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ORIGINAL PAPER

Modification of Hippocampal Markers of Synaptic Plasticity by Memantine in Animal Models of Acute and Repeated Restraint Stress: Implications for Memory and Behavior

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Abstract Stress is any condition that impairs the balance of the organism physiologically or psychologically. The response to stress involves several neurohormonal consequences. Glutamate is the primary excitatory neurotransmitter in the central nervous system, and its release is increased by stress that predisposes to excitotoxicity in the brain. Memantine is an uncompetitive N-methyl D-aspartate glutamatergic receptors antagonist and has shown beneficial effect on cognitive function especially in Alzheimer's disease. The aim of the work was to investigate memantine effect on memory and behavior in animal models of acute and repeated restraint stress with the evaluation of serum markers of stress and the expression of hippocampal markers of synaptic plasticity. Forty-two male rats were divided into seven groups (six rats/group): control, acute restraint stress, acute restraint stress with Memantine, repeated restraint stress, repeated restraint stress with Memantine and Memantine groups (two subgroups as positive control). Spatial working memory and behavior were assessed by performance in Y-maze. We evaluated serum cortisol, tumor necrotic factor, interleukin-6 and hippocampal expression of brain-derived neurotrophic factor, synaptophysin and calcium-/calmodulin-dependent protein kinase II. Our results revealed that Memantine improved spatial working memory in repeated stress, decreased serum level of stress markers and modified the hippocampal synaptic plasticity markers in both patterns of stress exposure; in ARS, Memantine upregulated the expression of synaptophysin and brain-derived neurotrophic factor and downregulated the expression of calcium-/calmodulin-dependent protein kinase II, and in repeated restraint stress, it upregulated the expression of synaptophysin and downregulated calcium-/calmodulin-dependent protein kinase II expression.

Keywords Restraint · Memantine · Memory · Behavior · Synaptic plasticity

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Introduction

Stress is a term describes a wide range of strong stimuli that may be internal or external and can cause a physiological response called the general adaptation syndrome. Exposure to both chronic and acute stress has substantial effects on learning and memory (Kim and Diamond 2002).

Acute stress initiates the release of corticotrophin releasing factor (CRF) from CRF neurons in the cortico-limbic areas of the brain. CRF then activates the locus coeruleus, the main noradrenergic nucleus in the brain, in addition to the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland and glucocorticoids from the adrenal cortex (Gray 1993). Although chronic stress produces different responses according to the coping strategies developed by the subject (Kant et al. 1987), chronic stress frequently results in a hypersecretion of adrenal glucocorticoids and a sustained activation of the central and peripheral sympathetic systems (Irwin et al. 1991).

As a part of stress response, cell products from an activated immune system, predominately the cytokines tumor

necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6, stimulate CRH secretion and, hence, activate both the HPA axis and the sympathetic nervous system (Elenkov et al. 2000).

The effects of stress on the central nervous system are prominent on the hippocampus where it substantially influences neuronal excitability and long-term potentiation (LTP) (Diamond et al. 1994). The hippocampus has a high density of glucocorticoid receptors (McEwen 1999). These receptors exert a variety of effects besides the autoregulation of the stress response, such as influencing mood, learning and memory (de Kloet 2000). Moreover, stress-related increases in glucocorticoids have been associated with cell death and cognitive impairments (Sapolsky et al. 1984; McEwen 1999).

The N-methyl-D-aspartate (NMDA) glutamate receptor is the predominant molecular device for controlling synaptic plasticity and memory function (Cull-Candy 2007). NMDA receptor activity is modulated by the phosphorylation catalyzed by several protein kinases including protein kinase A, protein kinase C and calcium-/calmodulin-dependent protein kinase II (CaMKII) (Gardoni et al. 1999).

CaMKII was identified as a major postsynaptic density protein with two isoforms: CaMKIIα and CaMKIIβ (Gaertner et al. 2004).CaMKII activity is essential for normal NMDA receptor-dependent forms of LTP in the hippocampal CA1 region and hippocampus-dependent behaviors, such as spatial learning and memory (Lisman et al. 2002).

Neurons in the hippocampus are maintained by proteins called neurotrophins, particularly brain-derived neurotrophic factor (BDNF). BDNF is a small dimeric protein that binds with a tyrosine kinase receptor and tropomyosin-related kinase B (TrkB) (Yan et al. 1997). In the hippocampus, a similar distribution of BDNF mRNA was described for rodents and primates (Phillips et al. 1990). Similar to this pattern of BDNF mRNA expression, BDNF protein can be detected throughout the CNS, with highest expression levels in the hippocampal formation and cerebral cortex (Conner et al. 1997).

Furthermore, synaptophysin (SYP) is an integral Ca2+-binding synaptic vesicle membrane protein (Rehm et al. 1986) abundant in synapses of the vertebrate brain (Wiedenmann and Franke 1985), which can be used as a general marker protein of presynaptic nerve endings (Masliah et al. 1994), and it is required for vesicle fusion (Greengard et al. 1993). Decrease in SYP is consistent with a decrease in synaptic density (Eastwood and Harrison 2001). Synaptic vesicle proteins have also been identified as possible factors involved in the pathophysiology of psychiatric disorders, such as schizophrenic psychoses and depression (Horner et al. 1999).

Excitotoxicity is the pathological process by which nerve cells are damaged by excessive stimulation by neurotransmitters such as glutamate and similar substances (Mehta et al. 2013). Stress is supposed to increase glutamate neurotransmission predisposing to excitotoxicity and neuronal damage (Jezova 2005).

The aim of this study was to investigate the effect of blocking NMDA receptors by Memantine on working spatial memory, serum markers of stress and hippocampal expression of BDNF, CaMKII and synaptophysin as markers of synaptic plasticity in animal models of acute and repeated restraint stress.

Materials and Methods

The protocol for this work (experimental steps, animal handling, sampling and scarification) was approved by the Institutional Ethics Committee. Animal housing and Y-maze test were performed at MSA university, while biochemical analysis of serum and brain samples from the experimental animals was done at Kasr Al Ainy Faculty of Medicine, Cairo University.

Forty-two male Wistar rats, 3–4 months old weighing about 100–130 gm, obtained from MSA university animal house constituted the animal model in this study. All rats were provided with standard laboratory chow and water and housed (3 rats/cage) in accordance with institutional animal care policies. Rats were habituated to the laboratory environment for 1 week before experimental work.

Experimental Groups

Rats were divided into the following groups (six rats/group):

Group I (control) Received distilled water per oral (p.o).

Group II; acute restraint stress (ARS) group Exposed to ARS.

Group III (Memantine + ARS group) Received Memantine (20 mg/kg, p.o) (Quan et al. 2011), obtained as commercial tablets (Memexa tablet, 10 mg/tablet, Copad Egypt for Trade and Pharmaceutical Industries, Egypt) starting from the habituation period till end of the work and exposed to ARS.

