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Research Report

Chronic restraint stress alters the expression and distribution of phosphorylated tau and MAP2 in cortex and hippocampus of rat brain

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ABSTRACT

Microtubule-associated proteins (MAPs) play a critical role in maintaining normal cytoskeletal architecture and functions. In the present study, we aim to explore the effects of the emotional stressor, chronic restraint stress, on the expression levels and localization of tau and MAP2. We found that after chronic restraint stress, soluble hyperphosphorylated tau was greatly increased, whereas MAP2 was decreased. Moreover, immunohistochemistry analysis demonstrated that phosphorylated tau and MAP2 displayed the similar subcellular distribution pattern after chronic restraint stress. Robust hyperphosphorylated tau immunolabeling was observed both in cortex and hippocampus of stressed animals and mainly located to perikaryal/dendritic elements. After stress, the MAP2 was mainly distributed in the perikaryal compartments, discontinuous dendrites and neuropil. Moreover, the distribution pattern of MAP2 in hippocampus significantly changed. Immunofluorescence double labeling indicated that hyperphosphorylated tau increased in the regions where there displayed an decrease of MAP2. These results suggest that the involvement of repeated restraint stress may not only induce phosphorylation state of tau and distribution of phosphorylated tau, but also alter the content and neuronal distribution of MAP2. Tau and MAP2 are most important MAPs for neuronal cells, the subcellular distribution change of them might be link to functional change of neurons after emotional stress.

1. Introduction

Several types of microtubule-associated protein (MAP) have been involved in eukaryotes, including microtubule motors, microtubule plus-end-binding proteins, centrosome-associated proteins, enzymatically active MAPs, and structural MAPs. The MAP2/Tau family of structural MAPs, which along with the MAP1A/1B family form one of the 'classical', well-characterized families of MAPs. In mammals, the family consists of the neuronal proteins MAP2 and Tau and the non-neuronal protein MAP4. Tau and MAP2 are best known for their microtubule stabilizing activity and for proposed roles

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regulating microtubule networks in the axons and dendrites of neurons. Contrary to this simple, traditional view, accumulating evidence suggests a much broader range of functions, such as binding to filamentous actin (F-actin), recruitment of signaling proteins, and regulation of microtubule-mediated transport (Dehmelt and Halpain, 2005) .

Tau, which is predominately located in axons in mature neurons, is also implicated in Alzheimer's disease (AD) and other dementias. Abnormal hyperphosphorylated tau protein in AD brain not only forms the neurofibrillary tangles (NFTs), but also sequesters normal tau, MAP1 and MAP2, and disrupts microtubules (Alonso et al., 1994, 1997). In contrast, MAP2 as well as MAP1 and tubulin deposit in Lewy bodies, a cytopathologic marker of Parkinson's disease. In addition, many observations confirm the notion of a role for increase in non-phosphorylated MAP2 and MAP1B at hippocampus in somatodendritic and cytoarchitectural abnormalities associated to schizophrenia (Benitez-King et al., 2004). Moreover, studies have indicated that the increase of hyperphosphorylated tau is concomitant with the decrease of MAP2, and hyperphosphorylated tau accumulation in neuropil may displace MAP2 (Ashford et al., 1998). Therefore, both tau and MAP2 were involved in machinery of neurodegeneration.

Epidemiological researches have suggested that a linkage between stress and neurodegeneration (Bissette, 2009; Friedl et al., 2009). The categorized stressors include "physiological" and "emotional" stressors (Dayas et al., 2001). Animal studies have showed that tau phosphorylation could be induced by physiological stressors such as food deprivation, forced swimming in cold water (Hartig et al., 2005, 2007; Yoshida et al., 2006). Recent studies also showed an elevated phosphorylated tau in emotional stress models (Rissman et al., 2007). However, the expression and distribution of MAPs in brain after stress are still not well elucidated. Restraint is a typical emotional stressor, which is related to the pathogenesis of depression (Yoshida et al., 2006). Acute restraint stress induces reversible increase of soluble phosphorylated tau in mouse brain, whereas chronic restraint stress induces both soluble and insoluble phosphorylated tau (Rissman et al., 2007).

In this study, we used the chronic restraint rat model, to investigate the effect of chronic restraint stress on the expression and distribution of phosphorylated tau and MAP2. We demonstrate that chronic restraint stress induced an increase in soluble phosphorylated tau and altered the distribution patterns of both phosphorylated tau and MAP2. The findings indicate that emotional stressor is able to cause phosphorylation of tau and abnormal distribution of tau and MAP2 in neuron.

2. Results

2.1. *Chronic restraint stress induces tau phosphorylation*

We initially determined whether the increase of hyperphosphorylated tau was observable in response to chronic restraint stress. Western analysis was used to detect phosphorylated tau at Ser202 site in the cortex and hippocampus extracts. We found that phosphorylated tau was significantly increased in soluble fraction extracted by RAB (Fig. 1), but did not change in RAB insoluble fraction which was subsequently extracted by RIPA buffer containing detergent (Fig. 2A). In addition, we did not find phosphorylated tau in the FA fraction which is considered as the NFT tau (Fig. 2B). These results indicate that after repeated restraint stress for 14 consecutive days, phosphorylated tau in rat brain was significantly increased in soluble form but not insoluble form which could be considered as pathological (paired helical filaments, PHF)-like tau.

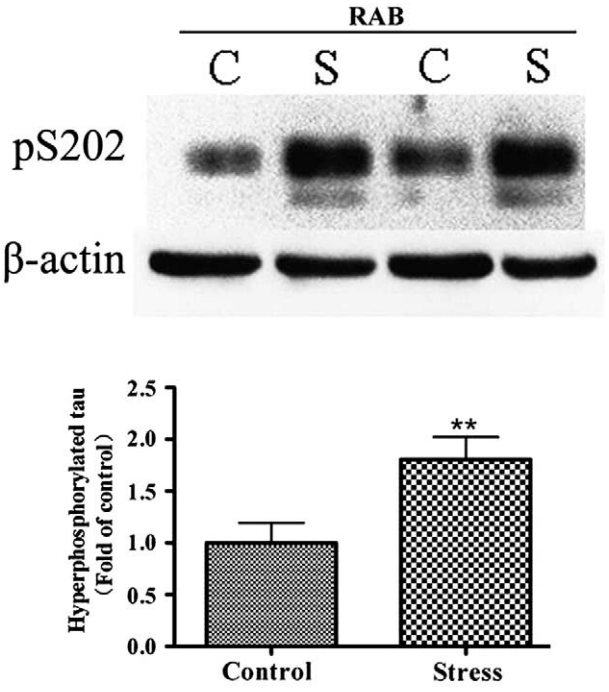

Fig. 1 – Chronic restraint stress increases soluble phosphorylated tau. Western blot analysis of cortex/hippocampus phosphorylated tau at Ser202 site under control (C) and chronic restraint stress (S) conditions. Quantitative analysis, expressed as mean ± S.E.M percentage of integrated intensity values of controls, reveals that stressed animals manifest comparably robust in phosphorylated tau at Ser202 site. Differs significantly from unstressed controls ($P < 0.01$), $n = 6$ rats per condition. β-Actin was used as a loading control.**

