

Chronic restraint stress promotes learning and memory impairment due to enhanced neuronal endoplasmic reticulum stress in the frontal cortex and hippocampus in male mice

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Received May 29, 2014; Accepted December 1, 2014

DOI: 10.3892/ijmm.2014.2026

Abstract. Chronic stress has been implicated in many types of neurodegenerative diseases, such as Alzheimer's disease (AD). In our previous study, we demonstrated that chronic restraint stress (CRS) induced reactive oxygen species (ROS) overproduction and oxidative damage in the frontal cortex and hippocampus in mice. In the present study, we investigated the effects of CRS (over a period of 8 weeks) on learning and memory impairment and endoplasmic reticulum (ER) stress in the frontal cortex and hippocampus in male mice. The Morris water maze was used to investigate the effects of CRS on learning and memory impairment. Immunohistochemistry and immunoblot analysis were also used to determine the expression levels of protein kinase C α (PKC α), 78 kDa glucose-regulated protein (GRP78), C/EBP-homologous protein (CHOP) and mesencephalic astrocyte-derived neurotrophic factor (MANF). The results revealed that CRS significantly accelerated learning and memory impairment, and induced neuronal damage in the frontal cortex and hippocampus CA1 region. Moreover, CRS significantly increased the expression of PKC α , CHOP and MANF, and decreased that of GRP78 in the frontal cortex and hippocampus. Our data suggest that exposure to CRS (for 8 weeks) significantly accelerates learning and memory impairment, and the mechanisms involved may be related to ER stress in the frontal cortex and hippocampus.

Introduction

Alzheimer's disease (AD) is an age-related, progressive, irreversible neurodegenerative illness characterized by memory deficits, neuronal loss, neurofibrillary tangles (NFTs) and β -amyloid (A β) deposits in amyloid plaques. The etiology of AD is largely unknown; however, there is growing evidence that chronic stress may increase the risk of developing AD (1,2). Stress initiates a sequence of events in the brain and peripheral systems that enable organisms to deal with and adapt to new and challenging situations (3). However, when stress is maintained for extended periods of time, most physiological systems are negatively affected by stress (3). It has been reported that chronic stress activates hippocampal glucocorticoid receptor, increases neuronal metabolism and decreases cell survival and neurogenesis. In addition, chronic stress promotes dendritic atrophy and causes long-term potentiation and cognitive deficits (4-6). In our previous study, we demonstrated that chronic restraint stress (CRS) and stress-level dexamethasone (5 mg/kg) exposure induced learning and memory impairment and hippocampal neuronal damage in mice (7,8).

Recent studies have indicated that the endoplasmic reticulum (ER) plays an important role in maintaining neurons in neuropathological situations (9). The ER is responsible for protein folding and the transport of newly synthesized proteins. Moreover, the ER is a target for two types of intracellular stresses: ER stress and oxidative stress (10,11). ER stress response involves three different signaling pathways: unfolded protein response (UPR), ER-associated protein degradation (ERAD) and ER overload response (EOR) (9,12). Under ER stress, unfolded proteins are accumulated within the ER lumen and trigger an adaptive response which acts to restore normal ER function. However, if ER stress persists for extended periods of time and cellular homeostasis cannot be restored, the ER stress response can lead to cell apoptosis (11,13,14). ER stress has been reported in different pathophysiological conditions, including neurodegenerative diseases, such as AD (15,16). Previous studies have demonstrated that chronic stress increases the accumulation of reactive oxygen species (ROS) and induces oxidative damage in the cerebral cortex and hippocampus (8,17). However, the effects of CRS on ER

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Key words: chronic restraint stress, endoplasmic reticulum stress, learning and memory impairment, GRP78, Alzheimer's disease

stress in the frontal cortex and hippocampus have not yet been established.

