Mifepristone Repairs Region-Dependent Alteration of Synapsin I in Hippocampus in Rat Model of Depression

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Clinical investigations present much evidence that the glucocorticoid receptor (GR) antagonist mifepristone leads to a rapid amelioration of depression. The molecular mechanisms of mifepristone involved in the treatment of depression are not fully understood. Depression is associated with hippocampal plasticity, for which increased excitatory amino acid (EAA) release in CA3 induced by chronic stress is responsible, and glucocorticoids have a permissive role and act synergistically with EAAs in producing neuronal damage. Moreover, glucocorticoids increase synapsin I, which has a key role in the release of neurotransmitter, including EAAs. Hereby, we hypothesize that major depression involves synapsin I alteration and that mifepristone blocks this alteration. In the present study, we observed both the expression of hippocampal synapsin I and depression-associated behavior in a rat model of depression induced by chronic unpredictable mild stress (CUMS). The result showed that a region-dependent synapsin I alteration occurs in the rat hippocampus after 21 days of CUMS, that is, it increases in dentate gyrus (DG)/CA3 and decreases in the CA1 region. Correlation analysis indicated that the decrease of synapsin I in CA1 is highly correlated with the increase in the DG/CA3 subfield. Simultaneously, the region-dependent alteration of synapsin I is correlated with depression-associated behaviors. Both the alteration of synapsin I and the depression-associated behavior were rapidly restored after treatment with mifepristone for 1 week. The result suggests that the molecular mechanism underlying the treatment of depression with mifepristone is associated with the rapid repair of the synaptic alteration.
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INTRODUCTION

Most of the antidepressants prescribed in the clinic are aimed at modulating monoamine neurotransmission and usually take at least 6 weeks to take effect. However, each drug is efficacious in only 60–70% of patients (Wong and Licinio, 2004). Many studies have therefore tried to find approaches other than monoamine-based therapies. Major alterations of the hypothalamus–pituitary–adrenal (HPA) system, which can be reversed by successful antidepressant therapy, are often seen in depressed patients (Holsboer and Barden, 1996). The direct use of drugs that block the stress system represents a new avenue for the treatment of types of depression that are resistant to classic antidepressants.

Glucocorticoid receptor (GR) antagonist mifepristone (RU-486) is now in Phase III clinical trials for psychotic major depression and might be the first nonmonoamine-based antidepressant in the market (Berton and Nestler, 2006). Several investigations suggest that mifepristone leads to the rapid amelioration of psychotic depression (Belanoff *et al*, 2001, 2002; Simpson *et al*, 2005; Flores *et al*, 2006). Another clinical study showed mifepristone improves cognition and mood in patients with bipolar disorder (Young *et al*, 2004). Use of mifepristone for only 1 week is efficacious in the treatment of depression, whereas the usual antidepressant drugs that modulate monoamine neurotransmission require 6–8 weeks to show effect. The molecular mechanisms involved are not fully understood, despite the many clinical trials.

Glucocorticoids have two receptors, for example, GR and mineralocorticoid receptor (MR). It is specifically GR activation that brings about the hippocampal damage, as MR has trophic effects (Gass *et al*, 2000) with regard to cognition and effect as well as to neuronal viability, plasticity, and gene expression. The hippocampus, a structure that plays a vital role in learning and memory, contextual fear conditioning, and neuroendocrine regulation, is a target for adrenal steroids that provides a model for studying neurobiological consequences of stress. Up until now, most studies showed that major depression is associated with a selective volume loss of hippocampus

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(Sheline et al, 1996; Bremner et al, 2000; Frodl et al, 2002), whereas one post-mortem study reported no effect of major depression or glucocorticoid treatment on the hippocampus (Muller et al, 2001). Besides cell loss induced by prolonged stress and glucocorticoid exposure (Uno et al, 1989; Sapolsky et al, 1990), another possibility underlying the decreased hippocampal volume is that chronic stress induces apical dendrite atrophy of CA3 pyramidal neurons, which is mediated by activation of endogenous excitatory amino acids (EAAs, eg glutamate) because the interference with the action of EAAs could prevent the atrophy (Watanabe et al, 1992; Magarinos et al, 1996). This result is consistent with the evidence that stress induces EAAs-mediated excitatory input to the hippocampal CA3 (Krugers et al, 1993; Bartanusz et al, 1995).

Glucocorticoids have a permissive role and act synergistically with EAAs in producing neuronal death in cell culture and in vivo (Magarinos et al, 1996). The adrenal steroids regulate the release of glutamate because adrenalectomy markedly reduces the magnitude of the EAA release evoked by restraint stress. Stress-induced hippocampal atrophy is blocked by treatment with the adrenal steroid synthesis blocker cyanoketone (McEwen, 1999). Moreover, corticosterone was found to increase the amount of rat hippocampal synapsin I (Nestler et al, 1981), a synaptic vesicle docking protein that plays a key role in the storage and release of neurotransmitters, including EAAs (De Camilli et al, 1990).

Hereby, we hypothesize that synapsin I alteration is involved in major depression and mifepristone blocks the alteration as a GR antagonist. To investigate the molecular mechanisms underlying the treatment of depression with mifepristone, both depression-associated behaviors and synapsin I changes were examined in the rat model of depression induced by chronic unpredictable mild stress (CUMS).

MATERIALS AND METHODS

Animals

A total of 32 male adult Sprague–Dawley rats were randomly assigned to four groups of equal size: unhandled control group (control); CUMS group (stress); 4.5 mg/kg/day mifepristone-treated CUMS group (4.5 mg/kg mifepristone + stress); 54 mg/kg/day mifepristone-treated CUMS group (54 mg/kg mifepristone + stress). Rats were housed eight per cage and acclimatized to the animal colony for 1 week before the start of the experimental procedures. At the start of the experiment, their body weight was 220–250 g. All rats received standard rodent diet and tap water ad lib under a 12 h light–dark cycle (lights on 0730–1930) and a constant temperature of 21–22°C and humidity of $55 \pm 5\%$. Animal housing, care, and application of experimental procedure were in accordance with all relevant local guidelines and legislation to minimize pain and suffering of the animals.

Chronic Unpredictable Mild Stress Procedure

Stressors were administered once daily between 0830 and 1030, except the 24 h duration stressors. Stressors consisted of (1) 1 h warm swim at 31°C; (2) 5 min cold swim at 8–10°C, after which they were toweled dry; (3) 5 min hot stress in oven at 42°C; (4) 1 min tail pinch; (5) 24 h food deprivation; (6) 24 h water deprivation; (7) 60 inescapable electric foot shocks at 1.5 mA intensity and 2 s duration with 1 s interval; (8) 24 h social isolation (one rat per cage); (9) 24 h social crowding (24 rats per cage) plus cage tilt (cages were tilted to 30° from the horizontal); (10) 1 h shaker stress (160 r.p.m.). The different stressors were distributed randomly at an interval of at least 7 days. All stressors were administered twice within 21 days, except the cold swim, which was administered three times.