2.2. *The distribution patterns of phosphorylated tau changes after repeated restraint stress*

Our previous finding indicates that the distribution of phosphorylated tau in rat brain after cold water stress was markedly different compared with controls (Feng et al., 2005). To probe the localization of phosphorylated tau in chronic restraint stressed animals and control animals, immunohistochemistry method was employed with a anti-phospho-tau pS202 which recognizes tau phosphorylated at Ser202 site. The staining showed an increase of phosphorylated tau in the cortex and hippocampus of stressed animals compared with control ones (Figs. 3 and 4).

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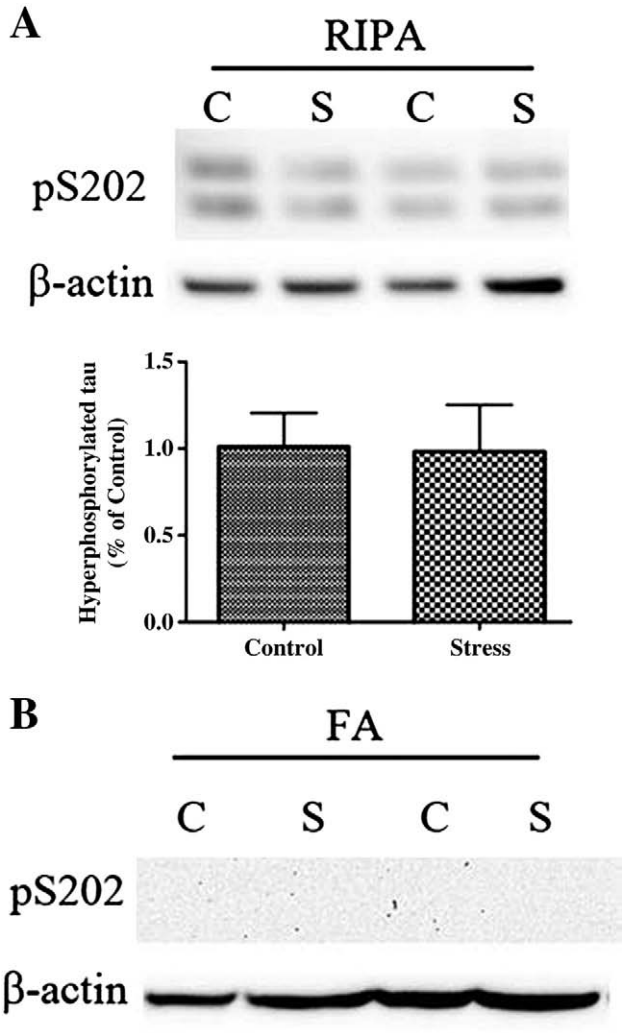

Fig. 2 – Chronic restraint stress has no effects on insoluble hyperphosphorylated tau. Western blot analysis of phosphorylated tau at pS202 site in brain extracts from rats subjected to chronic restraint stress and unstressed controls reveals that detergent-soluble hyperphosphorylated tau did not change at Ser202 site. $n=6$ rats per condition. β-Actin was used as a loading control.

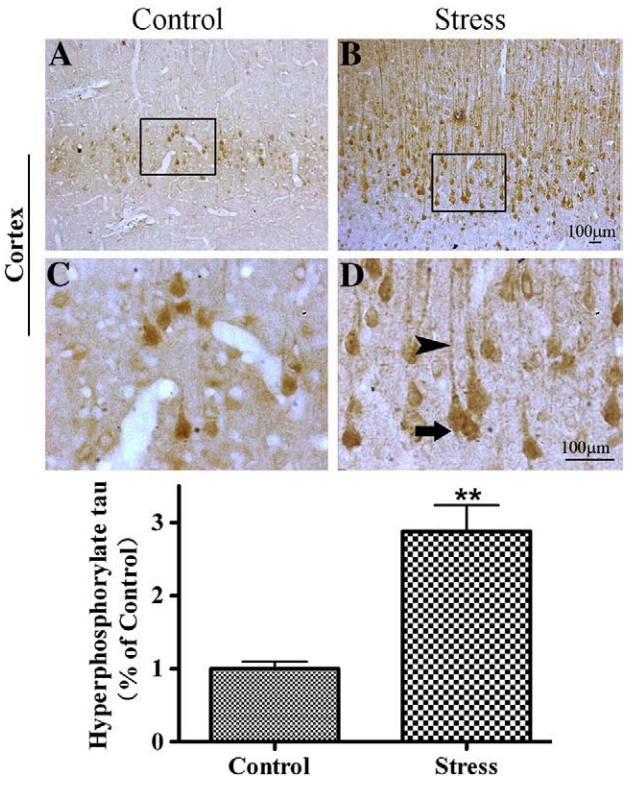

Fig. 3 – Cellular localization of stress induced tau phosphorylation in the cortex of rat brain. In control animals, hyperphosphorylated tau mainly distributed in soma, while phosphorylation is localized to distinct perikaryal/dendritic elements in stressed animals and consistent with the manner identical to that seen by western blot analysis, insets are shown at higher magnification in C, D. Scale bar = 100.