The model of CRS is most popular in the study of the mechanisms of cognitive deficits induced by chronic stress (18). We hypothesized that ER stress plays an important role in the neuronal damage induced by CRS. In the present study, male mice were repeatedly exposed to CRS for 8 weeks. Cognitive function and histological damage in the frontal cortex and hippocampus were assessed using the Morris water maze and H&E staining. Furthermore, we measured the expression levels of protein kinase C (PKC) α , 78 kDa glucose-regulated protein (GRP78), mesencephalic astrocyte-derived neurotrophic factor (MANF) and C/EBP-homologous protein (CHOP) in the frontal cortex and hippocampus. The data presented in this study may contribute to a more complete understanding of the mechanisms through which chronic stress affects the development and progression of AD.

Materials and methods

Animals and treatment. The animal experiments were approved by the Committee on the Care and Use of Laboratory Animals of Anhui Medical University, Anhui, China. KM strain male mice (20–25 g) were used in this study. The animals were obtained from the Anhui Laboratory Animal Center. The mice were housed in standard cages (6 animals per cage), maintained under a 12-h dark/light cycle and provided with food and water *ad libitum*.

The mice were randomly divided into the control group and CRS group. The mice in the CRS group were placed in a 50-ml conical centrifuge tube with multiple punctures to allow ventilation in their home cages for 2 h per day between 14:00 to 16:00 for 8 weeks (6 days each week) as previously reported (19,20). The mice in the control group were not subjected to stress and were allowed unrestricted access to food and water.

Morris water maze test. The Morris water maze test was carried out as previously described (21). The Morris water maze consisted of a black circular pool (diameter, 120 cm; height, 60 cm) filled with water and the pool was divided into 4 quadrants. The animals (10 mice in each group) were placed into the pool (facing the wall of the pool) and were allowed to circumnavigate the pool in search of the escape platform (in the center of a quadrant submerged 2 cm below the water surface) for 4 trials (60 sec per trial) per day from day 52 to 55 during exposure to CRS. The escape latency (sec) was recorded to indicate the learning results. After the final trial, the platform was removed from the tank and each mouse was subjected to a 60-sec swim probe test. The number of platform crossings (NPCs) and the swimming time in the quadrant of the platform (STP) were recorded to indicate the memory results.

Histological examination. Twenty-four hours after the final exposure to restraint stress, the animals were sacrificed by cervical dislocation. The brains were removed (5 animals in each group) and fixed in 4% paraformaldehyde and embedded in paraffin. The brain was cut into 5 μ m-thick sections using a section cutter (Leica Biosystems, Wetzlar, Germany). The sections were stained with hematoxylin and eosin (H&E) and

examined under a light microscope (Olympus IX71; Olympus, Tokyo, Japan).

Immunohistochemistry. The brain paraffin sections were passed through a gradient of xylene and ethyl alcohol to hydrate the tissue (5 sections in each group). The sections were treated with 0.3% hydrogen peroxide to block endogenous peroxidase activity, then washed with PBS. The sections were treated with primary antibody overnight at 4°C. The primary antibodies to PKC α (BS646; 1:200), GRP78 (BS6479; 1:200), CHOP (BS6814; 1:200) were from Bioworld Technology Co. The sections were incubated with a biotinylated anti-rabbit secondary antibody and subjected to diaminobenzidine oxidation using the ABC kit (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). The sections were re-stained with hematoxylin and examined under a microscope (Olympus IX71). The positive cells were stained brown. Three non-overlapping fields (x400) in each area of the hippocampus CA1 region and the frontal cortex of each section were analyzed in a blinded manner. The integral optical density of immunopositive neurons in each section was measured using the JD801 Image analysis system (Jiangsu Jieda Technology, Jiangsu, China) to indicate the expression of PKC α , GRP78 and CHOP.

