuronal excitability (Leuner and Shors, 2013). Namely, morphological changes in spines impact synapse strength, calcium ($Ca^{2+}$) transients, and AMPA receptor contents in the parent dendrite and PSD (Segal and Andersen, 2000; Yuste *et al*., 2000; Nimchinsky *et al*., 2002; Kasai *et al*., 2003).

In the present studies, we explored structural changes in dendritic spines in the BLA and mPFC in a chronic restraint stress-induced depression model (Table 1). In the BLA, the number of dendritic spines was significantly enhanced in the LAT and this effect was reversed by imipramine. Furthermore, reduced mushroom type proportions and a larger proportion of stubby spines distributed on the parent dendrites of both the LAT and BA in restrained mice were observed, and these responses were also blocked by imipramine (Fig. 2). In the mPFC, in contrast, the structural changes of spines were different from those observed in the BLA. Decreases in spine density and the proportion of stubby and mushroom spines were detected after repeated stress and this effect was reversed by imipramine (Fig. 3). Thus, although chronic stress differentially altered structural plasticity in different brain regions. these stress-induced structural alterations were reversed by imipramine in most subregions, suggesting that imipramine ameliorate depression-related behaviors by modulating structural change of spines.

Previous studies have demonstrated that chronic stress leads to decreased dendritic spine density, especially spine type-specific reduction, as evidenced by decreased and increased numbers of mushroom and thin spines, respectively, on mPFC layer II/III neurons (Radley *et al*., 2008; Luczynski *et al*., 2015). In addition, the 7 out of 9 consecutive days of restraint stress caused increases in spine number on LAT and BA neurons in the BLA (Padival *et al*., 2013). The present study showed the similar results. In mPFC the proportion of mushroom spines and thin spines was reduced and increased, respectively. In BLA, in contrast, the proportion of both mushroom and thin type was reduced while that of stubby spines was increased. Thus, reduction of mushroom type, known as the most functional spine was consistent, but changes of two other types were differential depending on brain areas examined. Thus, our data correspond to previously published studies and support the hypotheses that chronic stress causes the differential change of dendritic spine structure in the BLA and mPFC and this may contribute to depressive-like behaviors.

Structural remodeling of spines are important components of synaptic plasticity. In addition, structural plasticity is coupled with functional plasticity, and most divergent forms of synaptic potentiation depend on $Ca^{2+}$ influx and local cyclic adenosine monophosphate (cAMP) levels. Spines with stubby or mushroom shapes promote $Ca^{2+}$ transients and activate $Ca^{2+}$-dependent signaling in the parent dendrites (Nimchinsky *et al*., 2002). CAMKII, which serves as a major neuronal CREB kinase, is activated by local $Ca^{2+}$ increases and participates

**Table 1.** Alterations of spine density, spine phenotypes and synaptic molecules after chronic stress and modulation by imiparamine

| | BLA | | | | | | mPFC | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LAT | | | BA | | | | | | |
| | Control | RST | RST+Imi | Control | RST | RST+Imi | | Control | RST | RST+Imi |
| Spine density | 5.46 ± 0.07 | 7.06 ± 0.10** | 5.03 ± 0.22$^{\#\#}$ | 6.09 ± 0.29 | 6.00 ± 0.05 | 5.75 ± 0.06 | Apical | 12.0 ± 0.16 | 9.18 ± 0.13** | 11.0 ± 0.50$^{\#}$ |
| | | | | | | | Basal | 12.9 ± 0.66 | 9.21 ± 0.17** | 11.7 ± 0.13$^{\#}$ |
| Spine types | | | | | | | | | | |
| Stubby | 50.8 ± 2.88 | 62.4 ± 3.12** | 52.4 ± 1.65$^{\#}$ | 48.2 ± 1.14 | 65.0 ± 3.36** | 47.4 ± 3.79$^{\#\#}$ | Apical | 61.7 ± 0.85 | 61.3 ± 1.63 | 60.3 ± 1.22 |
| | | | | | | | Basal | 60.5 ± 1.99 | 53.9 ± 0.99** | 59.7 ± 2.20$^{\#}$ |
| Mushroom | 28.3 ± 2.59 | 22.5 ± 2.59 | 31.2 ± 1.87$^{\#}$ | 34.7 ± 0.74 | 23.5 ± 3.34* | 41.9 ± 4.64$^{\#\#}$ | Apical | 33.9 ± 0.94 | 24.8 ± 0.68** | 29.0 ± 0.93$^{\#\#}$ |
| | | | | | | | Basal | 31.1 ± 1.82 | 21.2 ± 1.95** | 28.2 ± 1.06$^{\#\#}$ |
| Thin | 20.9 ± 2.03 | 15.1 ± 1.31* | 16.5 ± 1.62 | 17.1 ± 0.82 | 11.5 ± 2.34 | 10.7 ± 1.97 | Apical | 4.44 ± 0.88 | 13.9 ± 1.84*** | 10.7 ± 1.63 |
| | | | | | | | Basal | 8.46 ± 1.93 | 24.9 ± 1.98*** | 12.1 ± 1.85$^{\#\#\#}$ |
| Synaptic molecules | | | | | | | | | | |
| P-CREB | 1,090 ± 47.2 | 1,470 ± 108** | 1,010 ± 17.7$^{\#\#}$ | 553 ± 6.61 | 794 ± 67.2** | 561 ± 20.5$^{\#\#}$ | | 6,850 ± 207 | 2,650 ± 100 | 6,190 ± 357$^{\#\#}$ |
| P-CAMII | 35.8 ± 2.74 | 96.5 ± 9.28** | 56.9 ± 9.39$^{\#\#}$ | 49.8 ± 9.07 | 201 ± 34.4** | 81.2 ± 12.6$^{\#\#}$ | | 151 ± 31.0 | 79.2 ± 19.3* | 146 ± 6.95$^{\#}$ |
| PSD-95 | 100 ± 5.50 | 129 ± 4.39** | 105 ± 6.05$^{\#}$ | 100 ± 6.50 | 141 ± 7.83** | 106 ± 4.27$^{\#\#}$ | | 100 ± 4.65 | 71.9 ± 4.73** | 90.3 ± 3.76$^{\#}$ |

