



# Endoplasmic reticulum stress is involved in restraint stress-induced hippocampal apoptosis and cognitive impairments in rats



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## HIGHLIGHTS

- RS induces hippocampal apoptosis and cognitive impairments.
- RS triggers the UPR in hippocampus.
- The ERS-induced apoptotic pathway is activated in hippocampus of RS rats.
- Inhibition of ERS alleviates the cognitive impairments induced by RS.

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## ABSTRACT

Long-term exposure to stressful stimuli can reduce hippocampal volume and cause cognitive impairments, but the underlying mechanisms are not well understood. Endoplasmic reticulum stress (ERS) is considered an early or initial response of cells under stress and linked to neuronal death in various neurodegenerative diseases. The present study investigated the involvement of ERS in restraint stress (RS)-induced hippocampal apoptosis and cognitive impairments. Using the rat RS model for 21 consecutive days, we found that the hippocampal apoptotic rate was significantly up-regulated as compared with unstressed controls, and salubrinal (ERS inhibitor) pretreatment effectively reduced the increase. As the marker of ERS, the 78-kDa glucose-regulated protein (GRP78) and the target molecule of the unfolded protein response (UPR), the splice variant of X-box binding protein 1 (sXBP-1) were also markedly increased in RS rats. Furthermore, in the three possible signaling pathways of ERS-induced apoptosis, the protein and mRNA levels of C/EBP homologous protein (CHOP) were significantly up-regulated, and caspase-12 was activated and cleaved, which suggested that these two pathways crucially contributed to hippocampal cell death. However, we found no changes in protein levels of phosphorylated JNK, implying that the JNK pathway was not the primary pathway involved in hippocampal apoptosis. It is more important that the cognitive impairments caused by RS were also effectively alleviated by salubrinal pretreatment. The present results suggested that ERS in hippocampus was excessively activated under stress, and amelioration of ERS could be a novel strategy to prevent and treat impaired cognitive function induced by RS.

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**Abbreviations:** ATF6, activating transcription factor 6; CHOP, C/EBP homologous protein; CNS, central nervous system; ER, endoplasmic reticulum; ERS, endoplasmic reticulum stress; GRP78, 78-kDa glucose-regulated protein; IRE1, inositol-requiring enzyme 1; JNK, c-JUN NH<sub>2</sub>-terminal kinase; PERK, protein kinase RNA (PKR)-like ER kinase; RS, restraint stress; UPR, unfolded protein response; XBP-1, X-box binding protein 1.

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## 1. Introduction

Stress is characterized by a combination of physiologic, neuroendocrine, behavioral, and emotional responses to novel or threatening stimuli [1]. Activation of the stress response leads to alterations that improve the ability of an organism to adjust its homeostasis and minimize the potential impact of a threat [2]. However, excessive stress can produce damaging physiological effects and is accompanied by various psychological and cognitive changes [3,4].

The hippocampus, which plays a vital role in learning and memory, contextual fear conditioning, and neuroendocrine regulation, is an important brain region susceptible to stress. It has been demonstrated

that chronic exposure to stress changes hippocampal function and structure [5,6] and reduces hippocampal volume [7]. Also, a reduction in the hippocampal volume has been reported in animal models of stressful events that have all been associated with memory loss in humans [8]. Furthermore, clinical studies have shown that individuals with depression suffer from hippocampal-dependent cognitive impairments and show reductions in hippocampal volume [9,10]. It is believed that the neuronal loss is one of the important factors involved in the reduction of hippocampal volume [11]. However, the underlying pathophysiological mechanisms of stress-induced hippocampal neuronal loss and cognitive impairments are complex and still not completely established.