Immunoblot analysis. Protein was extracted from the frozen tissue of the frontal cortex and hippocampus (4 animals in each group). The protein concentration was determined using the BCA Protein Assay kit (Shanghai Sangon Biotechnology, Shanghai, China) and equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked at room temperature for 1 h with 5% dry skim milk in Tris-buffered saline containing 1% Tween-20 (TBS-T). The membranes were treated with antibodies to GRP78, CHOP and MANF (provided by Professor Yuxian Shen, Anhui Medical University) and β -actin (1:1,000) overnight at 4°C. The membranes were then incubated with anti-rabbit IgG antibody conjugated to HRP (1:10,000) for 1 h. After extensive washes, the protein bands were detected using chemiluminescence reagents (ECL kit; Amersham Biosciences, Little Chalfont, UK). The Tanon4500 Imaging System (Shanghai Tanon Technology, Shanghai, China) was used to visualize the protein bands, and densitometry was performed using ImageJ software. The relative density of the immunoreactive bands was normalized to the density of the corresponding bands of β -actin.

Data analysis. The data were analyzed by using SPSS 16.0 statistical analysis software. Statistical differences between the two treatment groups were determined using an unpaired two-tailed Student's t-test, and a value of $P < 0.05$ was considered to indicate a statistically significant difference. Data are presented as the means \pm SD.

Results

CRS accelerates behavioral impairment in male mice. In the memory training experiment, the mean escape latencies (day 1 and 2) differed significantly between the 2 groups (day 1, 51.85 \pm 17.74 vs. 59.19 \pm 2.44 sec; day 2, 38.65 \pm 10.70 vs. 49.35 \pm 8.36 sec for mice in the control group and mice exposed to CRS, respectively) (Fig. 1A). In the probe trial, the average

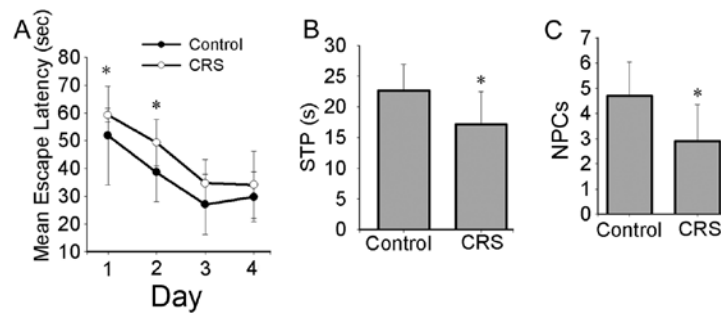


Figure 1. Exposure to chronic restraint stress (CRS) results in learning and memory impairment in male mice (Morris water maze). (A) Mean escape latency. (B) The swimming time in the quadrant of the platform (STP). (C) The average number of platform crossings (NPCs). Data represent the means \pm SD (n=10). *P<0.05 compared to the control group.