Pharmacological Treatment

For the last 7 days of the procedure, the animals were administered a daily oral dose of mifepristone (oral mifepristone was generously provided by Beijing Zizhu Pharmaceutical Company, diluted in milk) each morning. For this purpose, each animal was grabbed gently, and a bulb-headed probe was inserted into its mouth, slid over the tongue, and then slipped carefully down the esophagus. With this procedure, the dose was swallowed completely and the stress caused to the animals was minimized, because control rats that received milk in the same way showed no stress-induced behavior change. Mifepristone is an antagonist of both progesterone receptor and GR (Cadepond et al, 1997). In the clinic, a low dose of 50 mg/day mifepristone had an obvious antiprogesterone effect but no effects on cortisol, and only the high dose of 600 mg/day could antagonize GR (Belanoff et al, 2002). Using body–surface area for dose calculation, two dosages of mifepristone, 4.5 and 54 mg/kg, were selected according to the clinical dose.

Sucrose Preference Test

After a 12 h period (between 10 and 12 h of the light phase) of water deprivation, the sucrose preference test was performed. Animals were given free access to two bottles containing water and 1% sucrose solution. After 2 h the volumes of water and sucrose consumed were measured. The percentage of sucrose solution from the total liquid ingested represented the parameter of hedonic behavior. Sucrose preference was trained during four sessions. Before the start of the procedure, and during the chronic variable stress procedure, the test was applied once a week.

Open-Field Test

The open-field test was performed 12 h after ceasing the chronic variable stress procedure between the second and fifth hours of the dark phase. The apparatus consisted of a rectangular area of 81×81 cm surrounded by a 28 cm high wall. The area was divided into 16 squares of 20×20 cm by painted white lines. The field was lighted with a 40 W bulb fixed 50 cm above the field. Light was focused on the center of the field with the periphery remaining dark. The rat was placed in one corner of the open field and its activity during the subsequent 5 min was assessed. Horizontal locomotion (number of times crossings of the white lines), frequency of rearing or leaning (sometimes termed vertical activity),

frequency of grooming (protracted washing of the coat), and defecation (number of fecal pellets) were observed.

Tissue Preparation

Twenty-four hours after the last stressor had been administered, the rats were deeply anesthetized with chloral hydrate and decapitated. Their brains were rapidly removed from the skull. On ice, the whole right side of the brain was dissected, followed by 48 h of fixation in 4% paraformaldehyde at 4°C, and the hippocampus on the left side was quickly isolated. CA1 and DG/CA3 regions were quickly dissected from the hippocampal fissure, then dipped into liquid nitrogen and stored at −80°C. The fixed right side of the brain was dehydrated and embedded in paraffin. Serial 6 μm coronal sections were cut on a Leica microtome (Leica RM 2135). Five sections (1 out of every 20 serial sections) containing the dorsal hippocampus were examined per rat.

Immunohistochemistry

The sections were hydrated, rinsed in Tris-buffered saline (TBS) for 10 min, and treated with 1% hydrogen peroxide in TBS/0.3% Triton for 1/2 h at 37°C to quench endogenous peroxidase activity. After having been washed in TBS (3×5 min), the sections were treated with microwaves (700 W) in 0.05 M citrate-buffered saline (pH 6.0) for 2×10 min for antigen retrieval. Subsequently, the sections were washed in TBS (3×5 min) and (1) incubated in 5% normal goat serum (Vector Laboratories, Burlingame, CA) in TBS for 1 h at 37°C to block nonspecific staining; (2) incubated with the primary antibody for 1 h at 37°C and overnight at 4°C. Polyclonal (rabbit) antibody against synapsin I (Sigma) was diluted 1:600 in TBS/0.3% Triton (pH 7.4); (3) washed in TBS (3×10 min); (4) incubated with the biotinylated goat anti-rabbit Ig G (Vector Laboratories) diluted 1:200 in TBS for 1 h at 37°C; (5) washed in TBS (3×10 min); (6) incubated with avidin–biotin peroxidase complex (Vector Laboratories) diluted 1:200 in TBS for 1 h at 37°C; (7) washed in TBS (3×10 min); (8) incubated in 0.05% 3,3'diaminobenzidine (Sigma Chemicals, St Louis, MO) in TBS containing 0.01% H_2O_2 for 10 min; (9) washed in TBS (3×5 min); and (10) dehydrated in ethanol and mounted in Entellan.

Image Analyses

Image analyses were performed using a blinded procedure: the investigator did not know the experimental treatment. An image analysis system was used for quantitative analysis of synapsin I-immunoreactive synaptic button density (button number/1 mm^2) in the CA3. The system includes MetaMorph image acquisition and processing software (Universal Imaging Corp.), a spot cooled color digital camera (Diagnostic Instruments Inc.), a Nikon E800u microscope (Nikon Corporation, Japan) equipped with a Prior scanning stage (Prior Scientific Instruments Ltd, UK), and a HP computer. Digitized images of synapsin I DAB staining were captured from the different layers of hippocampus. The layers analyzed in the various hippocampal subfields included stratum pyramidal (SP) and stratum lucidum (SL) in the CA3. The main principle and the procedure of the measurements have been extensively described before (Liu et al, 2000; Hu et al, 2002, 2003). Briefly, each subfield of the hippocampus was manually outlined at low magnification ($\times 4$ objective). Its total area was measured, and a grid of fields was superimposed. From this grid 50% of the fields were randomly selected and stored. Then, at high magnification ($\times 40$ objective), each selected field was retrieved automatically. The number of synapsin I immunoreactive synaptic button was measured by setting the specific uniform thresholds.

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from the frozen hippocampus CA1 and DG/CA3 using the Trizol (Invitrogen, USA) method and determined by spectrophotometer measurements at 260 nm. For synapsin I, the sense primer was 5'-ccc ttc att gat gct aaa tac g-3' and the antisense primer was 5'-gtt gac cac aag ttc cac gat-3' to yield a 321 bp product of synapsin I cDNA. The endogenously expressed rat β-actin mRNA was used as an internal control. Synthetic sense primer 5'-GTA ACC CTC ATA GAT GGG CAC A-3' and the antisense primer 5'-ACC GTG AAA AGA TGA CCC AGA T-3' were used to detect the β-actin mRNA, which yielded a cDNA product of 165 bp. cDNAs were synthesized by using the manufacturer's protocol of RevertAidTMFirst Strand cDNA Synthesis kit (MBI Fermentas, USA). For polymerase chain reaction (PCR) amplification, different PCR cycle numbers and different amounts of synthesized cDNA were first examined to evaluate the linearity of the reaction. Amplification was carried out by the following cycle parameters after 95°C for 5 min: for synapsin I, 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s; for β-actin 94°C for 30 s, 59°C for 30 s, and 72°C for 20 s. After 30 cycles of amplification, a final extension step was performed at 72°C for 5 min. The PCR products were electrophoretically separated on 2% agarose gels. By staining with ethidium bromide, the cDNA bands were visualized and subsequently analyzed with Eaglesight software (Stratagene, USA). The relative amount of mRNA was expressed as the ratio of the gray value of synapsin I to β-actin cDNA band.

Statistical Analyses

The results were expressed as mean $\pm$ SEM. Statistical significance was determined by one-way analysis of variance (ANOVA), followed by the Tukey test when appropriate. The Pearson's correlation test was used to analyze the correlation between the depression-associated behavior and the level of synapsin I protein and mRNA. The p values < 0.05 were considered significant. All analyses were conducted by statistical software, SPSS 12.0 for Windows.