Group IV (Repeated restraint stress group) Exposed to repeated restraint stress.

Group V (Memantine + Repeated restraint stress group) Treated by Memantine (20 mg/kg, p.o) starting from the habituation period till end of the work and exposed to repeated restraint stress.

Group VI (Memantine group) Treated by Memantine (20 mg/kg/day, p.o). This group was further subdivided into two subgroups (positive control):

VIa: treated by Memantine for 8 days. This group represented the positive control for ARS groups

VIb: treated by Memantine for 15 days. This group represented the positive control for repeated stress groups.

Acute and Repeated Restraint Stress

Rats were restrained once for 2 h (10:00 a.m. to 12:00 p.m.) in ARS groups and restrained daily (2 h) for 7 days in repeated restraint stress groups (Yuen et al. 2013) by tightly tying all four limbs to a grid using a quartz-pasted tape (Kumar et al. 2010).

Assessment of Spatial Memory

Working spatial memory was evaluated by Y-maze twice for every group: at the start of the work and at the end of the work (for acute and repeated stress groups, the test was performed in the second time 24 h after exposure to the acute stress in ARS groups and 24 h after last day of restraint stress in repeated stress groups).

Y-Maze Task

Rodents tend to choose alternate arms in Y-maze spontaneously, and this tendency can be used to test spatial working memory.

Y-maze used in the current study was constructed using wood and the three arms made of equal size (60 cm × 12 cm × 25 cm). During test, we put the rat in the center of the maze and allow for exploration of the three arms for 8-min duration. Activity of the rat during the test period was recorded by windows movie maker (Windows 7) via web camera, and the recorded videos were analyzed manually.

Any three consecutive choices of three different arms were considered as a correct choice. The alternation score was calculated by dividing the total number of alternations by the total number of choices minus 2 × 100 (Arai et al. 2001). In addition to calculating the alternation score, we monitored rearing, grooming and freezing behavior during the 8 min of the test.

Biochemical Analysis

At the end of experimental period just after Y-maze test, animals were anesthetized by ether inhalation, and blood samples were collected from retro-orbital venous sinuses for biochemical measurement of serum cortisol, IL-6 and TNF-α. Serum TNF-α and IL-6 were measured by using ELISA (Quantikine R&D system USA), while cortisol was measured by kit supplied by (Cusabio USA) according to the manufacturer's instructions.

Rats were sacrificed and then decapitated, and brains were extracted and dissected to the level of the hippocampus for mRNA expression by polymerase chain reaction (PCR) and protein expression by Western blot analysis for CaMKII, BDNF and synaptophysin.

Detection of CaMKII, BDNF and Synaptophysin Gene Expression by Real-time PCR

RNA Extraction and cDNA Synthesis

Total RNA was isolated from brain tissue using (RNeasy; Qiagen Inc., Valencia, CA), according to the manufacturer's protocols. The RNA concentration was determined at 260 nm, and its integrity was checked by 1 % agarose gel electrophoresis in the presence of ethidium bromide with UV visualization. First-strand cDNA was synthesized from 4 μg of total RNA using an oligo (dT) 12–18 primer and Superscript™ II RNase Reverse Transcriptase (Invitrogen, USA). Samples were stored at −20 °C.

Real-time PCR

The quantification of the selected genes by real-time PCR was performed on ABI PRISM 7900 HT detection system (Applied Biosystems) using the primers shown in Table 1. Every reaction consisted of 2 μl cDNA, 1 μl of each primer (400 nM) and 21 μl reaction buffers (Platinum SYBR Green) (total reaction volume 25 μl) (Invitrogen). Real-time PCR cycles consisted of 2 min at 50 °C, 4 min at 95 °C for polymerase activation, 40 cycles of 94 °C for 15 s and 60 °C for 1 min (40 cycles). β-Actin of each sample served as intrinsic control. The threshold cycle (CT) of each sample was normalized to β-actin. Relative quantification analysis was carried out with the Applied Biosystem software version 1.7. The results are expressed as a normalized ratio.

Protein Expression of CaMKII, BDNF and Synaptophysin by Western Blot Analysis

Protein Extraction

For preparation of samples for Western blotting, tissues were homogenized in the homogenization buffer containing Tris–HCl 50 mM (pH: 7.4), 2 mM EDTA, 10 mM NaF, 10 mM β-glycerol phosphate, 1 mM PMSF and complete protease inhibitor cocktail using polytron homogenizer in ice. After centrifugation at 10,000×*g* for 15 min at 4 °C, supernatants were collected on ice, protein contents were determined using Bio-Rad Protein Assay Kit and all concentrations were

Table 1 Primer sequences used for RT-PCR

Primer	Sequence
Synaptophysin	Forward primer 5′-TCAGGACTCAACACCTCAGTGG-3′
	Reverse primer 5′-AACACGAACCATAAGTTGCCAA-3′
Calmodulin	Forward primer 5′-TATGCCACGCCCTTTGAG-3′
	Reverse primer 5′-CACAGCAGGATGTAGGAGATGA-3′
BDNF	Forward primer 5′-ACC CTG AGT TCC ACC AGG TG-3′
	Reverse primer 5′-TGG GCG CAG CCT TCA T-3′
β-actin	Forward: 5′-CTA CAA TGA GCT GCG TGT GG-3′
	Reverse: 5′-CAG TCA GGA TCT TCA TGA GG-3′

BDNF brain-derived neurotrophic factor

adjusted to 10 mg/ml. Equal volumes of SDS sample buffer containing 4 % w/v SDS, 10 % v/v 2-ME, 100 mM Tris-base, 0.2 % w/v BPB and 20 % v/v glycerol were added to the samples and incubated in boiling water for 5 min. Homogenates were stored at −80 °C until use.

Western Blot

Immunoblotting analysis was performed on the prepared samples to assess the levels of tau, MAPK and p-CREB. Briefly, samples containing equivalent amounts of 50 μg of total protein were loaded to SDS-PAGE gel and then transferred to PVDF membrane by electrophoresis. Blots were blocked with 5 % nonfat dry milk in TBST for 3 h at room temperature. After blocking, blots were probed with specific primary antibodies: mouse monoclonal anti-serum against calmodulin (Cell Signaling, USA), rabbit polyclonal antiserum against synaptophysin (Abcam, USA) and BDNF (Abcam, USA), and mouse and rabbit monoclonal anti-serums against β-actin (Cell Signaling, USA) at 1:1,000 dilutions for 2 h at room temperature. Membranes were washed 3 times with 0.1 % Tween and TBST.