In normal rat cortex, the phosphorylated tau was weakly stained, and was mainly localized to the cell body of neurons (Fig. 3C). In contrast, the phosphorylated tau was significantly increased, distributed in soma and extended along the large dendritic processes (Fig. 3D black arrow and black arrowhead, respectively). In hippocampus of normal control rat, phosphorylated tau was also weakly stained. The phosphorylated tau positive cells were scatted in stratum pyramidal layer of CA regions, hilus and inner granular layer (Figs. 4A, C, E). Meanwhile, the mossy fibers were immunopositive for phosphorylated tau (Fig. 4C). In the stressed animals, phosphorylated tau positive cells were seen throughout the pyramidal cell layer, in hilus and granular layer(Figs. 4B, D, F). In CA1 region, phosphorylated tau was distributed in neuronal cell bodies and thick, short dendritic processes (Fig. 4B). In the CA3 of hippocampus, strong positive staining was observed in both pyramidal cells and their dendrite processes (Fig. 4D). Radially oriented processes representing dendritic labeling were also seen in the stressed animals (Fig. 4D). The phosphorylated tau was increased about 7 folds after repeated stress which is compatible with western blot analysis. In the dentate gyrus of stressed animals, immunolabeling cell bodies and cell processes were present in the granule cell layer and in the hilus (Fig. 4F).

2.3. *Expression of MAP2 is modified after repeated restraint stress*

To examine whether repeated restraint stress altered the expression of MAP2, we performed immunohistochemistry studies in the cortex and hippocampus of the rat brain in both control and stressed animals. In the cortex of normal control rats, MAP2 staining was seen primarily in the long and continuous dendrites and cell bodies (Fig. 5C, black arrow), while in the stressed cortex, MAP2 labeled long dendrites decreased (Fig. 5B). Some neurons accumulated MAP2 both in cell bodies and discontinuous dendrites. The MAP2 immunolabeling in the dendritic processes displayed a distinct pattern of beady patches along the thick processes (Fig. 5D black

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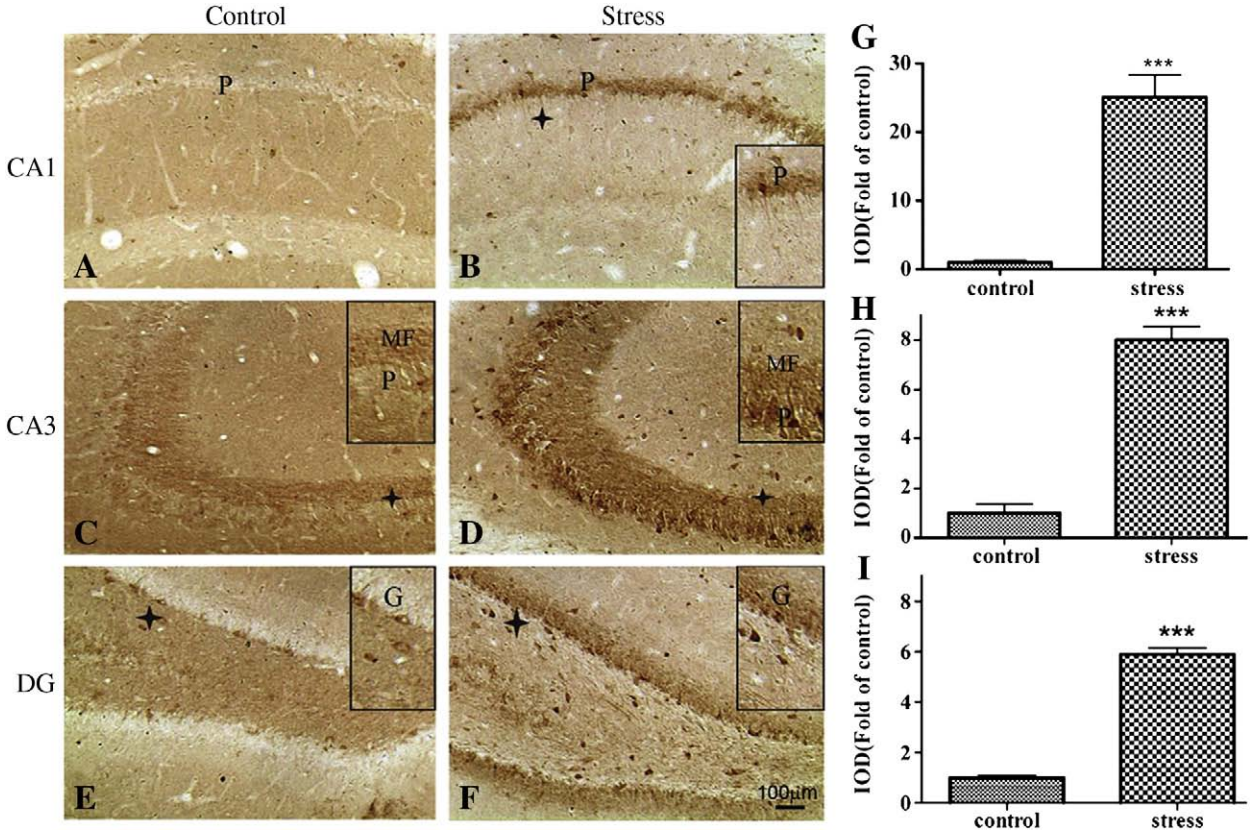

Fig. 4 – Stress induced hyperphosphorylated tau in hippocampus in the rat brain. Representative pictures show hyperphosphorylated tau immunopositive cells in CA1 (A,B), CA3 (C,D), and DG (E,F). Normal rat brain served as control. Image J was used to quantify the integrated intensity values of different cell layers. Quantification analysis revealed that hyperphosphorylated tau was greatly increased in CA1 and CA3 pyramidal cell layers and in granule cell layers of DG respectively. P, pyramidal cell layer , MF, mossy fiber, G, granule cell layer. Scale bar = 100 μm (means ± S.E.M;$^{***}P < 0.001$ *vs* normal rat brain, $n = 4$ in each group).

arrow), and strong immunoreactivity was also found in discontinuous dendrites processes (Fig. 5D black arrowhead) and neuropil (Fig. 5D black rhombus).