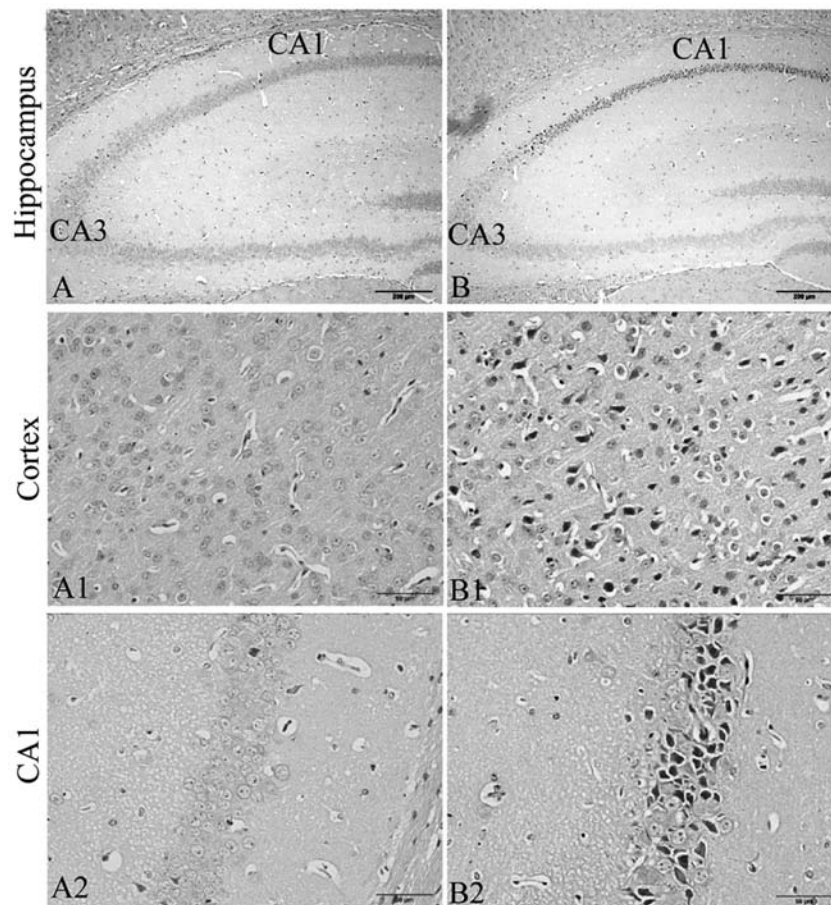


Figure 2. Exposure to chronic restraint stress (CRS) enhances neuronal degenerative changes in the frontal cortex and hippocampus CA1 region (H&E staining, hippocampus, magnification, $\times 100$; cortex and CA1, $\times 400$; n=5). In the degenerating cells, acidophilic degeneration and nuclear pyknosis was observed. In the CRS group, more degenerative neurons were observed in the frontal cortex and hippocampus CA1 areas. (A) Hippocampus, (A1) frontal cortex, and (A2) hippocampus CA1 region in the control group. (B) Hippocampus, (B1) frontal cortex, and (B2) hippocampus CA1 region in the group exposed to CRS.

number of platform crossings (NPCs) and the swimming time in the quadrant of the platform (STP) also differed significantly between the 2 groups (NPCs, 4.70 ± 1.34 vs. 2.90 ± 1.45 ; STP, 22.62 ± 4.36 vs. 17.19 ± 5.32 STP for mice in the control group and mice exposed to CRS, respectively) (Fig. 1B and C).

CRS enhances neuronal degeneration in the frontal cortex and hippocampus CA1 region. In order to investigate the effects of chronic stress on cortical and hippocampal neurons, we examined the neuronal histomorphological changes in the frontal

cortex and hippocampus (Fig. 2). In the control group mice, no obvious neuronal abnormalities were observed in the frontal cortex and hippocampus CA1 region (Fig. 2A, A1 and A2). However, in the CRS group, more degenerative neurons were observed in the frontal cortex and hippocampus CA1 region (Fig. 2B, B1 and B2). Acidophilic degeneration and nuclear pyknosis was observed.

Effects of CRS on the expression of PKC α , GRP78, CHOP and MANF in the frontal cortex and hippocampus. To evaluate the