Then, blots were incubated with antimouse and rabbit horseradish peroxidase labeled IgG (Cell Signaling, USA) as secondary antibodies at 1:3,000 dilutions for 1 h at room temperature. Finally, protein bands were visualized using an enhanced chemiluminescence reagent (Pierce ECL Western blotting substrate) and Alliance Gel-doc (Alliance 4.7 Gel doc, UVtec UK). UV Tec software (UK) was used to semi-quantify protein bands intensities. All blots were normalized against intensities of corresponding β-actin protein bands.

Statistical Analysis

All statistical calculations were done using computer programs Microsoft Excel 2010 (Microsoft Corporation, NY, USA) and SPSS 21(IBM SPSS Statistics 21; IBM Corporation, New York, USA) for Microsoft Windows. Data were analyzed and expressed as mean ± standard deviation (Mean ± SD). Comparison of quantitative variables between the studied groups was done using analysis of variance (ANOVA) with Bonferroni post Hoc test or Kruskal–Wallis test with Wilcoxon signed-rank test according to the data normality of distribution. Correlations between the measured parameters have been calculated by Spearman's correlation Results were considered statistically significant at $p \leq 0.05$ (Altman 2005).

Results

Cognitive and Behavioral Parameters Measured by Y-Maze

- The group exposed to ARS showed significant (*P* value ≤0.05) decrease in alternation score (Fig. 2) and significant (*P* value ≤0.05) increase in freezing frequency compared to control group (Fig. 3). Memantine therapy with ARS resulted in significant (*P* value ≤0.05) increase in number of arm entries (Fig. 1) and grooming frequency (Fig. 3) compared to control group and ARS groups and showed significant decrease (*P* value ≤0.05) in freezing frequency (Fig. 3)compared to ARS group.
- Repeated stress group showed significant decrease (*P* value ≤0.05) in alternation score (Fig. 2) and rearing frequency (Fig. 3) and significant increase (*P* value ≤0.05) in freezing frequency (Fig. 3) compared to control group.
- Memantine therapy with repeated stress caused significant increase (*P* value ≤0.05) in number of arm entries (Fig. 1), alternation score (Fig. 2), rearing frequency (Fig. 3), grooming frequency (Fig. 3) and significant decrease (*P* value ≤0.05) in freezing frequency (Fig. 3) compared to repeated stress group.

Serum Markers of Stress

As Revealed from Table 2

- ARS and repeated stress groups showed significant increase (*P* value ≤0.05) in serum cortisol, IL-6 and TNF-α compared to control group.

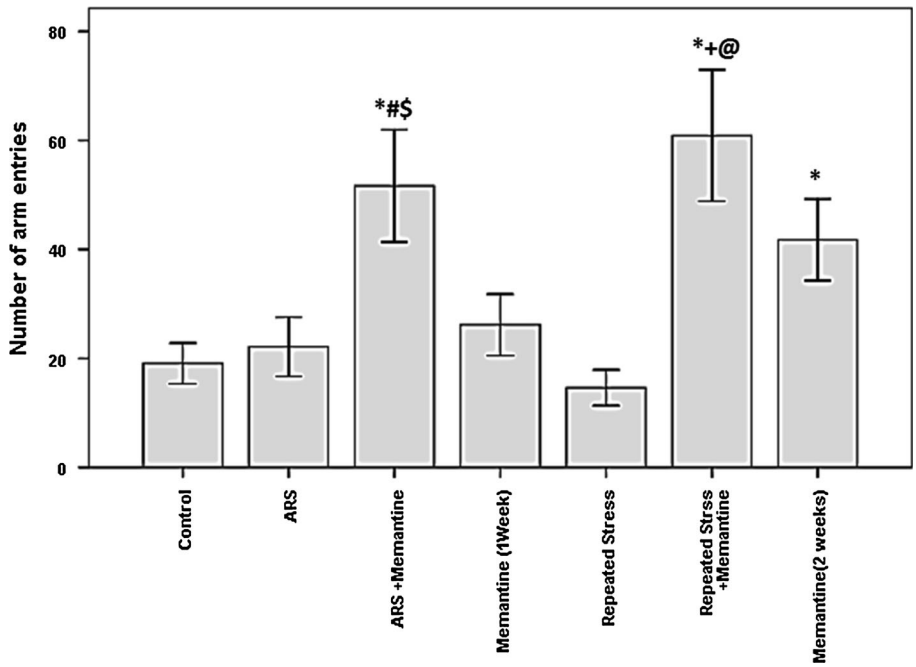

Fig. 1 Number of arm entries in Y-maze task among the studied groups. *Significant compared to control, #significant compared to ARS, $significant compared to Memantine (1 week), +significant compared to repeated stress, @significant compared to memantine (2 weeks) at *P* value ≤0.05. Data are presented as mean ± SD

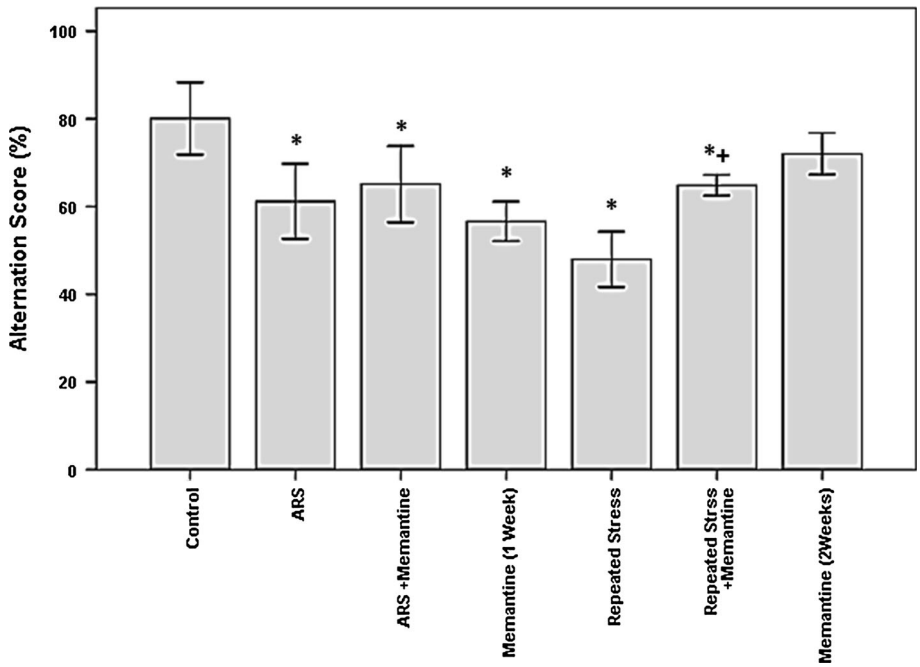

Fig. 2 Alternation score in Y-maze task among the studied groups. *Significant compared to control, +significant compared to repeated stress at *P* value ≤0.05. Data are presented as mean ± SD

- Group treated with Memantine with exposure to ARS showed significant decrease (*P* value ≤0.05) in serum cortisol, IL-6 and TNF-α compared to ARS group.
- Memantine therapy with repeated stress resulted in significant decrease (*P* value ≤0.05) in serum cortisol, IL-6 and TNF-α compared to repeated stress group.