As shown in Fig. 6, in normal hippocampus, somatic compartment of neurons throughout stratum pyramidal layer and granule cell layer contained high level of MAP2, from which MAP2 positive dendritic processes extended in stratum radiatum and distributed in stratum moleculare and molecular layer of dentate gyrus with a high immunoreactive pattern. In stressed rats, MAP2 staining was dramatically decreased in stratum pyramidal layer, and granule cell layer. The distribution of MAP2 in neurons of stratum pyramidal layer was confined in the perikaryal compartment of cell bodies sparing nuclear (Fig. 6B, D black arrow). The MAP2 stained dendritic neurites in stratum radiatum of CA1 region appeared thin and discontinuous (Fig. 6B). In CA3 region, the stratum lucidum located in the inner-side of the pyramidal cell layer of CA3 was lightly stained of MAP2 and displayed significant discontinuous fibers and beady patches (Fig. 6D). However, the MAP2 staining in stratum radiatum, stratum moleculare and stratum oriens of CA3 region was enhanced (Fig. 6D and Fig. 7B). MAP2 staining were greatly decreased in granule cell layer of dentate gyrus, meanwhile the MAP2 positive neurites within hilus were increased (Fig. 6F). The overall distribution pattern of MAP2 in hippocampus was shown in Fig. 7.

We also studied the distribution of hyperphosphorylated tau and MAP2 induced by chronic restraint stress employing the double immunofluorescence labeling. We observed a marked increase of hyperphosphorylated tau and decrease of dendritic MAP2 in the cortex and hippocampus as compared with normal control animals, the results are consistent with the immunohistochemistry staining. The most dramatic decrease staining of MAP2 was seen in the cortex and stratum radiatum of CA1 sector and stratum lucidum of CA3 sector of hippocampus where hyperphosphorylated tau was increased strikingly (Figs. 8 and 9).

2.4. *Chronic restraint stress induced decrease of MAP2 in the rat brain*

To confirm the expression of MAP2 after chronic restraint stress, the raw protein extracts (homogenized) were loaded on

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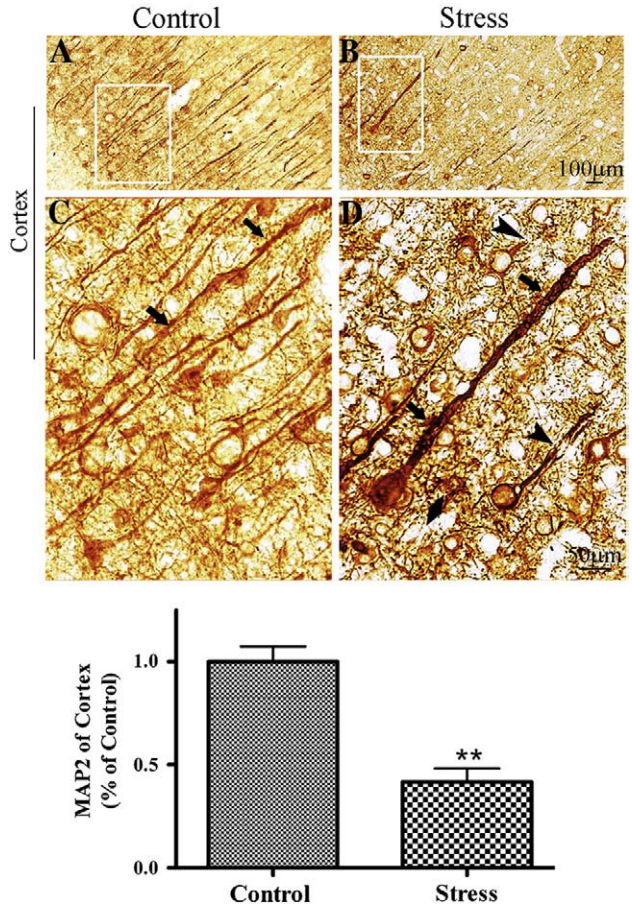

Fig. 5 – MAP2 staining patterns of neurons in the cortex from control (A) and stressed (B) rat brain, insets (C,D) show high magnification of A and B respectively. The photograph C shows that long, thin, continuous processes (arrow) in control animals, and there are discontinuous, thick processes (arrow) and beady patches along the processes (arrowhead) in the stressed rat brain sections. Scale bar = 100 μm (A, B) Scale bar = 50 μm (C, D).

the SDS-PAGE, and performed western blot to detect MAP2. The result showed that MAP2 was significantly decreased (Fig. 10).

3. Discussion

Previously we demonstrated that forced cold water swimming cause phosphorylation of tau in the mouse brain. In this study, we examined the effects of chronic restraint stress on neuronal morphology and distribution of hyperphosphorylated tau and MAP2 in the rat brain. Restraint is classified as an emotional stressor (Dayas et al., 2001; Rissman et al., 2007). Epidemiological researches have showed that chronic stress is related to neurodegenerative diseases. Moreover, it was reported that acute restraint stress induced the increase of soluble hyperphosphorylated tau in mice in a reversible way, while chronic restraint stress increased insoluble hyperphosphorylated tau in the mouse model (Rissman et al., 2007). It appears plausible that a stress-related dysregulation of tau phosphorylation in the hippocampus and cortex can manifest as memory impairment in the development of diseases such as Alzheimer disease. In our rat model, we detected tau phosphorylation at Ser202 which is among the prominently phosphorylated sites of PHF-tau in AD brains. We observed that the cytosolic soluble phosphorylated tau was elevated in stressed rat, but the insoluble phosphorylated tau did not change much, which might imply requirement of stronger stress stimulation to induce insoluble phosphorylated tau for rat model.

Stress leads to specific changes in the electrophysiological properties and axonal/dendritic morphology in brain (McEwen, 1999), especially in hippocampus, which is particularly vulnerable because of high density of glucocorticoid receptors. Chronic stress induces dendritic atrophy in hippocampal neurons (Magarinos and McEwen, 1995; Sousa et al., 2000), alters mossy fiber synaptic terminal structure (Magarinos et al., 1997) and proliferative capacity of brain cells, repeated restraint stress can cause retraction of the apical dendritic arbor in layer II/III pyramidal cells in the prefrontal cortex. Consistent with these reports, in our 1 h/14 days restraint stress model, the morphology changes of hyperphosphorylated tau in the cortex and hippocampus mostly appeared in the pyramidal cell layer dendrites of CA1 and CA3 and the mossy fiber, which indicated the potential role of hyperphosphorylated tau in the dendritic atrophy. Chronic stress may affect hippocampal function through such mechanisms as CA3 neuronal remodeling (Conrad, 2006), suppression of synaptic activity (Kim and Diamond, 2002; Stewart et al., 2005), and altered neurogenesis.