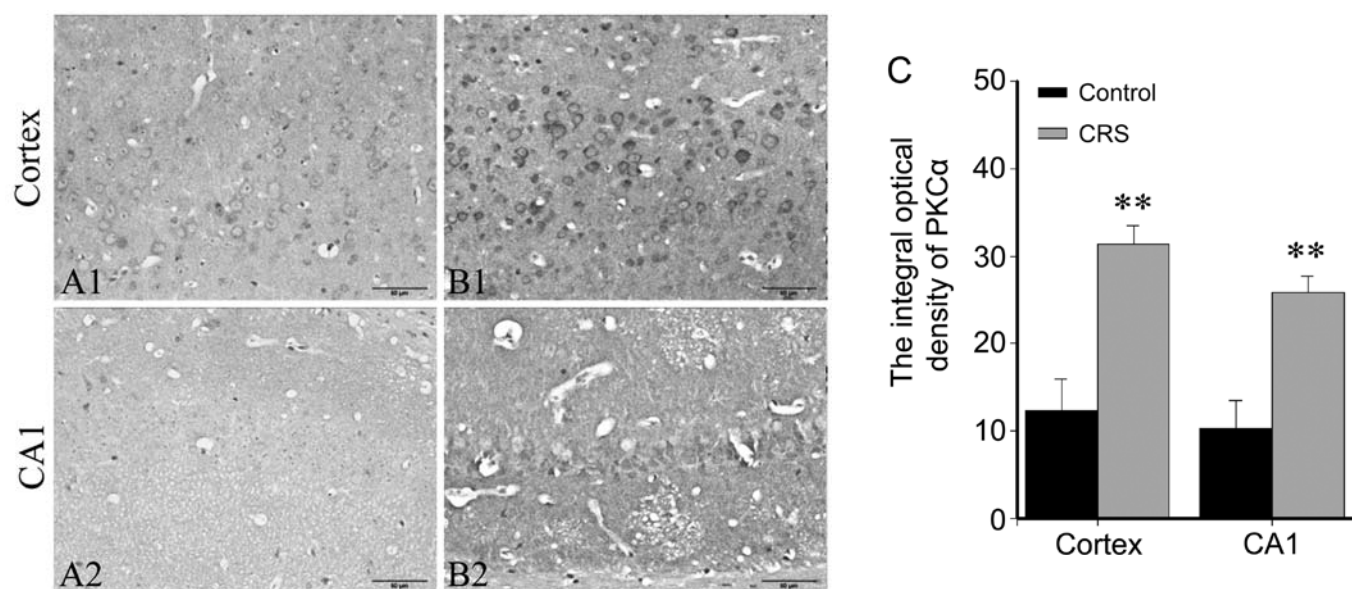


Figure 3. Exposure to chronic restraint stress (CRS) increases the expression of protein kinase C (PKC) α in the frontal cortex and hippocampus CA1 region (immunohistochemistry, magnification, x400). (A1) Frontal cortex, (A2) hippocampus CA1 region in the control group. (B1) Frontal cortex, (B2) hippocampus CA1 region in the group exposed to CRS. (C) Quantitative assessment of the data from (A and B). Data represent the means \pm SD (n=5). **P<0.05 compared to the control group.