Hippocampal Expression of Synaptic Plasticity Markers

As revealed from Tables 3 and 4 and Figs. 4, 5, 6

- ARS caused significant increase (*P* value ≤0.05) in relative expression of CaMKII and significant decrease

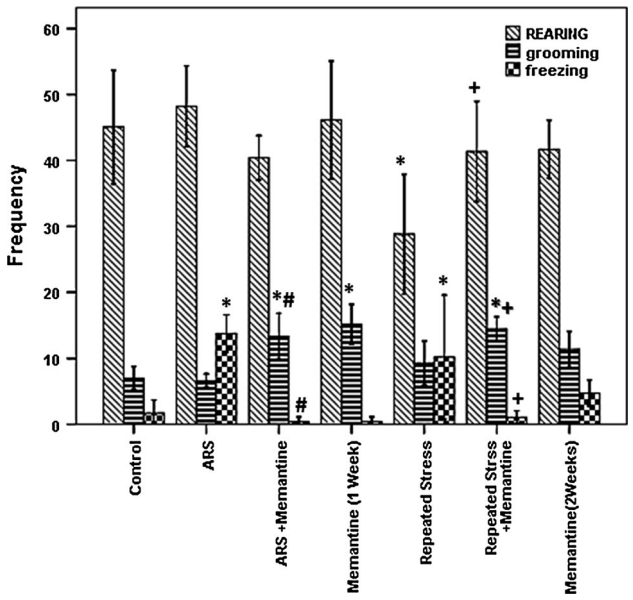

Fig. 3 Rearing, grooming and freezing frequencies in the studied groups. *Significant compared to control, #significant compared to ARS, +significant compared to repeated stress at *P* value ≤0.05. Data are presented as mean ± SD

(*P* value ≤0.05) in relative expression of BDNF and synaptophysin.

- Memantine administration with ARS resulted in significant increase (*P* value ≤0.05) in relative expression of BDNF and synaptophysin and significant decrease (*P* value ≤0.05) in relative expression of CaMKII compared to ARS group.
- Repeated stress caused significant increase (*P* value ≤0.05) in relative expression of CaMKII and significant decrease (*P* value ≤0.05) in relative expression of synaptophysin compared to control group; however, BDNF did not show significant difference compared to control group.
- Memantine treatment with exposure to repeated stress caused significant decrease (*P* value ≤0.05) in relative expression of CaMKII and significant increase (*P* value ≤0.05) in relative expression of synaptophysin. The relative expression of BDNF did not show significant difference when compared to repeated stress group.
- No statistical significant difference was found in the expression of the three markers (CaMKII, BDNF and synaptophysin) in Memantine groups compared to control group.

Correlations Between the Measured Parameters

- In the present study, we showed significant negative correlation between alternation score and serum cortisol, IL-6 and TNF-α ($r = -0.519$, -0.531, -0.520, respectively; *P* value ≤0.001).

Table 2 Serum markers of stress in the studied groups

	Control	Acute stress	Acute stress + memantine	Memantine (1 week)	Repeated stress	Repeated stress + memantine	Memantine (2 weeks)
Serum cortisol (ng/ml)	4.47 ± 1.9	18.1 ± 3.1*	10.8 ± 1.64$^{\#,\$}$	6.3 ± 2.16	28.1 ± 7.73*	11.4 ± 2.5$^{+,@}$	5.60 ± 2.07
Serum IL-6 (pg/ml)	42.3 ± 1	151.42 ± 33*	82.2 ± 16.4$^{\#,\$}$	45.18 ± 11.97	195.7 ± 17.33*	122.9 ± 11.70$^{+,@}$	54.9 ± 18.4
Serum TNF-α (pg/ml)	39.69 ± 9.43	104.51 ± 15.89*	76.54 ± 20.58$^{\#,\$}$	47.75 ± 10.80	164.43 ± 31.59*	103 ± 10.32$^{+,@}$	48.08 ± 10.82

Data are presented as Mean ± SD (n = 6/group)

TNF tumor necrotic factor

*Compared to control

Compared to ARS

$ Compared to Memantine (1 week)

+ Compared to repeated stress

@ Compared to Memantine (2 weeks) at *P* value ≤0.05

Table 3 Hippocampal expression of synaptic plasticity markers in the studied groups measured by **p**olymerase chain reaction

	Control	Acute stress	Acute stress + memantine	Memantine (1 week)	Repeated stress	Repeated stress + memantine	Memantine (2 weeks)
Relative expression of CaMKII mRNA	.113 ± .018	.750 ± .132*	.275 ± .0413$^{\#,\$}$	.135 ± .039	1.655 ± .417*	.720 ± .147$^{+,@}$	.118 ± .036
Relative expression of BDNF mRNA	1.31 ± .251	.27 ± .132*	.73 ± .125$^{\#,\$}$	1.18 ± .213	1.26 ± .281	.57 ± .214$^{+,@}$	1.19 ± .492
Relative expression of synaptophysin mRNA	10.233 ± .136	2.566 ± .372*	5.593 ± .654$^{\#,\$}$	9.473 ± .712	1.960 ± .705*	7.063 ± .952$^{+,@}$	10.1 ± .502

Data are presented as Mean ± SD (n = 6/group)

CaMKII calcium-/calmodulin-dependent protein kinase, *BDNF* brain-derived neurotrophic factor

*Compared to control

$^{\#}$ Compared to ARS

$^{\$}$ Compared to Memantine (1 week)

$^{+}$ Compared to repeated stress

$^{@}$ Compared to Memantine (2 weeks) at *P* value ≤0.05

- We revealed a significant positive correlation between relative expression of BDNF and synaptophysin and alternation score ($r = .363$ and .581, respectively; $P \leq .05$) and significant negative correlation between relative expression of CaMKII and alternation score ($r = -.586$; $P \leq .01$).
- There was a significant positive correlation between relative expression of CaMKII and serum cortisol, TNF-α and IL-6 ($r = .856$, .906, .875, respectively; $P \leq .01$ for all). However, there was significant negative correlation between relative expression of BDNF and serum cortisol, TNF-α and IL-6 ($r = -.721$, $-.769$, $-.719$, respectively; $P \leq .01$ for all). Furthermore, there was significant negative correlation between relative expression of synaptophysin and serum cortisol, TNF-α and IL-6 ($r = -.797$, $-.862$, $-.816$, respectively; $P \leq .01$ for all).