Values indicate mean +/– standard error of mean.
Significant difference between control and RST (*$p$<0.5, **$p$<0.01, ***$p$<0.001) or between RST and RST+Imi ($^{\#}p$<0.5, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001).
BA, basal nuclei of the BLA; BLA, basolateral amygdala; LAT, lateral nuclei of the BLA; mPFC, medial prefrontal cortex.

in the induction of neuroplasticity (Lisman *et al*., 2002; Vaynman *et al*., 2007). CREB regulation of cAMP-triggered gene expression was proposed to affect spine density and morphology; the phosphorylation of CREB at serine 133 plays a crucial role in spine structure changes (Sargin *et al*., 2013). To assess the relationship between spine structure and synaptic plasticity-related molecules following chronic stress, we evaluated pCAMKII/CREB levels and PSD-95 expression. We found that pCAMKII/CREB levels increased in both the BA and LAT of the BLA following chronic stress, and this was reversed by imipramine. The increase in this signaling activation is considered to be attributed to enhanced $Ca^{2+}$ transients by enhanced spine density and/or altered spine morphology. Interestingly, pCAMKII/CREB levels increased in both the BLA subregions of restrained mice regardless of spine density. This result suggests that CAMKII/CREB phosphorylation may be more closely associated with spine morphological change than with spine density in BLA following chronic stress. In the mPFC, pCAMKII/CREB was decreased by stress, and this was reversed by imipramine. Unlike BLA, this result is considered that chronic stress caused the reduced ratio of both functional spine forms and spine density, thereby down-regulating $Ca^{2+}$-dependent signaling in mPFC. These findings suggest that spine structure and CAMKII/CREB activation after chronic stress were differentially altered in BLA and mPFC.

PSD-95 is expressed in the PSDs of excitatory synapses and regulates basal and activity-dependent AMPA receptor synaptic recruitment (Ehrlich and Malinow, 2004; Kim and Sheng, 2004). A growing body of evidence has demonstrated that increased synaptic AMPA receptor expression is required for LTP, and enhanced levels of PSD-95 expression lead to increased numbers of synaptic AMPA receptors, thus elevating the synapse's AMPA receptor-mediated channel conductance (Ehrlich and Malinow, 2004; Liu and Zukin, 2007). As expected, the optical densities of PSD-95 were paralleled by changes in CAMKII/CREB signaling in the mPFC and BLA. That is, PSD-95 expression levels in the BLA of restrained mice were higher than those in control mice, while reduced expression was observed in the mPFC following chronic stress. Altered PSD-95 expression after chronic stress was reversed by imipramine in both the mPFC and BLA. Repeated stress-induced hyperactivity of the amygdala is hypothesized to be due to increased membrane excitability or elevated tonic excitatory afferent drive (Rosenkranz *et al*., 2010; Padival *et al*., 2013). Based on previously published results, our data at least in part supports the hypothesis that chronic stress elevates AMPA receptor-mediated synaptic transmission via enhanced PSD-95 expression in BLA, whereas chronic stress oppositely impacts synaptic transmission in the mPFC.

Anatomically, mPFC neurons modulate the activity of BLA excitatory afferent neurons via inhibitory and dopaminergic mechanisms (Rosenkranz and Grace, 2002). Dopamine-regulated BLA neurons project excitatory inputs to the mPFC (McDonald, 1991; Kröner *et al*., 2005). One probable explanation for the opposite direction in synaptic plasticity in the mPFC and BLA after chronic stress is that reduced mPFC activity after chronic stress may decrease the inhibitory effect of the mPFC on BLA neurons, with the net effect of increasing BLA neuronal activity. Alternatively, decreased dopaminergic transmission in the BLA due to chronic stress may attenuate its excitatory projections to the mPFC, thus causing decreased mPFC neuronal activity. Accordingly, chronic stress may alter neural circuitry and eventually induce altered behaviors and functional consequences such as a depressive phenotype.

In conclusion, the current study demonstrated that chronic stress differentially affects dendritic spine structural and functional plasticity in the BLA and mPFC in a region-specific manner, consequently inducing a depressive phenotype. Albeit that, imipramine, antidepressant counter-regulated chronic stress-induced change of spine structure and $Ca^{2+}$-related molecules in BLA and mPFC.

## CONFLICT OF INTEREST

The authors declare no financial and non-financial competing interests.

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