Strong and long-lasting stress may result in the death or loss of neurons through apoptosis [12]. The endoplasmic reticulum (ER) is the primary site for secretory protein synthesis and maturation,  $\text{Ca}^{2+}$  storage and lipid biosynthesis. Various stimuli can disturb ER homeostasis and result in the accumulation of unfolded and misfolded proteins and pathological consequences, namely endoplasmic reticulum stress (ERS) [13]. Meanwhile, the accumulating unfolded proteins cause dissociation of 78-kDa glucose-regulated protein (GRP78) from the three major ER transmembrane effector proteins: protein kinase RNA (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) and a resultant launching of the unfolded protein response (UPR) [14]. Increased expression of GRP78 serves as a good marker for ERS [15]. Moderate ERS can relieve cellular dysfunction and enhance the chance for survival, but prolonged and/or severe stress leads to cell apoptosis. ERS is considered an early or initial response of cells under stress or damage and linked to neuronal death in various neurodegenerative diseases [16]. In these processes, ERS causes cell damage and apoptosis, and inhibiting ERS response pathways may provide nervous system protection.

Restraint stress (RS), a common animal model for the production of chronic stress, is one of the well accepted stressors used in experimental stress research, which elicits psychological frustration and physiological stress accompanied with vigorous struggle to escape [17]. In the present study, using the rat RS model for 21 consecutive days, we explored the effects of repeated RS on hippocampal apoptosis *in vivo*. Salubrinal, a selective inhibitor of eukaryotic translation initiation factor 2 subunit  $\alpha$  (eIF2 $\alpha$ ) dephosphorylation, is known for its ability to protect cells from ERS-induced apoptosis [18], and pretreated by intracerebroventricular injection to examine the relationship between ERS and neuronal apoptosis, even cognitive impairments. Furthermore, the possible signaling pathways of ERS-induced apoptosis were assessed to clarify the mechanism of ERS-induced hippocampal apoptosis and cognitive impairments.

## 2. Materials and methods

### 2.1. Animals

All animal care and experimental protocols were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Institutional Animal Care and Use Committee of Hebei Medical University. Adult male Sprague–Dawley (SD) rats weighing 250–280 g were obtained from the Experimental Animal Center of Hebei Medical University (Shijiazhuang, Hebei, China). Every attempt was made to reduce the number and to minimize pain and suffering of animals.

### 2.2. Lateral ventricle cannula surgery and microinjections

After 7 days of acclimatization, all rats were housed individually in polypropylene cages under hygienic and standard environmental conditions ( $26 \pm 2 \text{ }^\circ\text{C}$ , humidity 60%–70%, 12 h light/dark cycle). Surgical implantation of cannula was used for the intracerebroventricular injection and conducted in an aseptic environment. All rats were

anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneally) and placed in a stereotaxic apparatus (Benchmark Stereotaxic Instruments, USA). After the surface of the skull was exposed, a single hole was drilled through the skull above the left lateral ventricle (from the bregma to AP, 0.92 mm; ML, 1.65 mm). A stainless steel guide cannula was implanted 3.31 mm ventrally beneath the surface of the skull. To prevent occlusion, a dummy cannula was inserted into the guide cannula. Dental cement was used to fix the guide cannula to the skull. After surgery, all animals were treated with penicillin (1000 u/day i.m.) for 3 days and allowed to recover for at least 7 days.

Each microinjection was made with a 10  $\mu\text{l}$  syringe (Hamilton, USA) attached to PE tubing connected to the injection cannula and was given at a rate of 0.5  $\mu\text{l}/\text{min}$  in a volume of 2  $\mu\text{l}$  using a syringe pump (KD Scientific, USA). The injection cannula extended 0.2 mm beyond the guide cannula and was left in place for 5 min following microinjections to minimize backflow of the drug.

### 2.3. Experimental design

All rats were divided into five groups: unstressed normal control group (Con group), restraint stress group (RS group), restraint stress combined with salubrinal (Sal, Santa Cruz Biotechnology, Santa Cruz, CA, USA) administration group (RS + Sal group), restraint stress combined with normal saline administration group (RS + NS group), and unstressed with salubrinal administration group (Sal group). Salubrinal (75  $\mu\text{M}$ ) or the same dose of normal saline respectively was injected into the lateral ventricle 30 min before restraint stress for 21 consecutive days, using the injection cannula.