Discussion

Stress influences synaptic plasticity, neuron morphology, neurotoxicity and neurogenesis that affect learning and memory. Stress and stress hormones impair LTP in the hippocampus (Kim and Diamond 2002).

Corticosterone is the principal glucocorticoid synthesized by the adrenal cortex, and stress increases its synthesis and secretion. The hippocampus is enriched with both Type-I mineralocorticoid receptors (MR) and Type-II glucocorticoid receptors (GR), and corticosterone actions through these receptors mediate stress effects on hippocampal plasticity (Reul and de Kloet 1985).

Acute exposure to stress or administration of glucocorticoids rapidly increases glutamate release in these brain areas (Popoli et al. 2011), and also glutamatergic signaling is involved in the control of the HPA axis such that intraventricular administration of glutamate increases activity of the HPA system (Zelena et al. 2005) and contributes to stress-induced hormone release (Tokarev and Jezova 1997).

Our results revealed significant decrease in alternation score in both ARS and repeated stress groups. Memantine administration with stress had more beneficial effect on spatial memory in case of repeated stress as revealed from significant improvement of alternation score in Y-maze test. In agreement with our results; Bowman et al. (2001) demonstrated that rats exposed to chronic stress showed impaired performance in radial arm maze task with decrease in hippocampal apical dendritic branching and total dendritic length (Bowman et al. 2001).

Maekawa et al. (2009) reported that a single injection of Memantine in high doses promoted proliferation of neural progenitor cells and production of mature granule neurons in adult hippocampus in mice.

Our results regarding Memantine positive impact on spatial memory may be explained by previous findings that agents antagonists NMDA receptor induced increase in BDNF levels, suggesting the involvement mediated by

Table 4 Hippocampal expression of synaptic plasticity markers in the studied groups measured by Western Blot

	Control	Acute stress	Acute stress + memantine	Memantine (1 week)	Repeated stress	Repeated stress + memantine	Memantine (2 weeks)
Relative expression of CaMKII mRNA	1.37 ± .35	8.3367 ± 1.422*	3.07 ± .749$^{\#,\$}$	1.1 ± .104	12.26 ± .83*	4.63 ± 0.866$^{+,@}$	1.07 ± .08165
Relative expression of BDNF mRNA	1.05 ± .037	.303 ± .065*	.725 ± .113$^{\#,\$}$	1.266 ± .250	1.11 ± .1	.53 ± .13$^{+,@}$	1.21 ± .177
Relative expression of synaptophysin mRNA	1.42 ± .379	.338 ± .087*	.758 ± .121$^{\#,\$}$	1.233 ± .326	.121 ± .023*	.58 ± .157$^{+,@}$	1.045 ± .036

Data are presented as Mean ± SD (n = 6/group)

CaMKII calcium-/calmodulin-dependent protein kinase, *BDNF* brain-derived neurotrophic factor

*Compared to control

Compared to ARS

$ Compared to Memantine (1 week)

+ Compared to repeated stress

@ Compared to Memantine (2 weeks) at *P* value ≤0.05

glutamate acting through NMDA receptors (Marvanová et al. 2001; Rogóz et al. 2008).

Opposite to our result, Quan et al. (2011) concluded that treatment with Memantine impaired spatial memory in rats. However, it improved chronic unpredictable stress-induced changes in prefrontal cortical synaptic plasticity and reversal learning. Also, Babic et al. (2012) demonstrated negative effects of Memantine under stress conditions.

Anxiety behaviors of the animal and working memory are related both anatomically and functionally: The posterior hippocampus has a preferential role in spatial learning and memory, and the anterior hippocampus has a preferential role in anxiety-related behaviors (Bannerman et al. 2004). Anxiety impairs spatial working memory. Attention may well represent the point overlap between anxiety and spatial working memory (Lavric et al. 2003). Bannerman et al. (2014) proposed that hippocampal NMDARs have a crucial role within a comparator behavioral inhibition system for detecting and resolving conflict that might occur during anxiety.

The current study demonstrated significant decrease in rearing frequency in group exposed to repeated stress. Memantine therapy caused significant increase in rearing frequency in repeated stress group and significant increase in number of arm entries in both ARS and repeated stress compared to corresponding groups exposed to the same pattern of stress without drug therapy.

Previous studies in agreement with our results showed that repeated stress reduces rearing frequency and exploratory behavior (Katz et al. 1981; Dubovicky and Jezova 2004; Daniels et al. 2008).

A study by Creeley et al. (2006) concluded that rearing was significantly increased in the 20 mg/kg Memantine group relative to saline controls at 60 and 90 min after treatment.

In contrast to our results, Mercier et al. (2003) found no change in exploratory activity after acute stress exposure. Also, Padovan and Guimaraes (2004) have reported that no difference is observed immediately after the stressor exposure in an elevated plus-maze but that it appears on the following day.

We demonstrated that ARS and repeated stress did not cause significant difference in grooming frequency compared to control. On the other hand, Memantine therapy with stress resulted in significant increase in grooming frequency compared to the corresponding group exposed to the same pattern of stress without Memantine therapy.

Beyond the primary purpose of hygiene and caring for the body surface, grooming serves a variety of other functions, including stimulation of the skin, thermoregulation, chemo-communication, social interaction, de-arousal and stress reduction (Smolinsky et al. 2009).