RESULTS

Sucrose Preference Test

Sucrose intake is as a measure of anhedonia, a core symptom of depression. There were significant decreases in sucrose preference in depressive animal induced by CUMS as expected ($p < 0.001$; Willner et al, 1987; De Vry and

Schreiber, 1997; Willner, 1997). Treatment with 54 mg/kg/day mifepristone in the last 7 of 21 days of CUMS significantly increased sucrose preference ($p < 0.01$),

whereas 4.5 mg/kg/day mifepristone had no obvious effect on sucrose preference (Figure 1).

Open-Field Test

Control rats were significantly more active than stressed rats in the open field. Rats showed approximately 10-fold decrease in the numbers of total zone crossings after CUMS for 21 days ($p < 0.001$). 54 mg/kg/day mifepristone significantly increased locomotion by about six times ($p < 0.001$), whereas 4.5 mg/kg/day mifepristone showed no obvious effect on locomotion (Figure 2a). Rearing was also decreased in stressed rats ($p < 0.001$), indicating decreased exploratory behavior. 54 mg/kg/day mifepristone increased the number of times of rearing ($p < 0.01$ vs stress group), whereas 4.5 mg/kg mifepristone showed no effect on rearing (Figure 2b). Rats showed a significantly decreased grooming frequency after CUMS ($p < 0.001$), but 54 mg/kg mifepristone repaired the behavior ($p < 0.001$, vs stress group), whereas 4.5 mg/kg/day mifepristone showed no effect on grooming (Figure 2c). The number of fecal pellets increased after CUMS ($p < 0.001$), and both 54 and 4.5 mg/kg/day mifepristone failed to decrease the defecation although 54 mg/kg/day mifepristone tended toward decreasing it
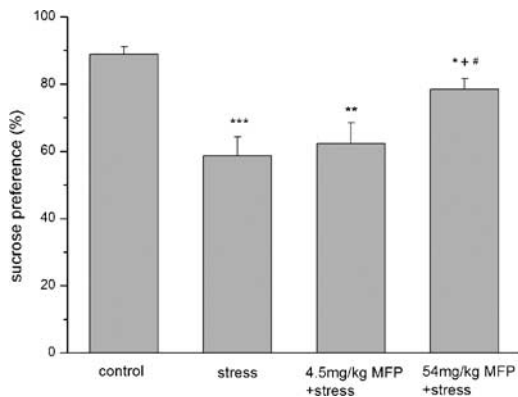
Figure 1 Percentage of sucrose solution from the total liquid in 2 h in sucrose preference test. The presented data are mean ± SEM (MFP, mifepristone). Statistical analysis was carried out by one-way ANOVA ($F(3,28) = 9.398$; $p < 0.001$) followed by Tukey test. *$p < 0.05$ vs control; **$p < 0.01$ vs control; ***$p < 0.001$ vs control; +$p < 0.01$ vs stress; #$p < 0.05$ vs 4.5 mg/kg MFP + stress.
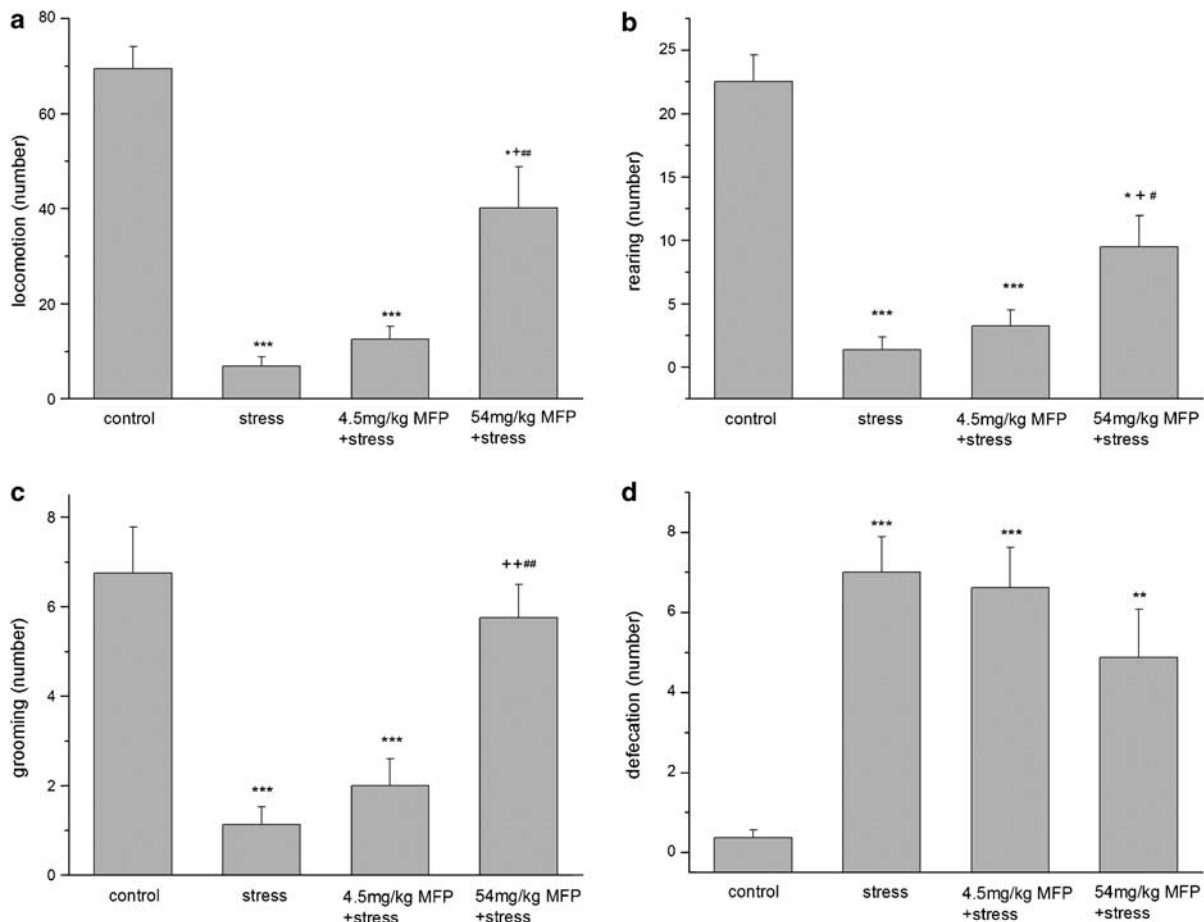
Figure 2 Behavioral data from open-field test. Data (mean ± SEM) shows the number of horizontal locomotion (a), rearing (b), grooming (c), and defecation (d) in 5 min in open field (MFP, mifepristone). Statistical analysis was carried out by one-way ANOVA (for locomotion, $F(3,28) = 30.218$; $p < 0.001$; for rearing, $F(3,28) = 28.242$; $p < 0.001$; for grooming, $F(3,28) = 14.236$; $p < 0.001$; and for defecation, $F(3,28) = 11.338$; $p < 0.001$) followed by Tukey test. *$p < 0.05$ vs control; **$p < 0.01$ vs control; ***$p < 0.001$ vs control; +$p < 0.01$ vs stress; ++$p < 0.001$ vs stress; #$p < 0.05$ vs 4.5 mg/kg MFP + stress; ##$p < 0.01$ vs 4.5 mg/kg MFP + stress.