It has been indicated that tau is mainly located in axon (Brion et al., 1988), and earliest hyperphosphorylated tau in AD is preferentially located within axonal dystrophic neurites (Su et al., 1993, 1994) and then phosphorylated tau extends to somatodendritic compartment, to form NFT (Trojanowski and Lee, 1994). In a previous study, we found a time dependent differential distribution of phosphorylated tau in hippocampus after stress stimulation by forced cold water swimming. At later phase after cold water stress, phosphorylated tau was distributed in somatodendritic compartment (Feng et al., 2005). In this study, the distribution of phosphorylated tau in chronic stressed brain also showed a somatodendritic pattern, which might be harmful for neurons or reflect a change of neuronal plasticity.

A cardinal feature of neurons is the morphological polarity of neurons with serious functional implications. Neuronal polarity is essential for the unidirectional signal flow from somata or dendrites to axons in neurons (Yoshimura et al., 2006). Researchers are accumulating a catalog of structural, molecular, and functional differences between axons and dendrites. In normal neuron, MAP2 is mainly in dendrites, whereas tau is mainly in axonal (Dehmelt and Halpain, 2005). In AD brain, evidence indicates an essential link between hyperphosphorylated tau and the loss of MAP2 (Ashford et al., 1998; Geddes et al., 1994). We demonstrated that the distribution of MAP2 changed as abnormally phosphorylated tau increased in our chronic restraint stress rat model. As described previously (Ashford et al., 1998; Lim and Halpain, 2000; Matus et al., 1986; Tourtellotte and Van Hoesen, 1991), we also showed

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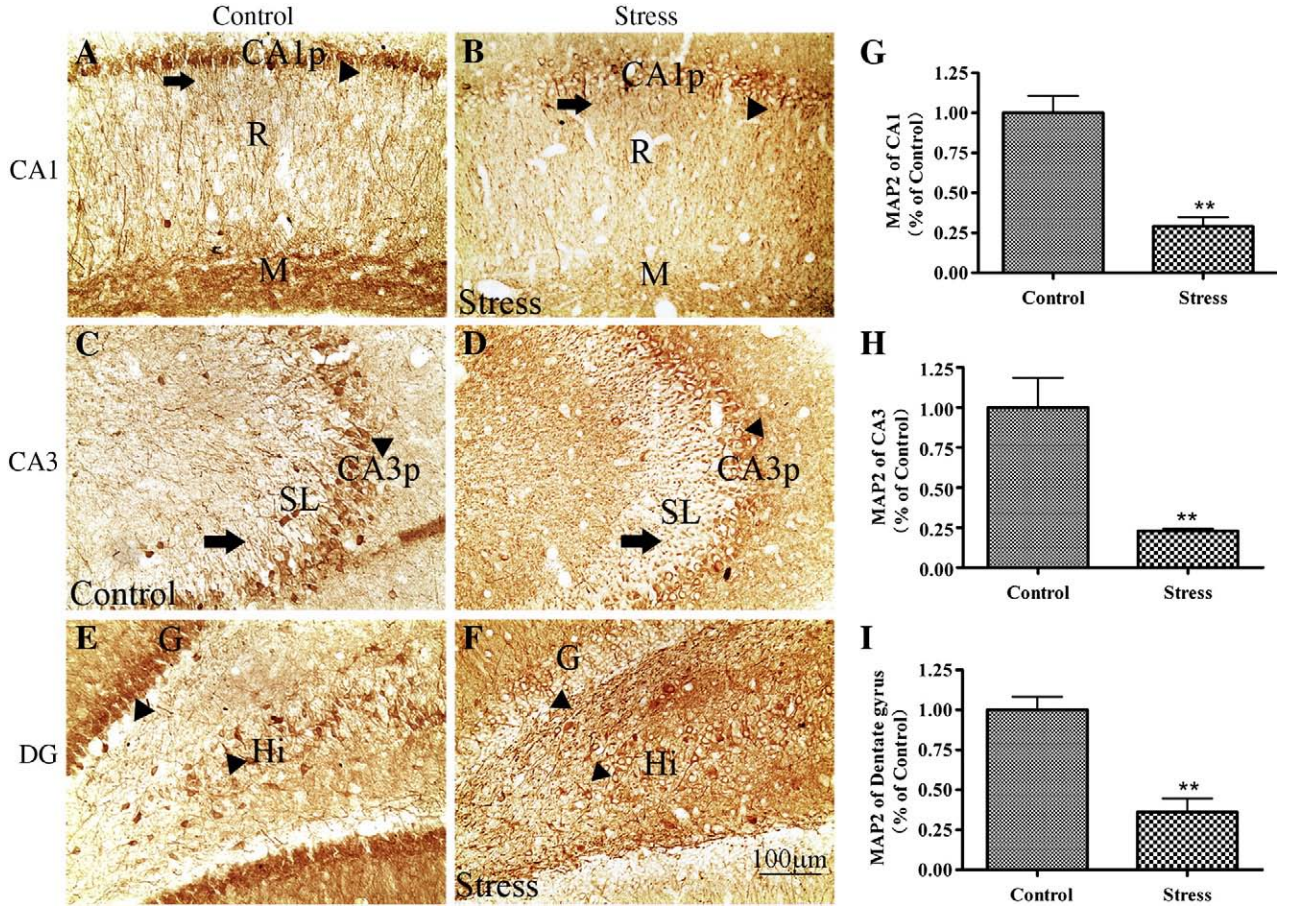

Fig. 6 – The change of MAP2 distribution in hippocampus after chronic restraint stress. Hippocampal layers: SL, stratum lucidum; M, stratum moleculare; O, stratum oriens; CA1p, stratum pyramidal of CA1; CA3p, stratum pyramidal of CA3; R, stratum radiatum. Components of the DG: G, granule cell layer; Hi, hilus; ML, molecular layer. Scale bar=200 μm.

MAP2 exclusively in the somatodendritic compartment in normal cortex and hippocampus. In stressed rats, the decrease in dendritic MAP2 was pronounced in the cortex and the hippocampus, although some neurons showed an accumulation of MAP2 in the soma and abnormal apical dendritic processes (Fig. 6). Interspersed MAP2 immunopositive dendritic segments were often beaded in appearance (Figs. 5D and 6D). In CA3, MAP2 positive dendrites had frequent short gaps with a bead-like or broken line appearance which was not observed in the control ones (Fig. 6D). In addition, the increase of MAP2 positive fibers and neuritic terminals in stratum oriens of CA3 region and in hilus of dentate gyrus after stress stimulation implies a redistribution of MAP2. However, we still could not conclude whether the increase of tau phosphorylation and the change of phosphorylated tau distribution in dendrites is prior to the loss of MAP2 or alternatively occurred after the change of MAP2 distribution.