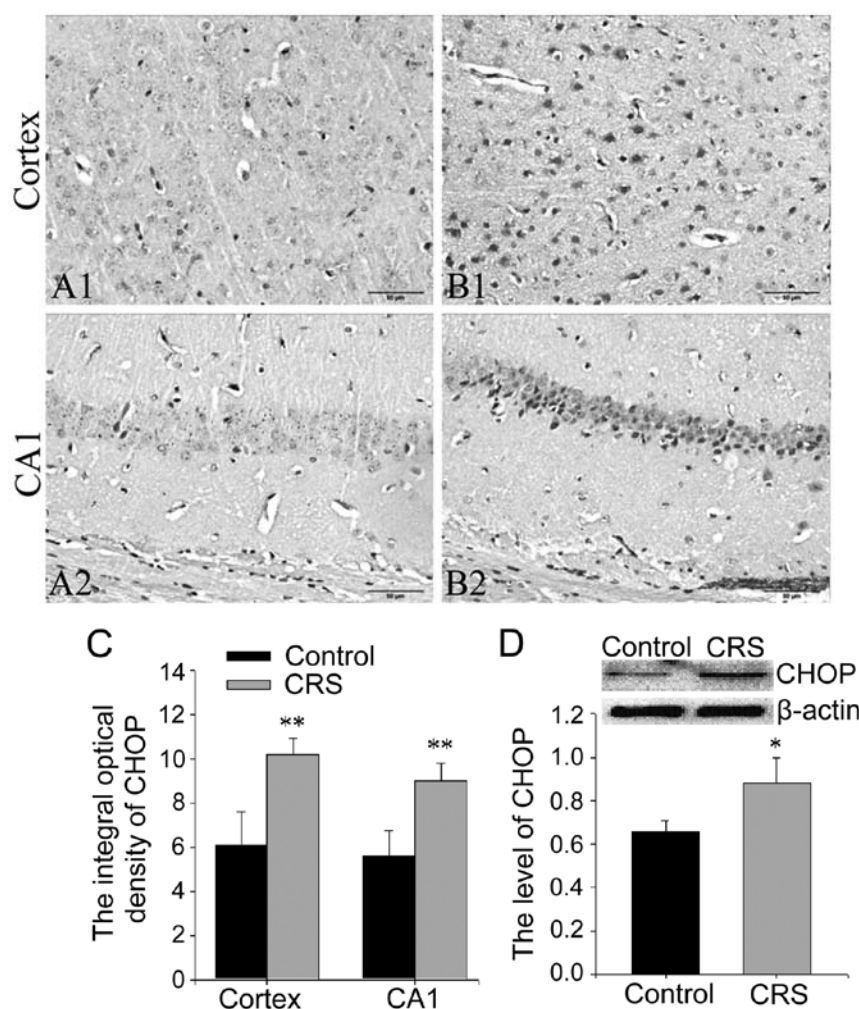


Figure 4. Exposure to chronic restraint stress (CRS) increases the expression of C/EBP-homologous protein (CHOP) in the frontal cortex and hippocampus CA1 region (immunohistochemistry, magnification, x400; immunoblot analysis). (A1) Frontal cortex, (A2) hippocampus CA1 region in the control group. (B1) Frontal cortex, (B2) hippocampus CA1 region in the group exposed to CRS. (C) Quantitative assessment of the data from (A and B). (D) Results of immunoblot analysis of CHOP expression in tissue of the frontal cortex and hippocampus. Data represent the means \pm SD (immunohistochemistry, n=5; immunoblot analysis, n=4). *P<0.05, **P<0.01 compared to the control group.