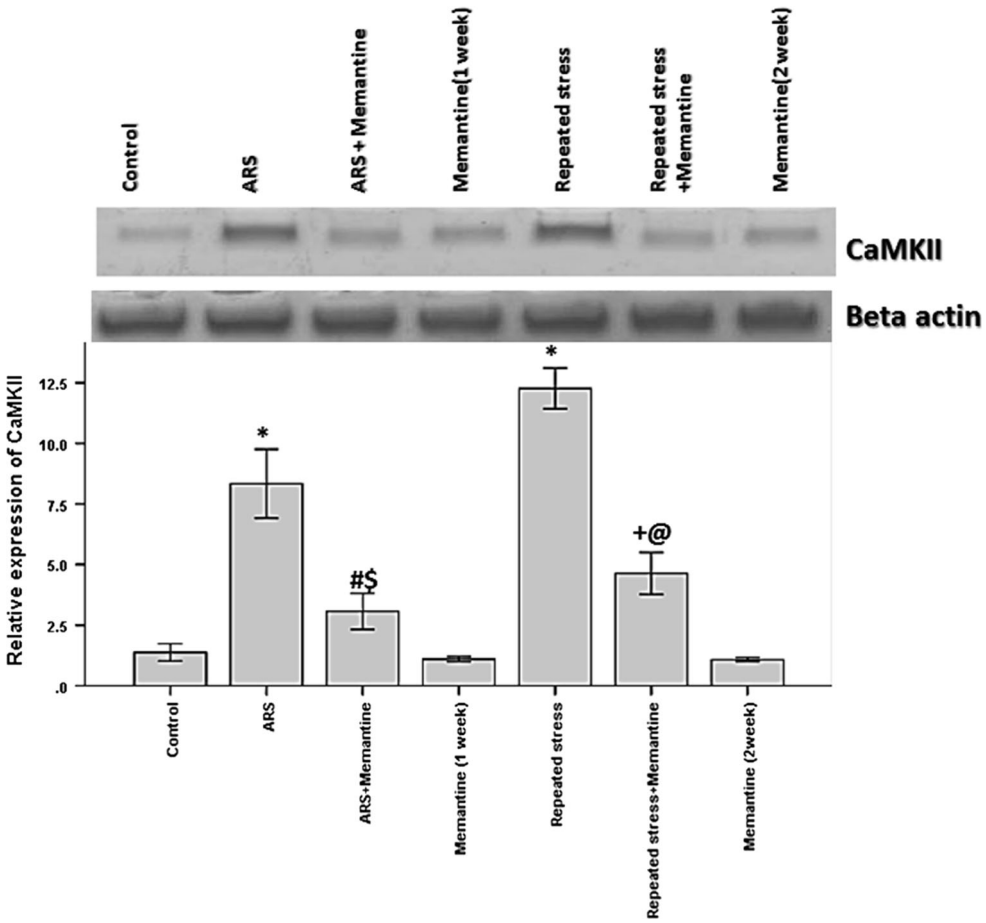

Fig. 4 Hippocampal CaMKII expression by Western Blot analysis in the studied groups. *Significant compared to control, #significant compared to ARS, $significant compared to Memantine (1 week), +significant compared to repeated stress, @significant compared to Memantine (2 weeks) at *P* value ≤0.05. Data are presented as mean ± SD

Genetic factors play an important role in the regulation of rodent grooming (Kalueff and Tuohimaa 2005).

Opposite to our observation, Van Erp et al. (1994) concluded that acute stress generally modulates rodent grooming activity and disrupts its sequencing. Chronic social stress is a powerful inducer of variances in grooming behavior (Denmark et al. 2010). Another study by Pan et al. (2006) showed that chronic social crowding stress inhibits frequency and chronic social isolation stress reduces both frequency and duration of rat grooming.

Contrary to our observation on Memantine effect on grooming, Kos and Popik (2005) showed that treatment with Memantine reduced grooming in the open field: the decrease of grooming as more apparent on the second measurement carried out 35 min after drug administration. However, in sub-chronically treated mice, Memantine continued to decrease grooming as measured 5 min after administration. Also, Hwa et al. (2013) demonstrated that Memantine significantly decreased self-grooming.

In the present work, there was significant increase in freezing frequency in ARS and repeated stress groups compared to control and Memantine therapy with stress resulted in significant decrease in freezing frequency compared to corresponding stress groups.

Freezing, defined as a species-specific defensive reaction characterized by lack of movement besides respiration and heartbeat, associated with crouching posture (Blanchard and Blanchard 1969; Bolles and Riley 1973). Exogenous CRF administration to rodents produces hypoactivity and freezing behavior in a novel environment (Britton et al. 1982).

Current results agree with previous studies that freezing in open-field test in stress group was more increased than that in control group (Steimer and Driscoll 2003; Nosek et al. 2008). Moreover, Costa et al. (2008) showed that Memantine therapy reduced percentage of freezing.

In the present study, there was significant increase in serum markers of stress: serum cortisol, IL-6 and TNF-α which is significantly decreased by Memantine therapy in both ARS and repeated stress groups compared to groups exposed to the same form of stress without Memantine therapy.

Acute and chronic stresses both have similar effects such as activating the hypothalamic-pituitary cytokines. Proinflammatory cytokines, including IL-1b IL-1b, IL-6

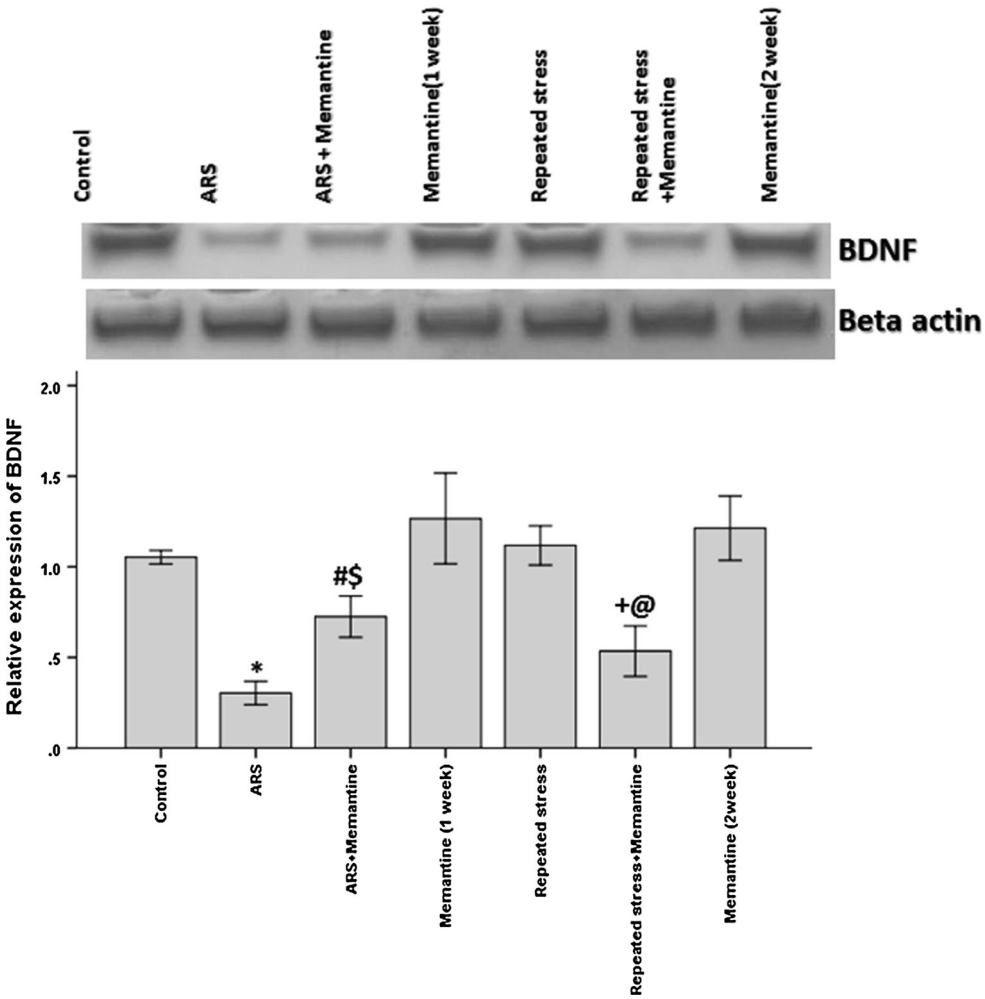

Fig. 5 Hippocampal BDNF expression by Western Blot analysis in the studied groups. *Significant compared to control, #significant compared to ARS, $significant compared to Memantine (1 week), +significant compared to repeated stress, @significant compared to Memantine (2 weeks) at *P* value ≤0.05. Data are presented as mean ± SD

and tumor necrosis factor-a TNF-α, are implicated in the etiologies of clinical depression and anxiety disorders (Dunn et al. 2005).