The fact that the tangle bearing neurons in Alzheimer disease seem to survive many years (Morsch et al., 1999) and that the cognitive deficiencies correlate with the appearance of soluble hyperphosphorylated tau (Santacruz et al., 2005) suggests that the soluble hyperphosphorylated tau is probably the key point. Previous reports suggest that the soluble hyperphosphorylated tau sequesters normal tau, MAP1A/MAP1B and MAP2 *in vitro* (Alonso et al., 1996; Iqbal et al., 2008). In AD, tau becomes hyperphosphorylated and accumulates in the somatodendritic compartment (Duff, 2006), and hyperphosphorylate tau sequesters MAP2 and detaches it from microtubules (Alonso et al., 1997). The removal of MAP2 from the dendritic microtubules could contribute to increase microtubule dynamics and thereby to induce the aberrant remodeling of dendrites noted at an early stage of the disease (Arendt, 2001). Tau is also a substrate for several kinases that phosphorylate MAP2 such as PKA, CaMKII, ERKs and JNK1 (Avila, 2006). By accumulating in the somatodendritic compartment, tau could compete with MAP2 for these kinases and alter MAP2 phosphorylation level. This alteration could lead to the aberrant remodeling of dendrites and ultimately to their retraction in AD brain. In our restraint stress model, we speculate that hyperphosphorylated tau may cause the loss of MAP2 by the mechanisms mentioned above. In our *in vivo* model, the results suggest the notion that cytosolic abnormally hyperphosphorylated tau but not paired helical filaments could be relevant to the changes of MAP2.

In conclusion, we demonstrated that chronic restraint stress induces soluble hyperphosphorylated tau and the soluble

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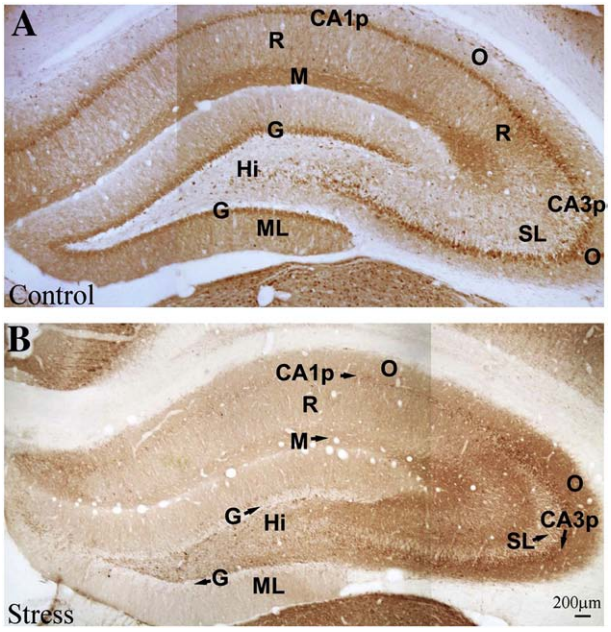

Fig. 7 – The overall distribution of MAP2 in hippocampus after chronic stress. MAP2 decreased dramatically in the pyramidal cell layers of CA1 and CA3 and the granule cell layer of DG. In the stratum molecular and stratum radiatum, the same distribution pattern was observed. Hippocampal layers: SL, stratum lucidum; M, stratum moleculare; O, stratum oriens; CA1p, stratum pyramidal of CA1; CA3p, stratum pyramidal of CA3; R, stratum radiatum. Components of the DG: G, granule cell layer; Hi, hilus; ML, molecular layer. Scale bar = 200 μm.

hyperphosphorylated tau is closely linked to the loss of MAP2. The results further illustrate that soluble hyperphosphorylated tau may play a pivotal role in some neurodegenerative diseases.

4. Experimental procedures

4.1. *Materials*

Rabbit polyclonal antibody (pAb) against phospho-tau (Ser202; 1:500 for immunohistochemistry) was obtained from LifeSpan Biosciences, WA, USA; 1:500 dilution); Rabbit pAb against MAP2 (1:200 dilution for immunohistochemistry) was obtained from Millipore, MA, USA; Mouse monoclonal antibody (mAb) against MAP2 (1:200 dilution for immunofluorescence) was obtained from Santa Cruz Biotechnology, Inc, CA, USA, Complete Protease Inhibitor was from Roche Diagnostics GmbH (Penzberg, Germany). Goat anti-Rabbit Alexa Fluor 594 and Goat anti-Mouse Alexa Fluor 488 were from Invitrogen (Beverly, MA, USA). All the other chemicals used were of the high grade available commercially.

4.2. *Restraint stress*

Male Sprague–Dawley rats (220–250 g) were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Science, and maintained in a temperature-controlled room (22–25 °C). All animal experiments were performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, PR China, 1998) and the guidelines of the Shanghai Medical Laboratory Animal Care and Use Committee. All rats were habituated to the colony and food and water were supplied *ad libitum*. The chronic restraint stress method was modified according to procedures described previously (Rissman et al., 2007). Repeated stress involved placing rats in plastic tubes (6×18 cm), provided with several holes, 1 h each day for 14 consecutive days. Slight manipulation may put on the rat in order to avoid physical stimulus in restraint stress process. The stress environment was a room with the same temperature, illumination and background noise as the animal house. They