(Figure 2d). Stressed rats never entered the center square of open field within 5 min, whereas control and high-dose mifepristone-treated rats entered occasionally (data not shown).

Protein Level of Synapsin I

Synapsin I-immunoreactive puncta (Figure 3) were distributed mainly in the SL in the subfields CA3 in all groups and revealed mossy fiber terminal (MFT) button distribution in CA3. Few synapsin I puncta were distributed in SP of CA3 regions in control rats (a), whereas a great deal of puncta were distributed throughout the SP in CUMS-induced depressed rats (b). The synapsin I-immunoreactive zone in hippocampal CA3 of depressed rats seems to be wider than that of control rats, because of the invasion of synapsin I-immunoreactive synaptic buttons from SL to SP. Compared to the depressed rats, the amount of synapsin I puncta density did not change in rats treated with 4.5 mg/kg mifepristone for 7 days (c), whereas an obvious suppression of the synapsin I puncta number occurred in SP of CA3 in rats treated with 54 mg/kg mifepristone (d). Although a trend toward suppression in the SL of CA3 was detected, there was no statistically significant difference (Table 1). Figure 3b and c shows that the invasion of the infrapyramidal layer with mossy fibers persists with irregularly oriented intrapyramidal mossy fibers forming a web between the suprapyramidal and infrapyramidal mossy fiber bundles. These aberrant fibers could have physiological consequences because the number of synapsin I-positive terminals in SP of the CA3 region remains 6–7 times greater than that in controls (Table 1).

A quantitative analysis of synapsin I-immunoreactive synaptic buttons density in subfield CA3 is shown in

Table 1. Data showed that a significantly increased number of synapsin I-inmmunoreactive synaptic buttons was induced after 21 days of CUMS in CA3 ($p < 0.001$ in SP, $p < 0.05$ in SL). Treatment with 54 mg/kg/day mifepristone in the last 7 days restored the alteration of the synaptic button number in SP of the CA3 ($p < 0.001$ vs stress group), whereas 4.5 mg/kg/day mifepristone did not change the alteration.

mRNA Level of Synapsin I

Using the RNA extracted from the hippocampus of an unhandled rat, different PCR cycle numbers and different

Table 1 A Quantitative Analysis of Synapsin I Immunoreactive Synaptic Button Density

	Synaptic button density in CA3 (number/mm^2)	
Group	SP	SL
Control	103.60 ± 21.39	3137.65 ± 160.60
Stress	750.52 ± 125.54**	3839.08 ± 192.17*
4.5 mg/kg MFP+stress	763.15 ± 118.48**	3783.17 ± 156.69*
54 mg/kg MFP+stress	146.59 ± 47.30[+#]	3323.90 ± 178.93

MFP, mifepristone; SL, stratum lucidum; SP, stratum pyramidale.
Data (mean ± SEM) show the density of the synapsin I-immunoreactive synaptic buttons (number/mm^2) in the SP and SL of the hippocampal CA3 subfield in control, stress, 4.5 mg/kg MFP+stress, and 54 mg/kg MFP+stress groups. Statistical analysis was carried out by one-way ANOVA (for SP, $F_{(3,28)} = 16.417$; $p < 0.001$; and for SL, $F_{(3,28)} = 3.977$; $p < 0.05$) followed by Turkey test. *$p < 0.05$ vs control; **$p < 0.001$ vs control; [+]$p < 0.001$ vs stress; [#]$p < 0.001$ vs 4.5 mg/kg MFP+stress.
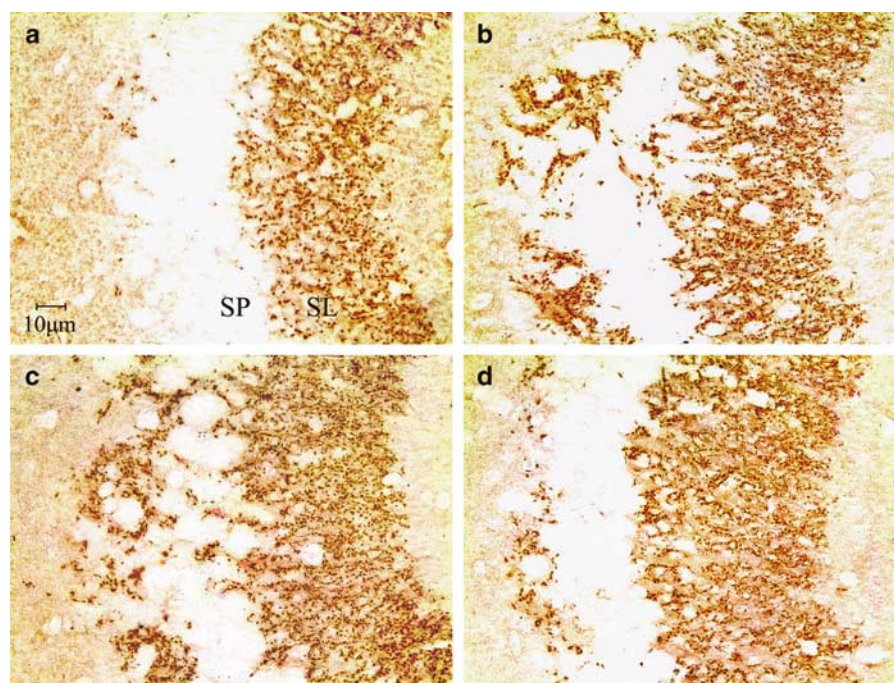
Figure 3 Presentation of synapsin I in hippocampal CA3 subfield. The figure shows synapsin I immunoactivity in control (a), stress (b), 4.5 mg/kg/day mifepristone + stress (c), and 54 mg/kg/day mifepristone + stress (d) groups, revealing the MFT buttons in CA3.

amounts of synthesized synapsin I cDNA were first examined to evaluate the linearity of the reaction, and the 30 cycles were chosen (Figure 4a and b). Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of synapsin I in CA1 and DG/CA3 subfields in hippocampus was shown in Figure 4c and d. Consistent with the increased protein level of synapsin I in CA3, the mRNA level in DG/CA3 was significantly increased in rats induced by 21 days CUMS ($p < 0.001$). The increase in mRNA level was repressed by treatment with 54 mg/kg/day mifepristone in the last 7 of 21 days ($p < 0.05$ *vs* stress group). In contrast to the increases in the DG/CA3 region, there are significantly decreased mRNA levels of synapsin I in the hippocampal CA1 region ($p < 0.01$). Although 54 mg/kg/day mifepristone restored the synapsin I mRNA level ($p < 0.05$ *vs* stress group), 4.5 mg/kg/day mifepristone showed no obvious effect on the mRNA level of synapsin I (Figure 4c). A semiquantitation of the mRNA level of synapsin I is shown in Figure 4d.