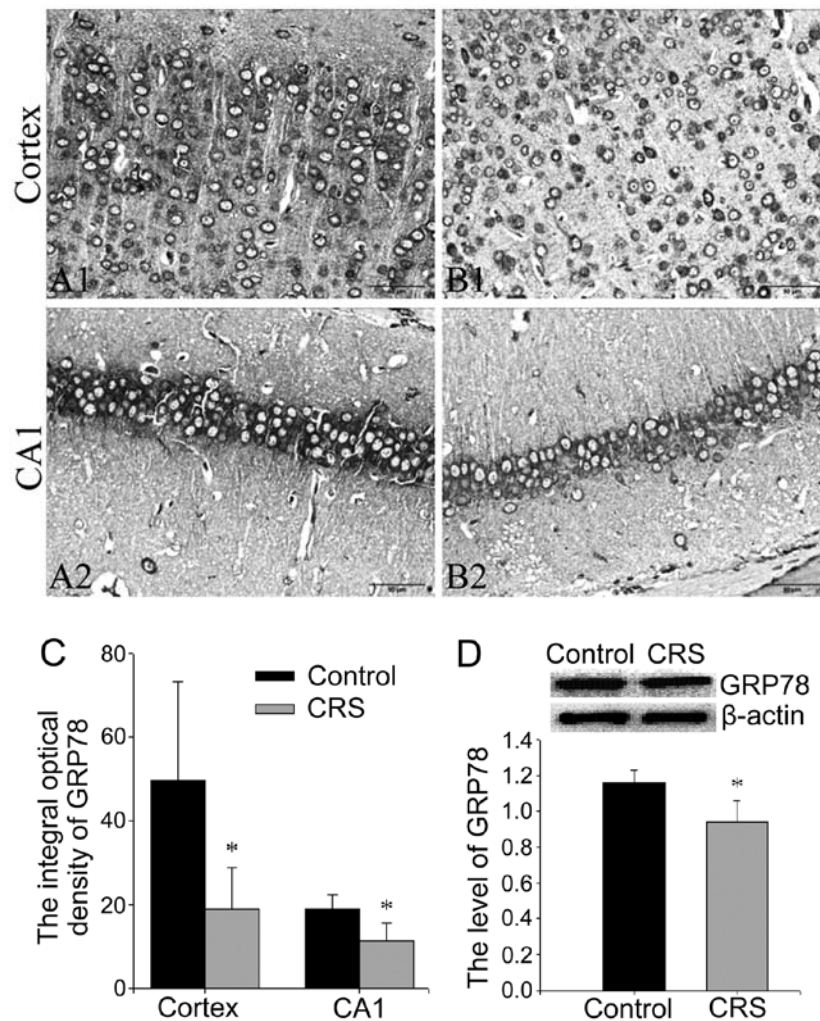


Figure 5. Exposure to chronic restraint stress (CRS) decreases 78 kDa glucose-regulated protein (GRP78) expression in the frontal cortex and hippocampus CA1 region (immunohistochemistry, magnification, x400; immunoblot analysis). (A1) Frontal cortex, (A2) hippocampus CA1 region in the control group. (B1) Frontal cortex, (B2) hippocampus CA1 region in group exposed to CRS. (C) Quantitative assessment of the data from (A and B). (D) Results of immunoblot analysis of CHOP expression in tissue of the frontal cortex and hippocampus. Data represent the means \pm SD (immunohistochemistry, n=5; immunoblot analysis, n=4). *P<0.05 compared to the control group.

effects of CRS on ER stress, we detected the expression of PKC α , GRP78 and CHOP in the frontal cortex and hippocampus by immunohistochemistry. We further detected the expression of MANF, GRP78 and CHOP in the tissue of the frontal cortex and hippocampus by immunoblot analysis. The results from immunohistochemistry revealed that exposure to CRS significantly increased the expression of PKC α (Fig. 3) and CHOP (Fig. 4A-C), and decreased the expression of GRP78 (Fig. 5A-C) in the frontal cortex and hippocampus CA1 region. The results of immunoblot analysis revealed that exposure to CRS significantly increased the expression of MANF (Fig. 6) and CHOP (Fig. 4D) and decreased the expression of GRP78 (Fig. 5D).

Discussion

The results of the present study demonstrated that exposure to CRS for 8 weeks caused a significant impairment in cognitive function and neuronal damage in the frontal cortex and hippocampus CA1 region of male mice. Moreover, exposure to CRS (for 8 weeks) significantly increased the expression of PKC α , CHOP and MANF, decreased the expression of GRP78.

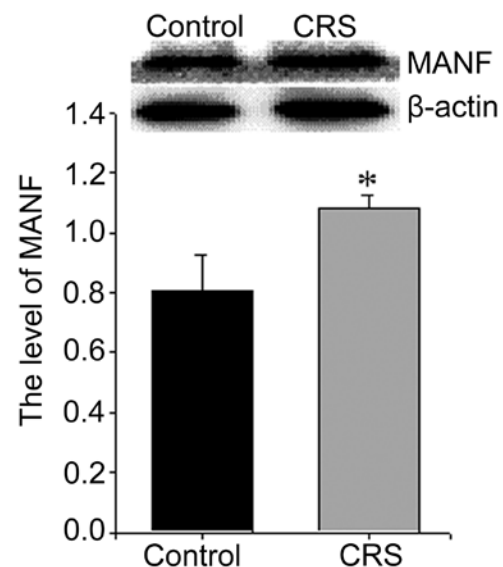


Figure 6. Exposure to chronic restraint stress (CRS) increases the expression of mesencephalic astrocyte-derived neurotrophic factor (MANF) in the frontal cortex and hippocampus CA1 tissue (immunoblot analysis). Data represent the means \pm SD (n=4). *P<0.05 compared to the control group.

To the best of our knowledge, this is the first study to describe the effects of chronic stress on ER stress in the frontal cortex and hippocampus.

Chronic stress has been reported to be associated with many neurodegenerative diseases, such as depression, AD and Parkinson's disease (22-24). The chronic stress-induced neurodegenerative diseases are an outcome of different mechanisms, such as central neurotransmitters, neurohormonal factors, particularly those linked with the hypothalamic-pituitary-adrenal (HPA) axis and free radical generation (25,26). It has been reported that exposure to acute restraint stress (150 min of immobilization) significantly increases neuronal damage in the frontal cortex and striatum both at 1 and 24 h following exposure to restraint stress (27). The present study demonstrated that exposure to CRS for 8 weeks caused a significant impairment in cognitive function and neuronal damage in the frontal cortex and hippocampus CA1 region in male mice. However, it remains unclear as to whether ER stress is involved in chronic stress-induced learning and memory impairment.