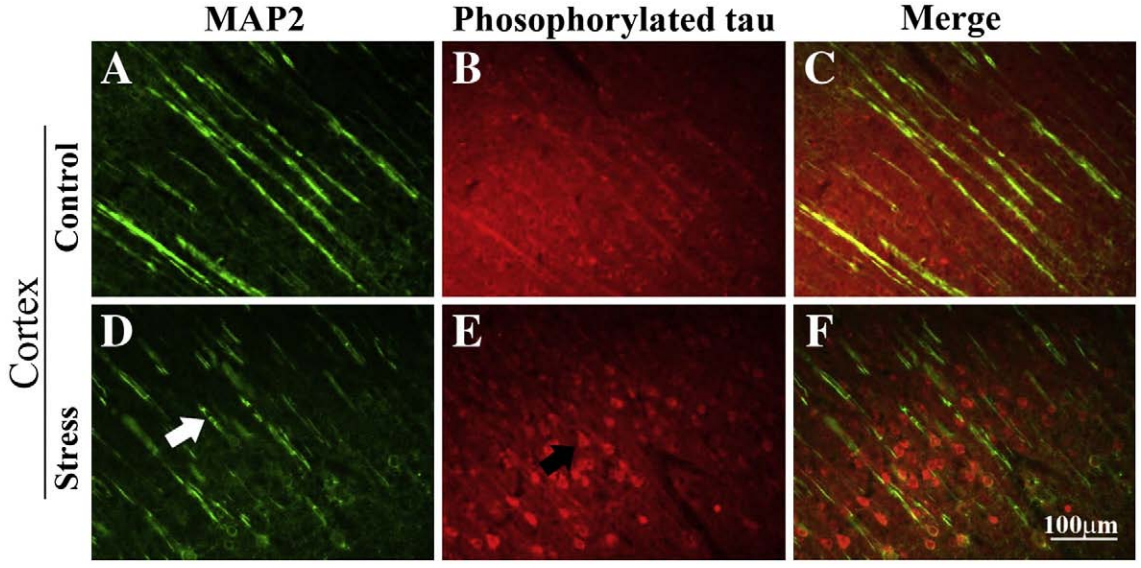

Fig. 8 – Expression of phosphorylated tau and MAP2 in cortex after chronic restraint stress. Immunofluorescence double labeling showed that phosphorylated tau (Red) increased where MAP2 (Green) decreased after chronic stress. MAP2 manifested a discontinuous distribution pattern. Scale bar = 100 μm.

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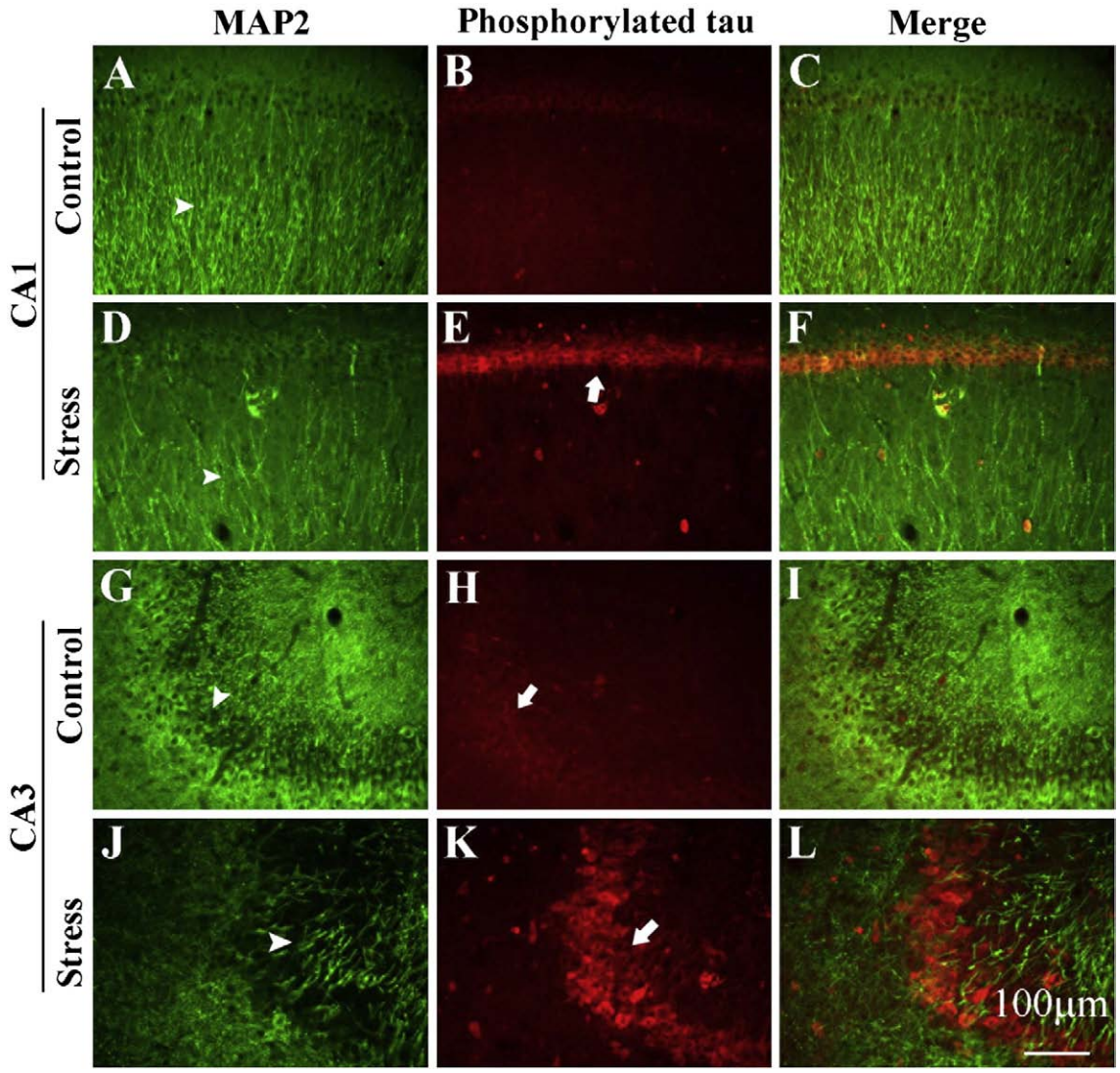

Fig. 9 – Expression of phosphorylated tau and MAP2 in hippocampus after chronic restraint stress. Immunofluorescence double labeling showed that phosphorylated tau (Red) increased where MAP2 (Green) decreased after chronic stress. Representative pictures showed the CA1 and CA3 of hippocampus respectively. Scale bar = 100 μm.

were sacrificed 4 h after the last restraint stress in order to avoid the effect of acute restraint stress .Control rats were handled comparably but not otherwise manipulated.