In total, 86 animals were used. Nine rats were excluded because of anesthesia accident or surgery failure. At the end of all behavioral experiments, the location of the cannula was examined histologically. Due to the mislocation or dropout of cannula, 17 rats were not included in the statistical analyses. At last, 60 rats (12 rats per group) were used in the analyses.

Morris water maze test was assessed at 24 h after the last restraint stress. Then, the rats were decapitated in deep anesthesia, and brains were rapidly removed. The left hippocampus was dissected for apoptosis examination and the right was examined for protein or mRNA analysis.

### 2.4. Restraint stress procedure

The chronic repeated restraint stress protocol was adapted from the previous procedure [19]. And it was carried out by placing the rats in the restrainer (25 cm  $\times$  7 cm) without supplying food and water for 8 h (from 8:00 AM to 16:00 PM) every day for 21 consecutive days. The restraint devices had multiple air holes and allowed animals to stretch their legs, but not to move within the restrainers. The unstressed rats were left in their cages for the same time without food and water. During the rest period food and water were provided *ad libitum*.

### 2.5. Morris water maze test

The water maze consisted of a circular water tank (180 cm in diameter, 70 cm in height) that was partially filled with  $24 \pm 1 \text{ }^\circ\text{C}$  water. The pool was divided virtually into four equal quadrants labeled N-S-E-W. A colorless escape platform (10 cm in diameter) was hidden 1.5 cm below the surface of the water in a fixed location. The platform remained in the same quadrant during the entire experiment. The maze was located in a quiet test room, surrounded by many visual cues outside of the maze which was visible from within the pool and could be used by the rats for spatial orientation. The movement of the animals was recorded by a TV camera located over the center of the pool and was connected to a personal computer. The experiments were conducted two sessions per day for 5 days, each session comprised four trials, with an intertrial interval of 60 s, and the intersession interval was  $>2$  h. In each trial, the

rat was gently placed into the pool at the middle of the circular edge in a randomly selected quadrant, with the nose pointing toward the wall. If rats failed to find the escape platform within 120 s by themselves, they were placed on the platform for 10 s by the experimenter and their escape latency was accepted as 120 s. After climbing onto the platform, the animal remained there for 30 s before the commencement of the next trial. On the sixth day, a probe trial without the platform was assessed, and the time spent in the target quadrant where the platform had been located was recorded. Finally, swimming speed was analyzed as cm/s on the probe trial. The test was performed from 10:00 AM to 17:00 PM to exclude variations in performance resulting from circadian rhythmicity.

## 2.6. Annexin V and propidium iodide staining assay

Apoptosis was detected by Annexin V/PI apoptosis detection kit according to manufacturer's protocol (MultiSciences Biotech, Hangzhou, China). After dissection, the hippocampal tissues were processed for preparation of a single-cell suspension using mechanical trituration. The diluted cell suspension was then sieved through a steel mesh (pore size, 75  $\mu\text{m}$ ) and centrifuged at 1000 rpm for 5 min, and the pellet was resuspended in 0.01 M phosphate-buffered saline (PBS). Cells were washed twice by resuspension in PBS and centrifugation. The final pellet was resuspended in 1 $\times$  binding buffer followed by incubation with 5  $\mu\text{l}$  of Annexin V (conjugated with FITC) and 10  $\mu\text{l}$  of PI in dark for 5 min. The fluorescence of the cells was then analyzed by flow cytometer (Epics-XLII, Becton Coulter). This test discriminates intact cells (Annexin V<sup>-</sup>/PI<sup>-</sup>), early apoptotic cells (Annexin V<sup>+</sup>/PI<sup>-</sup>) and late apoptotic cells (Annexin V<sup>+</sup>/PI<sup>+</sup>).