Relationships between Depression-Associated Behaviors and Levels of Synapsin I mRNA and Protein

Figure 5 shows a significant negative correlation between the behaviors (figure for sucrose preference was (a), for locomotion, (b), for rearing, (c), and for grooming, (d)) and synapsin I mRNA level in DG/CA3 ($p < 0.001$), as well as a positive correlation between behaviors and synapsin I mRNA level in CA1 of hippocampus ($p < 0.001$–0.01). The correla-

tions between sucrose preference ($r = 0.76$ in CA1, $r = -0.85$ in DG/CA3), locomotion ($r = 0.77$ in CA1, $r = -0.80$ in DG/CA3), rearing ($r = 0.75$ in CA1, $r = -0.77$ in DG/CA3) and synapsin I mRNA level seem to be a little more significant than those between grooming ($r = 0.66$ in CA1, $r = -0.71$ in DG/CA3) and the level of synapsin I mRNA.

Figure 6 shows a negative correlation between the behaviors (figure for sucrose preference was (a), for locomotion, (b), for rearing, (c), and for grooming, (d)) and synapsin I immunoreactive synaptic button density in both SP ($p < 0.001$–0.01) and SL ($p < 0.01$–0.05) of the hippocampal CA3 subfield. The correlations between depression-associated behaviors and synaptic button density in SP (for sucrose preference, $r = -0.82$; for locomotion, $r = -0.79$; for rearing, $r = -0.76$; and for grooming, $r = -0.72$) were more obvious compared to that between behaviors and synaptic button density in SL (for sucrose preference, $r = -0.57$; for locomotion, $r = -0.61$; for rearing, $r = -0.69$; and for grooming, $r = -0.42$). These data imply that the increased synaptic button in SP and SL of hippocampal CA3 subfield, especially in SP, has significant correlation with the depression-associated behaviors. Only one behavior variable, defecation, had no correlation with either mRNA or protein level of synapsin I (data not shown).
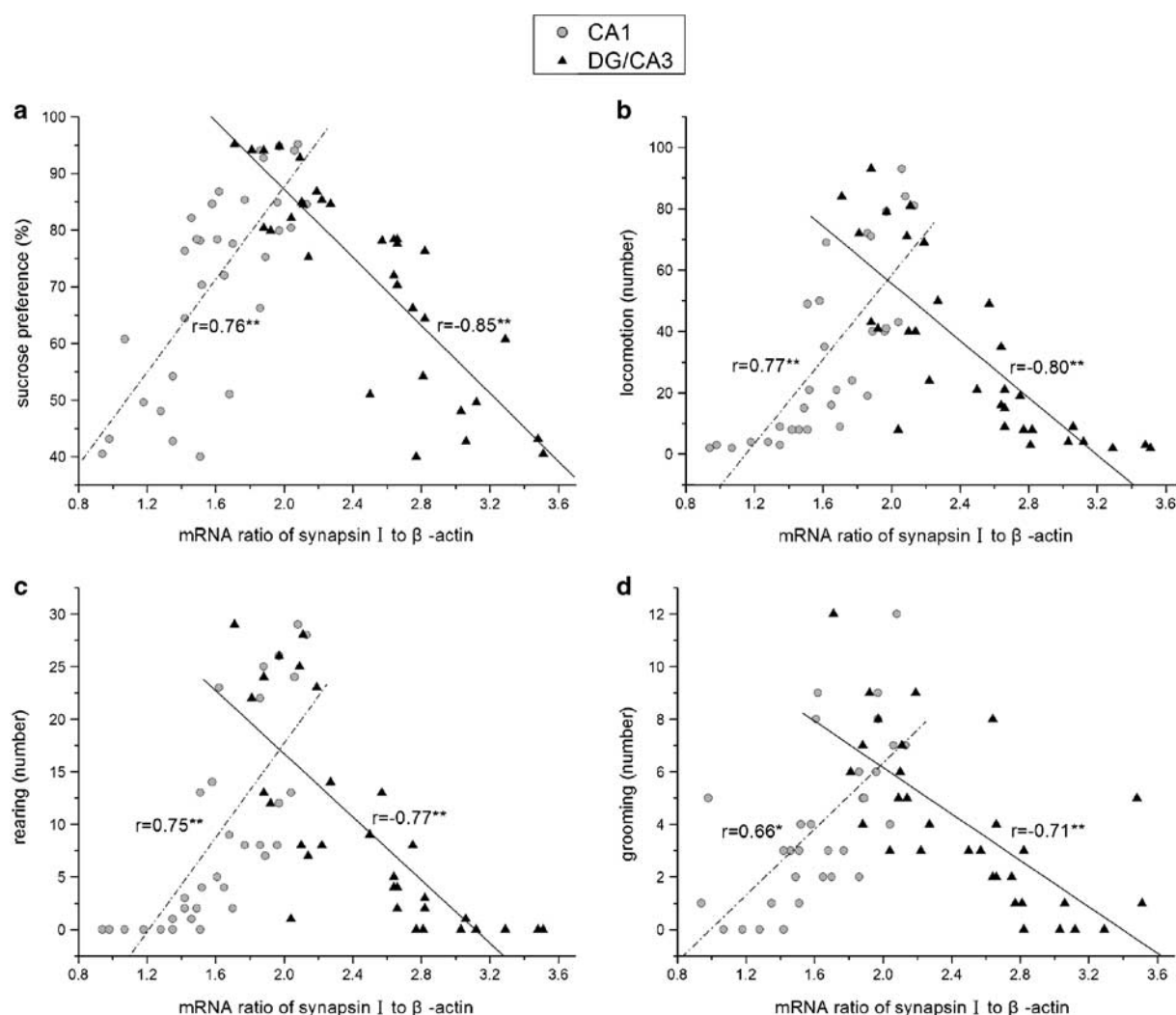
Relationships between Levels of Synapsin I mRNA in CA1 and in DG/CA3