4.3. *Tissue preparation and immunohistochemistry*

The procedures were carried out according to previous study with modifications(Xiong et al., 2008). Rats were anaesthetized with sodium pentobarbital which has been demonstrated to have no influence hyperphosphorylated tau here (Rissman et al., 2007) and perfused with 0.9% saline solution followed by 4% ice-cold phosphate-buffered paraformaldehyde (PA). The brains were removed and post-fixed for 4–6 h in the same solution of paraformaldehyde at 4 °C, and then cryoprotected by immersion overnight at 4 °C in 4% paraformaldehyde containing 20% sucrose, then immersed in 0.1 M PB containing 30% sucrose until sunk. Coronal sections were cut on a freezing microtome (Jung Histocut, Model 820-II, Leica, Germany) at a thickness of 30 μm, and stored at −20 °C in cryoprotectant solution. For phosphorylated tau at pS202 site and MAP2 staining, sections were incubated with the following primary antibodies: rabbit pAb against phospho-tau (LifeSpan Biosciences, WA, USA; 1:500 dilution); rabbit pAb anti-MAP2 (Millipore, MA, USA; 1:200 dilution). The sections were washed and incubated with corresponding biotinylated secondary antibodies (1:200 dilution) for 1 h at 37 °C. Subsequently, they were incubated with 1:200 avidin–biotin–peroxidase for 45 min at 37 °C, stained with 0.05% diaminobenzidine (DAB) in the presence of 0.03% H_2O_2. Controls were performed by omitting primary antibody, and showed no positive staining. The statistical software Image J was used for the statistical analysis.

4.4. *Double-labeling immunofluorescence staining*

Immunofluorescence staining was performed as described previously(Zhang et al., 2008). Rats were anaesthetized with

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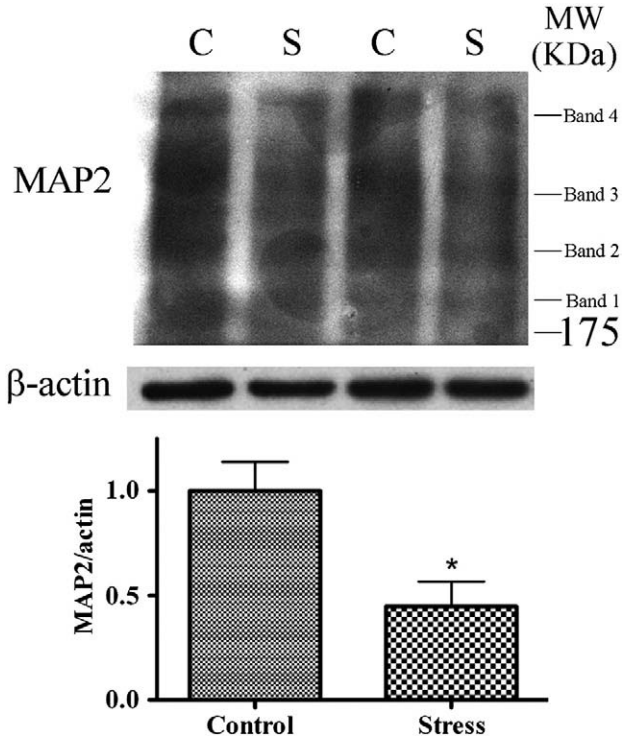

Fig. 10 – Decrease in the level of MAP2 expression after exposed to chronic restraint stress. Quantitative analysis, expressed as mean ± S.E.M percentage of integrated intensity values of controls, reveals that stress animals decreased in MAP2 expression.*Differs from unstressed controls($P < 0.05$), $n = 4$ rats per condition. β-Actin was used as a loading control.

sodium pentobarbital (40 mg/kg) and perfused through aorta with 100 ml 0.9% NaCl followed by 400 ml phosphate buffer containing 4% paraformaldehyde. Brains were removed and post-fixed in perfusate overnight and coronal brain sections of hippocampal tissue were cut at 30 μm with a freezing microtome(Jung Histocut, Model 820-II, Leica, Germany). For double-labeling immunofluorescence staining, free-floating slices were incubated at 4 °C overnight with rabbit polyclonal anti-tau pS202 and mouse polyclonal anti-MAP2. The immunoreactivity of anti-tau pS202 was probed using Alexa Fluor-594 conjugated goat anti-rabbit IgG (H+L). The immunoreactivity of MAP2 was detected using Alexa Fluor-488 conjugated goat anti-mouse IgG (H + L).

4.5. *Western blot analysis*

To determine tau solubility, sequential fractionation procedures were performed as described previously (Rissman et al., 2007). After dissection, the brains were immediately homogenized in high-salt reassembly buffer (RAB) (0.1 M MES, 0.75 M NaCl, 1 mM EGTA, and 0.5 mM $MgSO_4$) and centrifuged at 40,000 *g* for 40 min. Before homogenization, protease inhibitors cocktail tablet (1:50; Roche, BS, Switzerland), PMSF(2 mM), NaF (20 mM), aprotinin and pepstatin (1 μg/ml each) were added. The supernatant was collected (soluble RAB fraction), and pellets (insoluble in RAB) were resuspended in RIPA buffer (50 mM Tris–HCl, pH 7.4, 0.1% SDS, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA), added PMSF(2 mM), NaF (1 mM), aprotinin and pepstatin (1 μg/ml each) and cocktail as before. RIPA fractions were pelleted at 40,000 *g* for 20 min, and the supernatant was collected. The pellet were resuspended in 70% formic acid (FA), then centrifuged at 13,000 rpm for 15 min. Protein concentrations were determined using a BCA Protein Assay Kit (Beyotime, Jiangsu, China). Proteins were then boiled in sample buffer in boiled water for 5 min. 30 micrograms of protein were separated by 10% SDS-PAGE, and then transferred to nitrocellulose membrane (0.22 μm; Millipore, MA, USA;). Membranes were blocked with 5% BSA in TBST (Tris-buffered saline with 0.1% Tween) at room temperature for 1 h, followed by incubation with the primary antibodies diluted in 2% BSA-TBST overnight at 4 °C. Primary antibodies were detected with anti-rabbit horseradish peroxidase-linked secondary antibodies (1:3000; Santa Cruz, CA, USA) and immunoreactivity was visualized by ECL. (Santa Cruz, CA, USA).

4.6. *Statistical analysis*

Data were expressed as mean ± S.E.M. Statistical analysis was determined using student *t*-test. $P < 0.05$ was considered to be statistically significant.

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