## 2.7. Western blot analysis

Hippocampus tissues were homogenized in ice-cold homogenization buffer (1% Nonidet P-40, 0.1% Triton X-100, 30 mM sodium phosphate, PH 7.4, containing 1 mM sodium orthovanadate, 100 mM NaCl, 2.5 mM Tris-HCl, pH 7.5, and protease inhibitors), followed by centrifugation at 20,000 g for 20 min at 4 °C. After protein concentration was determined in supernatants by the Coomassie blue assay, protein extracts were resuspended in sample buffer containing 2% sodium dodecyl sulfate (SDS), 2%  $\beta$ -mercaptoethanol, 50 mmol/l Tris-HCl (PH 6.8), 10% glycerol, and 0.05% bromophenol blue. The protein mixture was then placed in boiling water for 10 min and briefly centrifuged at a low speed to collect the denatured proteins. Protein samples were loaded on 10% SDS-polyacrylamide gel electrophoresis (PAGE) in running buffer containing 25 mmol/l Tris, 192 mmol/l glycine, and 0.1% SDS. The proteins were then transferred to PVDF membranes (Millipore Corporation, Bedford, MA, USA) for 3 h at 4 °C and 200 mA with the transfer buffer containing 20 mmol/l Tris-HCl (PH 8.0), 150 mmol/l glycine, and 20% methanol. After blocking with 5% nonfat dried milk in TBS-T (20 mmol/l Tris-HCl (PH 7.6), 150 mmol/l NaCl, and 0.1% Tween 20) for 1 h at room temperature with agitation, the PVDF membranes were incubated with the primary antibodies anti-cleaved caspase-3 (1:1000; Cell Signaling Technology, Beverly, MA, USA), Bax (1:1000; Proteintech, Chicago, USA), Bcl-2 (1:500; Proteintech), GRP78 (1:1000; Santa Cruz), sXBP-1 (1:500; Santa Cruz), CHOP (1:1000; Cell Signaling Technology), caspase-12 (1:1500; Abcam, Cambridge, UK), total (T)-JNK (1:500; Epitomics, Burlingame, CA, USA), phosphorylated (p)-JNK (1:1000; Epitomics) and  $\beta$ -actin (1:2000; Santa Cruz) overnight at 4 °C. After washing with TBST three times, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) at room temperature for 2 h, followed by detection using enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA). Immunoreactive bands were visualized using X-ray films. For quantitative analysis, bands were evaluated with ImageJ software, normalized for  $\beta$ -actin density. All experiments were repeated at least three times.

## 2.8. Quantitative RT-PCR analysis

Total RNA was extracted from hippocampus with TriZol Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized from the total RNA (0.5  $\mu\text{g}$ ) using the PrimeScript<sup>TM</sup> RT reagent Kit following the instructions provided by the manufacturer (Takara Biotechnology, Dalian, China). Subsequently, the cDNA was subject to quantitative PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Each quantitative PCR reaction consisted of 2  $\mu\text{l}$  diluted RT product, 10  $\mu\text{l}$  SYBR Green PCR Master Mix (2 $\times$ ) and 250 nM forward and reverse primers in a total volume of 20  $\mu\text{l}$ . Reactions were carried out on 7500 quantitative PCR System (Applied Biosystems) for 40 cycles (95 °C for 30 s, 60 °C for 40 s) after initial 10 min incubation at 95 °C. The primers used for quantitative PCR were as follows: GRP78 forward: 5'-AAG GTG AAC GAC CCC TAA CAA A-3', reverse: 5'-GTC ACT CGG AGA ATA CCA TTA ACA TCT-3'; sXBP-1 forward: 5'-AGA GTA GCA GCA CAG ACT GCG CG-3', reverse: 5'-GGA ACT GG-GT-CCT TCT GGG TA-3'; CHOP forward: 5'-GCC TTT CGC CTT TGA GAC AGT-3', reverse: 5'-TGA GAT ATA GGT GCC CCC AAT T-3'; GAPDH forward: 5'-GGC ATG GAC TGT GGT CAT GA-3', reverse: 5'-TTC ACC ACC ATG GAG AAG GC-3'. The fold change in expression of each gene was calculated using the  $\Delta\Delta\text{Ct}$  method, with the housekeeping gene GAPDH mRNA as an internal control.