In humans, the circulating levels of the proinflammatory cytokine IL-6 increase with acute (Edwards et al. 2006) and chronic stress (Ranjit et al. 2007). Stressor-induced increases in plasma IL-6 concentrations may be mediated by increased sympathetic activity and concomitant release of adrenaline from the adrenal medulla (Deak et al. 2004).

Kirschbaum et al. (1996) study agrees with our study, and it demonstrated significant increase in cortisol in male when they exposed to psychological stress of public speaking and mental arithmetic in front of an audience. Also Khalaj et al. (2013) showed that the physical and psychological stress elevates plasma levels of several cytokines including TNF-α. Moreover, Gądek-Michalska et al. (2013) found that stress conditions are characterized by a complex release of several inflammatory mediators including cytokines, prostanoids, nitric oxide and transcription factors.

Opposite to our results, Adlard and Cotman (2004) showed that changes in blood cortisol level in rats after a single acute exposure to a stressor return to normal levels within 1 h after stress.

In agreement with our results regarding Memantine effect on cytokines, Lee et al. (2014) showed significant decrease in IL-6 level compared to placebo group. However, Lin et al. (2011) demonstrated that Memantine treatment did not affect IL-6 levels in the medial prefrontal cortex or nucleus accumbens.

In the present work, we assessed the hippocampal expression of three markers of synaptic plasticity: synaptophysin, BDNF and CaMKII trying to reveal the molecular mechanism by which Memantine works to improve spatial working memory in addition to the attenuating effect of excitotoxicity.

There was significant decrease in relative expression of synaptophysin in both ARS and repeated stress groups compared to control group. Memantine therapy with stress exposure significantly increased synaptophysin expression compared to stress groups which suggest possible role of Memantine in neurogenesis.

In agreement with our study, Thome et al. (2001) revealed that synaptophysin expression decreased significantly in

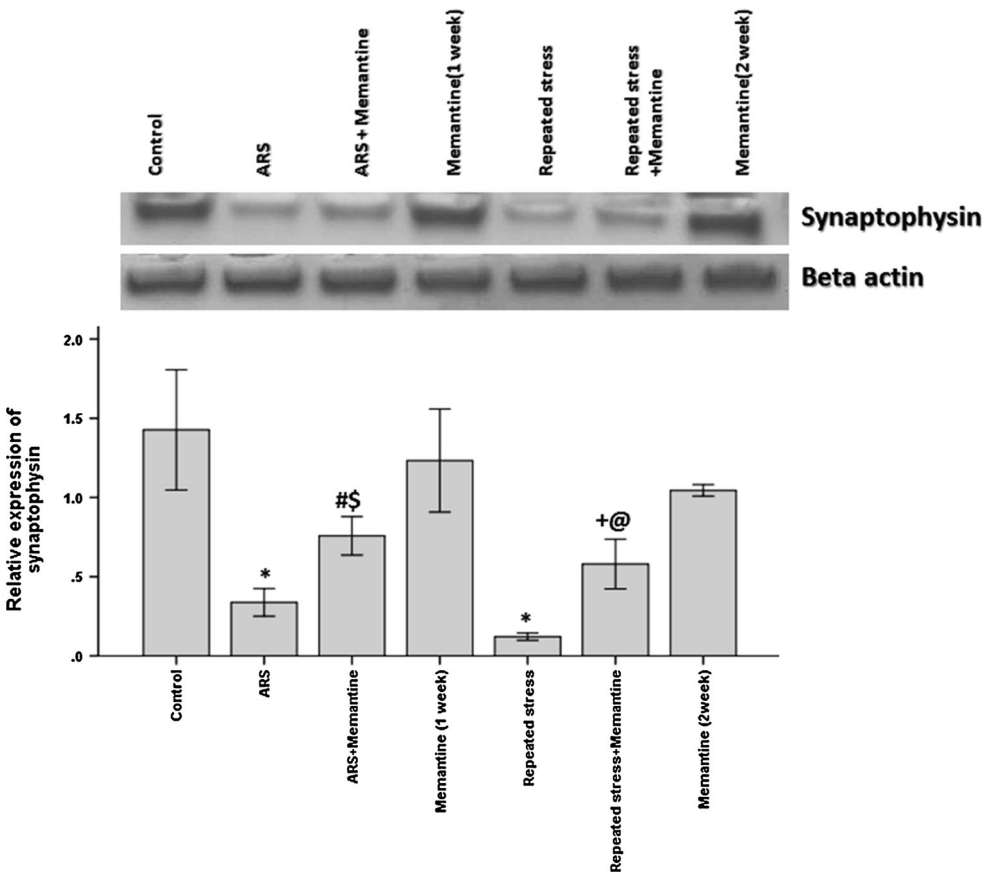

Fig. 6 Hippocampal synaptophysin expression by Western Blot analysis in the studied groups. *Significant compared to control, #significant compared to ARS, $significant compared to Memantine (1 week), +significant compared to repeated stress, @significant compared to Memantine (2 weeks) at *P* value ≤0.05. Data are presented as mean ± SD

hippocampus and cerebral cortex after acute and after chronic immobilization stress. Also Xu et al. (2004) reported significant decrease in hippocampal synaptophysin expression in rats exposed to repeated restraint stress.

However, Campos et al. (2013) study on predator stress exposure reported that synaptophysin mRNA expression was increased in the amygdaloid complex without significant changes in the expression in the dorsal hippocampus. Their findings is also related to previous studies suggesting that predator stress induces long-lasting potentiation of excitatory neural transmission in the basolateral amygdala (Adamec et al. 2012).

The current work demonstrated significant decrease in BDNF only in ARS not in repeated stress groups when compared to control groups and Memantine therapy with acute stress significantly increased BDNF expression compared to ARS group.

BDNF is a member of the neurotrophin family known to play a prominent role in the survival, growth, maintenance of neurons during development (Barde 1994) and the ability to modulate synaptic plasticity in the adult brain (Lo 1995) and to modulate hippocampal-dependent learning and memory (Lu 2003). BDNF can modulate synaptic efficacy either by changes in presynaptic transmitter release, or by increased postsynaptic transmitter sensitivity (Itami et al. 2003).