Figure 7 shows significant negative correlation between synapsin I mRNA levels in CA1 and in DG/CA3 ($r = -0.90$,
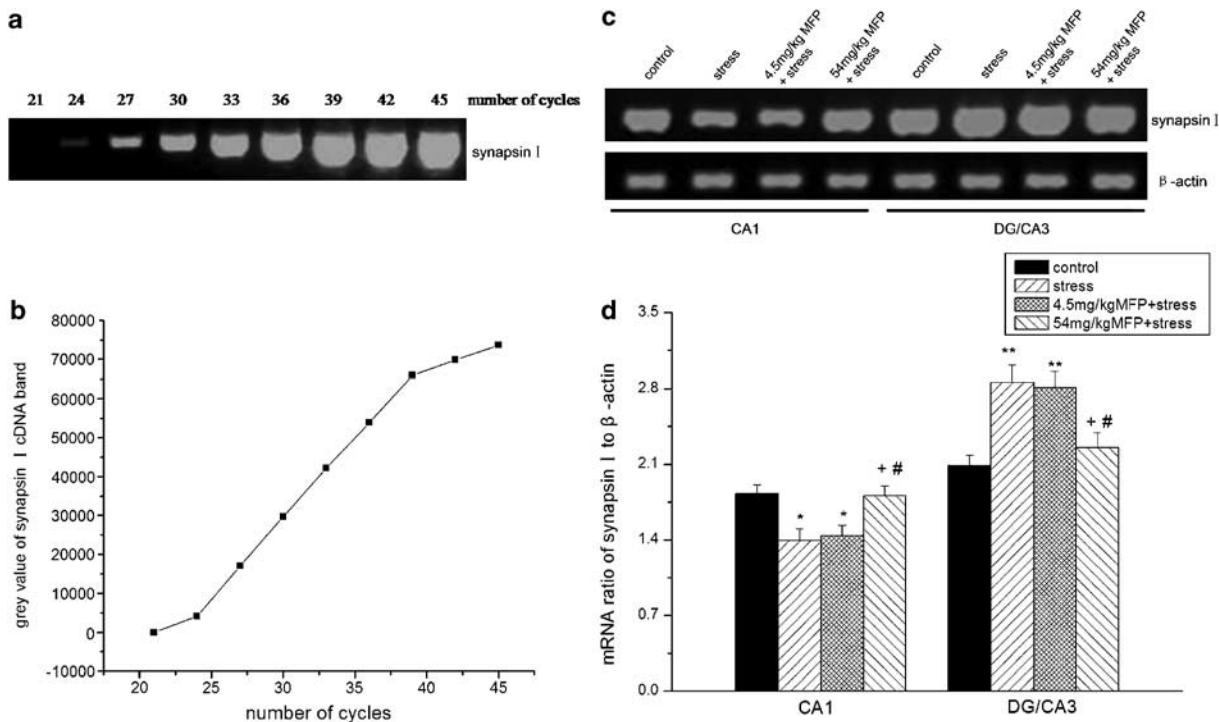
Figure 4 RT-PCR analyses of synapsin I in CA1 and DG/CA3 subfields in hippocampus. (a and b) Show the examination of different PCR cycle numbers and different amounts of synthesized cDNA to evaluate the linearity of the reaction are shown. (c) representative gel pattern of synapsin I and β-actin cDNA bands of CA1 (lane 1, 2, 3, 4) and DG/CA3 (lane 5, 6, 7, 8) subfields of hippocampus of control (lane 1, 5), stress (lane 2, 6), 4.5 mg/kg MFP + stress (lane 3, 7), and 54 mg/kg MFP + stress (lane 4, 8) groups. (d) relative amount (mean ± SEM) of synapsin I mRNA in the CA1 and DG/CA3 subfields of hippocampus is shown. (MFP, mifepristone). Data are expressed as the ratio of the gray level of synapsin I cDNA band to the β-actin cDNA band. Statistical analysis was carried out by one-way ANOVA (for CA1, F(3,28) = 6.114; $p < 0.01$; and for DG/CA3, F(3,28) = 8.372; $p < 0.001$) followed by Tukey test. *$p < 0.01$ *vs* control; **$p < 0.001$ *vs* control; +$p < 0.05$ *vs* stress; #$p < 0.05$ *vs* 4.5 mg/kg MFP + stress.

Figure 5 Correlations between the depression-associated behaviors and synapsin I mRNA levels in hippocampal CA1 and DG/CA3 subfields ($n = 32$). The correlation analysis between sucrose preference (a), locomotion (b), rearing (c), grooming (d), and the levels of synapsin I mRNA in hippocampal DG/CA3 and CA1 subfields was performed by the Pearson's correlation test. Asterisks (*) show the significance of the correlation (two tails). $*p < 0.01$; $**p < 0.001$.

$p < 0.001$). The data very much indicate that the decrease of synapsin I mRNA levels in hippocampal CA1 is correlated with the increase of synapsin I mRNA levels in DG/CA3 subfield.

DISCUSSION

The major finding of this study is that a region-dependent synaptic plasticity alteration occurred in the rat hippocampus after CUMS, with synapsin I increasing in DG/CA3 and decreasing in the CA1 region. Correlation analysis indicated that the synapsin I mRNA level in hippocampal CA1 is highly negatively correlated with that in the DG/CA3 subfield. At the behavioral level, this synaptic plasticity is accompanied by significantly reduced locomotion, rearing, and grooming behavior in open field and sucrose preference, a parameter of anhedonia. Correlation analysis showed that these depression-associated behaviors are related to the alteration of synapsin I. Both abnormal synaptic plasticity and behavior changes rapidly amelio-

rated due to treatment with 54 mg/kg/day mifepristone in the last 7 of 21 days of chronic stress.

The role of environmental stress (so-called 'life events') in triggering the early episodes of depression has long been appreciated. In the present study, we used the rat model of depression induced by CUMS, which mimic a series of life events. CUMS has shown to produce behavioral and neuroendocrine changes in rats similar to major depression in human (Willner *et al*, 1987; De Vry and Schreiber, 1997; Willner, 1997). Patients with psychotic major depression are characterized by abnormalities in the HPA axis activity, for example, elevated urinary free cortisol levels and plasma adrenocorticotropin hormone, as well as the highest rates of nonsuppression on the dexamethasone suppression test (Belanoff *et al*, 2002). The rat model of depression induced by CUMS also shows signs of increased activity in the HPA axis, including adrenal hypertrophy and corticosterone hypersecretion (Muscat and Willner, 1992; Ayensu *et al*, 1995; Willner, 1997), which may support the validity of the CUMS-induced rat model of psychotic depression. Repeated restraint stress was used to study the hippocampal plasticity
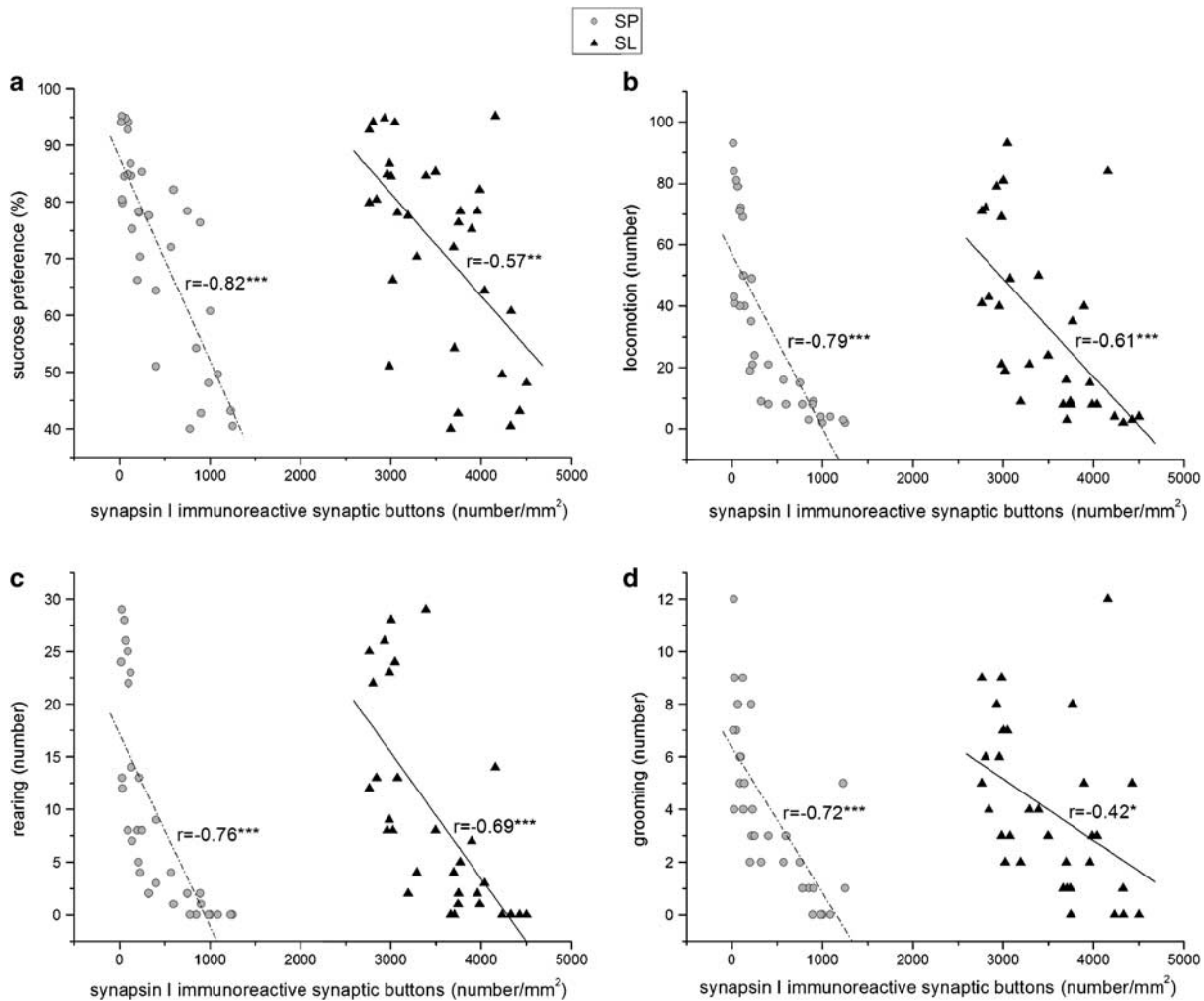
Figure 6 Correlations between the depression-associated behaviors and synapsin I protein levels in SP and SL of hippocampal CA3 subfield ($n = 32$). The correlation analysis between sucrose preference (a), locomotion (b), rearing (c), grooming (d), and synapsin I immunoreactive synaptic button density in SP and SL of hippocampal CA3 subfield was performed by the Pearson's correlation test. Asterisks (*) show the significance of the correlation (two tails). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