ER stress is originally an adaptive response that can be induced by various stimuli during normal ER function, such as the accumulation of unfolded or misfolded proteins and changes in ER Ca^{2+} homeostasis. ER stress triggers the activation of several signaling pathways to cope with the abnormal load in the ER lumen (28). The UPR pathway induces the upregulation of ER chaperones, such as GRP78 and the EOR pathway induces the activation of nuclear factor- κB (NF- κB), leading to the production of cytokines (29). However, ER stress may also contribute to cell suicide when abnormalities become extensive (30). Furthermore, studies have demonstrated that ER stress may play an important role in the development of neurodegenerative diseases (13) and ER stress may occur prior to neuronal cell death (31-33). Previous studies have also reported that oxidative stress contributes to ER stress (33-35). In our previous study, we demonstrated that exposure to CRS increased ROS accumulation and induced oxidative damage in the cerebral cortex and hippocampus (8). This suggests that ER stress is involved in the oxidative damage induced by chronic stress in the frontal cortex and hippocampus.

The activation of PKC isoforms has been shown to be associated with ER stress (28,36). PKC is involved in the induction of GRP78 (37), a major ER chaperone and a crucial regulator of ER homeostasis. PKC α , a classic PKC isoform, has been reported to mediate growth arrest in human embryonal rhabdomyosarcoma cells by inducing the activation of JNKs, p38 kinase and ERKs (38), which are involved in the induction of ER stress (37,39). GRP78 is a molecular chaperone only located in the ER. GRP78 facilitates protein folding, stabilizes calcium homeostasis and protects cells against oxidative stress and apoptotic death (40,41). With ER stress, the level of the ER marker, GRP78, has been shown to increase in a time-dependent manner, peaking at 24 h and decreasing at 48 h (42). CHOP, also known as growth arrest and DNA damage-inducible gene 153 (GADD153), is an important mediator of ER stress-induced cell death (43). CHOP is expressed at low levels under physiological conditions. It is upregulated during severe and prolonged ER stress and plays a crucial role in cell arrest and apoptosis (44,45). In a previous study, the apoptotic initiator, CHOP, showed the highest level at 48 h during ER stress (42). MANF is also an ER stress responsive protein with

neuroprotective effects in animal models of neurodegeneration. ER stress can cause the upregulation of MANF (46). In the present study, we found that exposure to CRS significantly increased the expression of PKC α , CHOP and MANF, and decreased the expression of GRP78 in the frontal cortex and hippocampus CA1 region. These data suggest that CRS induces the ER overload response in the cortex and hippocampus. Furthermore, ER-resident proteins, such as GRP78 are susceptible to oxidative damage (47). Oxidation of the ER proteins may dissociate GRP78 and cause further activation of the ER stress signals (48). CRS can promote ROS accumulation, which may dissociate GRP78 and induce the decrease of GRP78 in the frontal cortex and hippocampus.

In conclusion, to the best of our knowledge, the present study demonstrates for the first time that ER stress may be involved in the impairment of cognitive function and neuronal damage in the frontal cortex and hippocampus induced by CRS. It should be noted that the present study only examined the effects of CRS on cognitive function, neuronal damage and the expression of PKC α , GRP78, CHOP and MANF in the frontal cortex and hippocampus. The close interaction between chronic stress and ER warrants further investigation. Despite its preliminary character, this study clearly indicates that ER stress is implicated in the learning and memory impairment induced by CRS. Manipulation of the ER stress pathway signals may provide novel therapeutic interventions for chronic stress-related neurodegenerative diseases, such as AD.

Acknowledgements

The authors are grateful for the support provided by the National Natural Science Foundation of China (81173624) and the Doctor Foundation of Anhui Medical University (XJ201011). They also would like to thank Dake Huang, Shan Huang and Li Gui (Synthetic Laboratory of Basic Medicine College, Anhui Medical University) for their excellent technical assistance.

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