## 2.9. Statistical analysis

All data were expressed as the mean  $\pm$  SEM and analyzed using SPSS for Windows (Chicago, IL, USA). Significance of escape latencies in MWM was analyzed using a two-way analysis of variance (ANOVA). Other data were analyzed with one-way ANOVA followed by Duncan's post hoc test. The *P* value of <0.05 was considered statistically significant.

## 3. Results

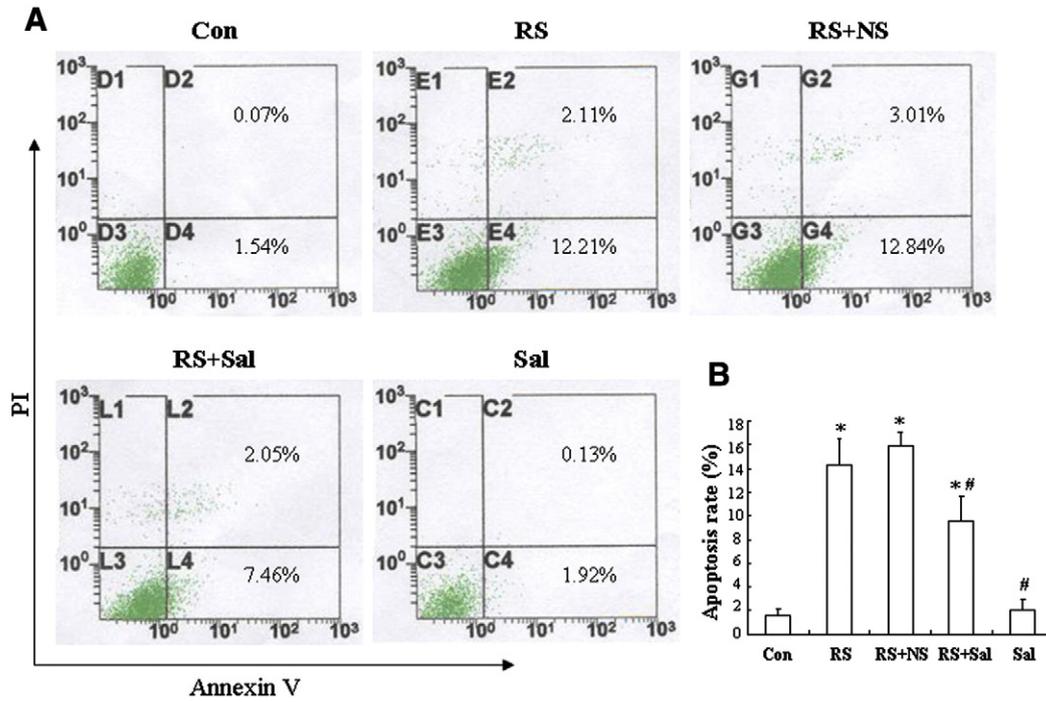
### 3.1. Restraint stress initiates hippocampal apoptosis

To investigate whether repeated restraint stress induced hippocampal apoptosis, Annexin V and PI staining was used to measure the apoptotic rate. This assay divided apoptotic cells into two stages: early apoptotic (Annexin V<sup>+</sup>/PI<sup>-</sup>) and late apoptotic (Annexin V<sup>+</sup>/PI<sup>+</sup>) cells, the two parts of cells represented the total cells of apoptosis. As shown in Fig. 1A and B, the percentage of hippocampal cells including early and late apoptotic cells was significantly increased when rats were subjected to restraint stress. Salubrinal, an inhibitor of eIF2 $\alpha$  dephosphorylation, was administered by intracerebroventricular injection before restraint stress, and effectively reduced the apoptotic rate, although it was not completely abolished ( $F_{(4,59)} = 109.85$ ,  $p < 0.001$ , post hoc Duncan's test).