induced by stress (Magarinos and McEwen, 1995a; Magarinos et al, 1997; McEwen and Magarinos, 1997; Pawlak et al, 2005). Compared to repeated restraint stress, CUMS produced the same degree of apical dendritic atrophy in CA3 pyramidal neurons (Magarinos and McEwen, 1995b). As an animal model of depression, the advantage of the CUMS model is that this procedure simulates anhedonia, a loss of responsiveness to pleasant events, which is a core symptom of depression and the defining feature of melancholia (Willner et al, 1987; De Vry and Schreiber, 1997; Willner, 1997). We chose the standard use of sucrose preference as the parameter of anhedonia, instead of sucrose consumption, because some data suggested that preference appears to be more sensitive to CUMS than intake (De Vry and Schreiber, 1997).

The initial activity of a rat placed in novel surroundings (ie open-field activity) has long been taken as an indicator of its emotional and psychological state (Hall, 1934); emotional rats show little motor activity, low levels of rearing and grooming, and high defecation. They avoid the center of the field. Their engagement in their immediate surroundings may be termed limited and passive. In

analogy, engagement of both a social and nonsocial nature is also typically disrupted in clinical depression (Hall, 1934; Katz et al, 1981). The effects of CUMS on open-field activity of the rat in our study implicated that the model of depression was established successfully. Amelioration of behaviors due to mifepristone showed, both in sucrose preference and in open-field test in depressed rats, that mifepristone has a curative effect on depression.

To make the mechanism underlying hippocampal dysfunction and its role in depression clear, hippocampal anatomy should be discussed. Granule cells in DG send mossy fibers to the CA3 neurons. The DG–CA3 pathway provides the major excitatory afferent to the hippocampal region inferior, by using glutamate as its major neurotransmitter. The proximal segments of apical dendrites of CA3 pyramidal neurons are covered with complex spines or excrescences that receive mossy fiber input from granule neurons of the DG. CA3 pyramidal neurons have recurrent projections that excite other CA3 neurons. Region CA3 combines the signals within the region with input from the DG and sends connections to the CA1 region through a set of fibers called the Schaffer collateral. The main output
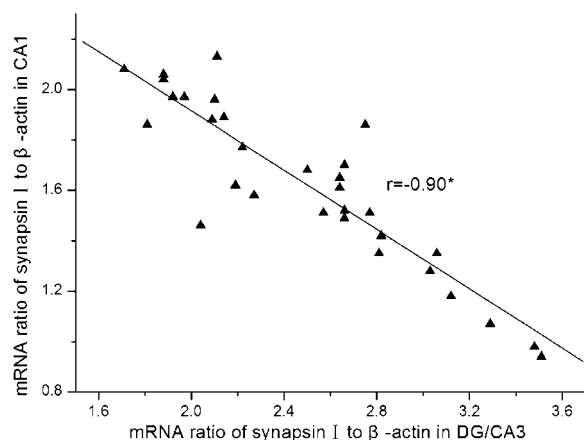
Figure 7 Correlation between the levels of synapsin I mRNA in hippocampal CA1 and DG/CA3 subfields (n = 32). The correlation analysis was performed by the Pearson's correlation test. Asterisk (*) shows the significance of the correlation (two tails) (*$p < 0.001$).

pathways of the hippocampus are the perforant path, the cingulum bundle, and the fimbria/fornix, which all arise from field CA1 and the subiculum (Traub and Miles, 1991; Magarinos et al, 1996). In this study, we found that synapsin I increased in DG/CA3 and decreased in CA1 region, suggesting that synaptic activity is excited within the hippocampal network and the output from the hippocampus is weakened in rats after 21 days of CUMS. Correlation analysis showed highly negative correlations between synapsin I mRNA levels in the CA1 and in the DG/CA3 subfield of the hippocampus. The correlation indicated that the reduced expression of synapsin I in CA1 possibly results from overexcited synaptic activity in the DG/CA3 region, because CA1 receives connections from region CA3 through Schaffer collateral fibers. In general, the hippocampus has an inhibitory role in the regulation of HPA activity. Hippocampal lesions produced elevated cortisol secretion under a variety of stressful and nonstressful conditions (Jacobson and Sapolsky, 1991). The weakened output from hippocampal CA1 is probably responsible for the HPA hyperactivity often observed in patients with depression.