BDNF regulates axonal and dendritic branching and remodelling, synaptogenesis in arborizing axon terminals (Alsina et al. 2001), the efficacy of synaptic transmission, and the functional maturation of excitatory and inhibitory synapses (Seil and Drake-Baumann 2000; Farmer et al. 2004).

Stressful experiences decrease hippocampal levels of BDNF (Smith et al. 1995; Ueyama et al. 1997; Gronli et al. 2006), and a positive association between BDNF and SYP immunoreactivity has been demonstrated (Koo et al. 2003). BDNF mRNA in the hippocampus increased after a radial maze training, and treatment with an antisense BDNF oligonucleotide led to impairment of not only the acquisition, but also the maintenance and/or recall of spatial memory (Mizuno et al. 2000).

Chronic stress may affect multiple neural systems, which may have counteracted the BDNF downregulation, in a concerted manner, during chronic stress period. It should be noted that cyclic AMP response element binding protein (CREB) plays an important role in the regulation of BDNF expression (Katoh-Semba et al. 2001). Chronic antidepressant administration increased the expression, phosphorylation and function of CREB in the hippocampus and cerebral cortex (Thome et al. 2000) and upregulated BDNF mRNA in the hippocampus (Coppell et al. 2003).

Marvanová et al. (2001) showed that Memantine increased BDNF mRNA levels in the limbic cortex in rat brain. The induction of BDNF may be due to an effect of Memantine on non-neuronal cells known to produce BDNF (Barde 1994) lack the NMDA receptor signal transduction. Another possibility is that Memantine activates extrasynaptic NMDA receptors and promotes neuronal function (Novelli et al. 2005).

Opposite of our results regarding the effect of repeated stress on BDNF expression, Adlard et al. (2004) found that chronic stress caused a decrease in BDNF expression.

Several studies have shown that Memantine promotes cell proliferation and production of mature granule neurons in the adult hippocampus (Volbracht et al. 2006; Jin et al. 2006). Memantine has also been demonstrated to stimulate the proliferation of hippocampal progenitor cells (Namba et al. 2010).

The present study revealed that ARS and repeated stress significantly increased CaMKII expression in the hippocampus and Memantine administration decreased CaMKII expression in groups exposed to stress.

The enhancement of both short-term and long-term memory is controlled at the molecular level in neurons (Carew 1996). Whereas short-term memory involves covalent modifications of preexisting proteins, long-term memory requires the synthesis of new mRNAs and proteins (Kelleher et al. 2004). Four major signaling pathways control this process: (i) cAMP-dependent protein kinase, (ii) calcium–calmodulin kinases, (iii) protein kinase C and (iv) mitogen-activated protein kinase. All four pathways converge to signal to CREB, a transcription factor which binds to the promoter regions of many genes associated with memory (Barco et al. 2006).

CaMKII is a multifunctional serine/threonine protein kinase with the α and β isoforms predominating in the brain (Hudmon, and Schulman 2002). CaMKII is a major mediator of the postsynaptic mechanisms of LTP (Lisman et al. 2012), and it acts as a molecular switch in mediating the all-or-none potentiation of synapses and is also capable of autophosphorylation in the absence of Ca^{2+}, creating a lifelong memory molecule (Fink and Meyer 2002) .

Excitatory synapses modify their efficacy to store information, either by strengthening through LTP or weakening through long-term depression (LTD) (Malenka and Bear 2004). Both LTP and LTD are expressed through the insertion or removal, respectively, of AMPA-type glutamate receptors (Kessels and Malinow 2009). Furthermore, LTD comes in both NMDAR- and metabotropic glutamate receptor (mGluR)-dependent forms (Collingridge et al. 2010).

CaMKII may also play a role in LTD at glutamatergic synapses and to regulate the function of inhibitory synapses (Marsden et al. 2010), and for NMDAR-dependent LTD, an involvement of CaMKII has been attributed to presynaptic mechanisms (Stanton and Gage 1996).

Sun et al. (2006) demonstrated that the expression of CaMKII and CREB mRNA in the hippocampus of the stressed group exposed to chronic multiple-stress was higher than that of the control group. However, Novak et al. (2013) showed that exposure to maternal deprivation stress caused no effect on expression of CaMKIIβ and CaMKIIα in the striatum. And another study by Zhang et al. (2013) on the effect of sleep deprivation stress revealed a reduction in BDNF and CaMKII in the hippocampus. Also Suenaga et al. (2004) showed that acute and repeated stress exposure increased phospho-CaMKII levels without affecting the levels of CaMKII.

There was a significant positive correlation between relative expression of CaMKII and serum cortisol, TNF-α and IL-6. Chen et al. (2012) showed that hippocampal glucocorticoid receptors were coupled to the activation of CaMKIIα by a non-genomic effect of glucocorticoid receptors.

However, there was significant negative correlation between relative expression of BDNF and serum cortisol, TNF-α and IL-6. In agreement with our results, Murakami et al. (2005) showed that the hippocampal BDNF mRNA is negatively correlated with plasma glucocorticoid (GC) levels. It is well documented that administration of GC decreases BDNF mRNA in the rat hippocampus (Smith et al. 1995; Schaaf et al. 1998; Hansson et al. 2003).

Hansson et al. (2003) analyzed the time course change of hippocampal BDNF expression after 10 mg/kg of corticosterone administration and demonstrated that hippocampal BDNF expression reached nadir at 4 h after corticosterone administration, suggesting that a time lag of several hours is necessary for corticosterone to cause reduction in hippocampal BDNF mRNA expression.

There are cross talks between steroids and BDNF and in animals; central administration of exogenous BDNF modified HPA axis function (Givalois et al. 2004). Moreover, BDNF is important for the regulation of synaptic proteins like synaptophysin (Lu 2003).

In contrast to our results, Saha et al. (2006) reported that TNF-alpha upregulates BDNF protein in primary astrocytes.

Furthermore, there was significant negative correlation between relative expression of synaptophysin and serum cortisol. In agreement with our findings, Thome et al. (2001) demonstrated that stress exposure leads to reduced expression of synaptophysin.

Conclusions

Data obtained indicate that Memantine provides neuroprotective effect in both acute and repeated stress with more protection offered in chronic stress. This neuroprotective

effect may be achieved through attenuating the activity of HPA axis and cytokines release. Also the positive impact of Memantine on spatial working memory and behavior may be explained by inducing neurogenesis as revealed from the increase of hippocampal synaptophysin and BDNF expression and by inhibiting LTD through downregulation of CAMKII expression in the hippocampus.

Conflict of interest There is no conflict of interest.

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