Furthermore, we examined the protein levels of cleaved caspase-3, Bax and Bcl-2, which are well recognized indicators of apoptosis [20]. Western blot analysis revealed that in restraint stress rat hippocampus, the protein levels of cleaved caspase-3 were significantly up-regulated as compared with unstressed controls, and the increase was effectively inhibited by salubrinal pretreatment ( $F_{(4,59)} = 34.12$ ,  $p < 0.001$ , post hoc Duncan's test) (Fig. 2A, B). Similarly, the ratio of Bax and Bcl-2 showed the same tendency with the protein level of active caspase-3 ( $F_{(4,59)} = 67.28$ ,  $p < 0.001$ , post hoc Duncan's test) (Fig. 2C, D). Notably, the hippocampal apoptotic rate by flow cytometry, the protein level of active caspase-3 or Bax/Bcl-2 ratio all did not change when salubrinal was administered in unstressed rats. Collectively, these data confirmed that exposure of hippocampus to restraint stress induced apoptotic cell death.

### 3.2. Restraint stress triggers the unfolded protein response in hippocampus

In order to examine the effect of restraint stress on the ER of hippocampal cells, induction of GRP78, a marker of ERS was determined using

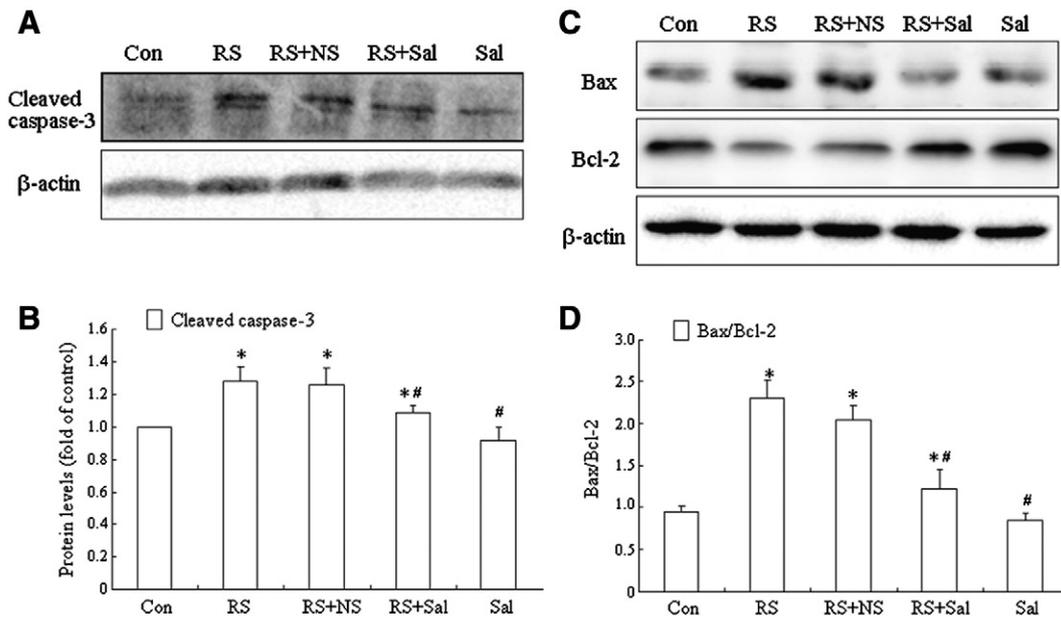


**Fig. 1.** Effect of restraint stress on hippocampal apoptotic rate. (A) Apoptotic rate was detected using Annexin V/PI for flow cytometry analysis. As shown, the cell populations in the lower left represented living cells, lower right represented early apoptotic cells, upper right represented late apoptotic cells and upper left represented damaged cells. (B) The total apoptotic rates were quantified and shown with a histogram. The data are expressed as mean  $\pm$  SEM ( $n = 12$  rats/group). Con, unstressed normal control group; RS, restraint stress group; RS + NS, restraint stress combined with normal saline administration group; RS + Sal, restraint stress combined with Salubrinal administration group; Sal, unstressed with Salubrinal administration group. \*  $p < 0.05$  compared with Con. #  $p < 0.05$  compared with RS.