Synapsin I, which represents approximately 6% of the total protein in the highly purified synaptic vesicle fraction, is usually a marker proteins of synaptic vesicles (Huttner et al, 1983). It is primarily associated with synaptic vesicles and involved in the storage and release of neurotransmitters (De Camilli et al, 1990; Thiel, 1993). Phosphorylation of synapsin I reduces its binding to vesicles and/or cytoskeletal structures, enabling vesicles to be released during a presynaptic depolarization (De Camilli et al, 1990). Synapsin I mutant mice exhibited impairment of synaptic vesicle clustering and of synaptic transmission. Release of glutamate from nerve endings was markedly decreased in the synapsin I mutant mice (Li et al, 1995). The total synapsin (Ia, IIa, and IIIa) protein was reduced in the hippocampus of patients with bipolar disorder, another type of depression besides major depression, implying that altered or reduced synaptic function in the hippocampus may be involved in this disorder (Vawter et al, 2002). In our study, we found that the density of synapsin I immunoreactive synaptic buttons increased significantly in the CA3

subfield after 21 days CUMS. Considering the corticosterone hypersecretion in the rat model of depression induced by CUMS (Ayensu et al, 1995), this finding is in accordance with the report that corticosterone increases the amount of synapsin I in rat hippocampus (Nestler et al, 1981). At the mRNA level, we found that synapsin I correspondingly increased in DG/CA3, but decreased in CA1. Correlation analysis between behaviors and synapsin I demonstrated that depression-associated behaviors are related to the alteration of synapsin I both in mRNA and protein levels. These behaviors are negatively correlated with synapsin I in DG/CA3 and positively correlated with synapsin I in the CA1 subfield of the hippocampus. The increased synapsin I immunoreactive synaptic buttons which reveal MFT distribution in CA3 induced by CUMS is consistent with the report from electron microscopy studies that repeated restraint stress causes a reorganization of synaptic vesicles within the MFT (Magarinos et al, 1997). Increased synapsin I in CA3 induced by CUMS is probably the reason for the increased glutamate release from the presynaptic terminal, as phosphorylated synapsin I enables vesicles to be released. The increased synapsin I may be an appearance of an increased EAAs transmitter cluster in the presynaptic vesicle, as synapsin I is a marker protein of synaptic vesicles and transmitter amino acids are significantly concentrated in synaptic vesicles. A decreased mRNA level of synapsin I in the CA1 subfield suggested that the output synaptic activity from the hippocampal CA1 to the neuroanatomically connected region (eg hypothalamus) was inhibited by 21 days of CUMS. This result is consistent with the finding that in the CA1 region expression of GAP-43, which usually acts as a marker for synaptic plasticity and is associated with axon regeneration, was decreased by chronic repeated restraint stress (Pawlak et al, 2005).

Although mossy fibers originating from granular cells of the DG provide a strong input of EAAs into the hippocampal CA3 subfield, MFTs are usually not considered to be the main source of EAAs toxicity to CA3 neurons because mossy fibers usually exist in SL, which does not express N-methyl-D-aspartate (NMDA) receptors (Monaghan et al, 1983). In our study, synapsin I immunohistochemistry showed that the abundant invasion of mossy fibers from SL to SP was induced by 21 days of CUMS. These aberrant fibers could have physiological consequences in CA3 SP neurons because the number of synapsin I immunoreactive synaptic buttons in SP of the CA3 region remains 6–7 times greater than that in controls. The correlation analysis in our study also implied that the increased synaptic button in SP rather than in SL of hippocampal CA3 is correlated with depression-associated behaviors. The abnormally abundant MFT within CA3 induced by CUMS in our study coincides with the finding that early life administration of corticotropin-releasing hormone, which reproduces the effects of early life stress, causes the abnormally abundant MFT (Brunson et al, 2001).

Our own results, as well as the studies of others led us to conclude that chronic stress induces synapsin I expression and synaptic formation in DG/CA3 and the prolongation of mossy fibers. Synergistically affected by increased glucocorticoids induced by chronic stress, a great deal of glutamate released from these synaptic terminals, which extensively activate the NMDA receptor and lead to an

influx of calcium through its channel into the postsynaptic terminals. Thus, the postsynaptic neurons in CA3 will be intracellularly calcium-overloaded and then damaged, including dendrite atrophy, as seen in other studies (Krugers et al, 1993; Magarinos and McEwen, 1995a; Magarinos et al, 1996). The synthesization of synapsin I was then reduced in the CA1 subfield, because CA1 receives connections from region CA3 through Schaffer collateral fibers. The negative correlation between synapsin I levels in CA1 and in DG/CA3 was supported by correlation analysis in our study. Alternatively, the decreased synthesization of synapsin I may be a direct result of the high level of glucocorticoid-mediated neuronal toxicity induced by chronic stress.

The steroid mifepristone is a remarkably active anti-progesterone and at high concentrations also an antagonist of GR action (Cadepond et al, 1997). The treatment of depression needs high dose of mifepristone, 600 or 1200 mg/day. The low dose, 50 mg/day, which is thought to have significant antiprogesterone effects but no substantial effects on cortisol and GR could not ameliorate the symptoms of depression (Belanoff et al, 2002). Corresponding to the dose of 50 and 600 mg/day for humans, the dose in rat is 4.5 and 54 mg/kg/day, respectively. As expected, 54 mg/kg/day mifepristone restored alteration in both behavior and synaptic plasticity in rats with depression induced by CUMS, whereas 4.5 mg/kg/day had no effect. The result suggests that glucocorticoids may participate in the hippocampal plasticity through manipulating synapsin I expression in the hippocampus. The blockade of GR restores the abnormal synaptic alteration. In a dose sufficient to block GR, mifepristone was able to repress the increases of synapsin I in DG/CA3. Considering the fact that the DG–CA3 pathway provides the major excitatory afferent to the hippocampal region inferior by using glutamate as its major neurotransmitter, the result is consistent with the view that glucocorticoids have a permissive role and act synergistically with EAAs in producing neuronal death in cell culture and in vivo (Magarinos et al, 1996). CUMS induced the decrease of the synapsin I mRNA level in CA1, which was repaired rapidly by treatment with mifepristone in the present study. This finding seems to present a molecular mechanism for the report that chronic stress hampers synaptic potentiation in hippocampal CA1 of brain slices, and mifepristone restores the ability of synapses to display synaptic potentiation (Krugers et al, 2006). There are a few reports about the effects of antidepressants on synapsin I in rat hippocampus. Chronic treatment with tranylcypromine, but not desipramine or fluoxetine, decreases synapsin I mRNA expression in rat hippocampal DG, CA3, and CA1 (Rapp et al, 2004), and imipramine or fluvoxamine decreases synapsin I immunoreactivity in rat hippocampus (Iwata et al, 2006). Another study showed that injecting rats with lithium for 30 days produces no changes in total synapsin in the hippocampus (Vawter et al, 2002). The effects of mifepristone on the mRNA level of synapsin I are not quite the same as tranylcypromine. Mifepristone decreases synapsin I in DG/CA3 and increases it in CA1 subfield in hippocampus, whereas tranylcypromine decreases synapsin I in both DG/CA3 and CA1 subfields (Rapp et al, 2004). This difference may be explained by using different animal models.

Contrasted with the normal unhandled rats used in the study of Rapp et al (2004), the rat model of depression induced by chronic stress was used in the studies of Krugers et al (2006) and in our own study. Considering EEAs in DG/CA3 is a major risk factor for the hippocampal atrophy induced by chronic stress (Watanabe et al, 1992; Magarinos et al, 1996), the selective inhibition of synapsin I by mifepristone may have a unique advantage compared with the general inhibition of synapsin I in both DG/CA3 and CA1, as shown by a classic antidepressant such as tranylcypromine.

Although depression is a psychiatric disorder that affects ~15% of the worldwide population, we know little about the underlying fundamental biology. From the angle of synaptic plasticity, the present experiments formulated a part of the molecular mechanism underlying the hippocampal dysfunction in the animal model of depression induced by CUMS and tried to explain the molecular mechanism with which mifepristone treats depression. Our study could help clarify the pathogenesis of depression and thus benefit clinical therapies.

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