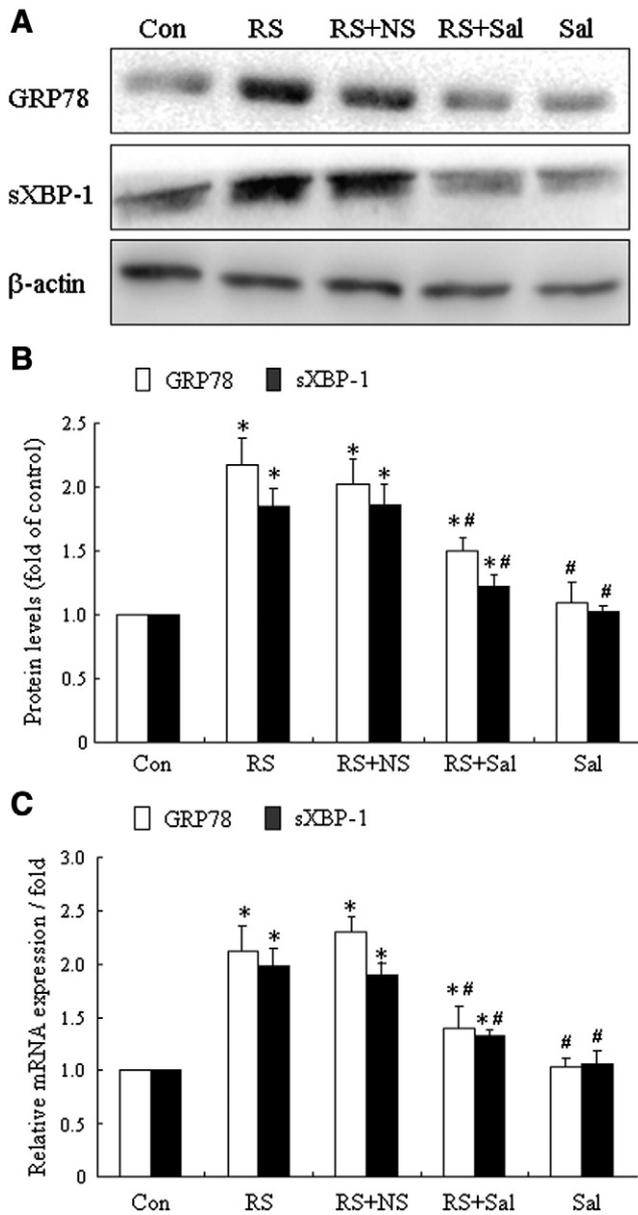
Western blot and quantitative RT-PCR analysis. As shown in Fig. 3A and B, the protein levels of GRP78 detected by Western blot were significantly increased in restraint stress rats, compared with unstressed control, and the increases were effectively inhibited by salubrinal administration ( $F_{(4,59)} = 71.20$ ,  $p < 0.001$ , post hoc Duncan's test). Furthermore, qRT-PCR analysis revealed that restraint stress increased the mRNA expression of GRP78 by 221%, and salubrinal markedly

declined the increases ( $F_{(4,59)} = 53.06$ ,  $p < 0.001$ , post hoc Duncan's test) (Fig. 3C). However, neither the protein nor the mRNA expression levels of GRP78 significantly changed when salubrinal pretreatment in unstressed rats.

Since induction of GRP78 is indicative of the activation of the UPR, we further examined the splice variant of XBP-1, as the target molecule of the branches of the UPR. The generated frame-shift splice



**Fig. 2.** Effect of restraint stress on hippocampal apoptosis. (A) The protein expression of cleaved caspase-3 was detected by Western blot analysis. (B) Protein levels of cleaved caspase-3. The level of each protein was quantified by densitometric analysis and normalized to the level of  $\beta$ -actin. (C) The protein expressions of Bax and Bcl-2 were detected by Western blot analysis. (D) The ratio for Bax/Bcl-2 protein levels was based on a densitometry reading, compared to  $\beta$ -actin. The data are expressed as mean  $\pm$  SEM ( $n = 12$  rats/group). Con, unstressed normal control group; RS, restraint stress group; RS + NS, restraint stress combined with normal saline administration group; RS + Sal, restraint stress combined with salubrinal administration group; Sal, unstressed with salubrinal administration group. \*  $p < 0.05$  compared with Con. #  $p < 0.05$  compared with RS.

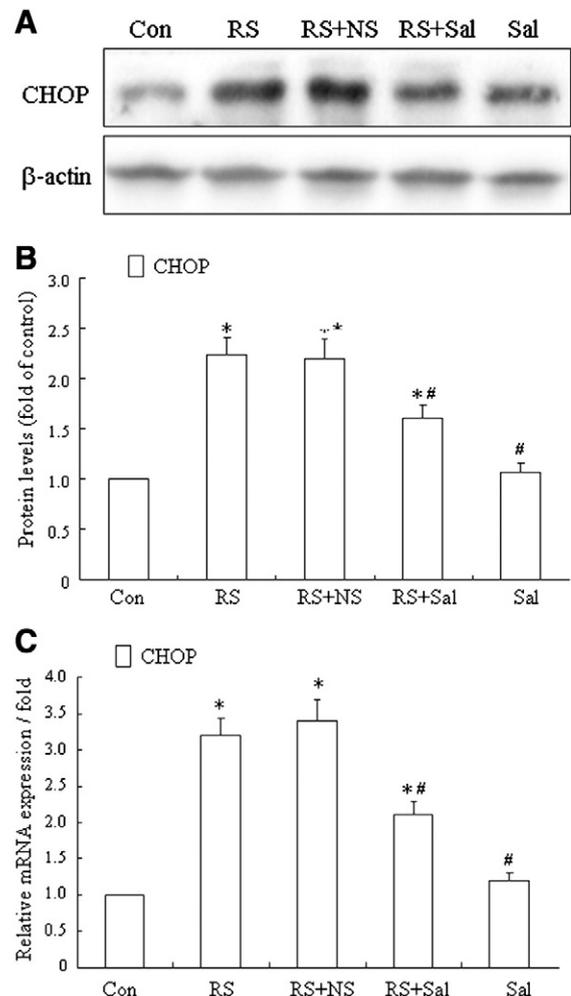


**Fig. 3.** Effects of restraint stress on the protein and mRNA levels of GRP78 and sXBP-1. (A) The protein expressions of GRP78 and sXBP-1 were detected by Western blot analysis. (B) Protein levels of GRP78 and sXBP-1. The level of each protein was quantified by densitometric analysis and normalized to the level of  $\beta$ -actin. (C) The relative mRNA levels of GRP78 and sXBP-1. Relative mRNA expression levels of GRP78 and sXBP-1 were measured by quantitative RT-PCR using the  $\Delta\Delta C_t$  method with the GAPDH mRNA as an internal control. The data are expressed as mean  $\pm$  SEM ( $n = 12$  rats/group). Con, unstressed normal control group; RS, restraint stress group; RS + NS, restraint stress combined with normal saline administration group; RS + Sal, restraint stress combined with salubral administration group; Sal, unstressed with salubral administration group. \*  $p < 0.05$  compared with Con. #  $p < 0.05$  compared with RS.

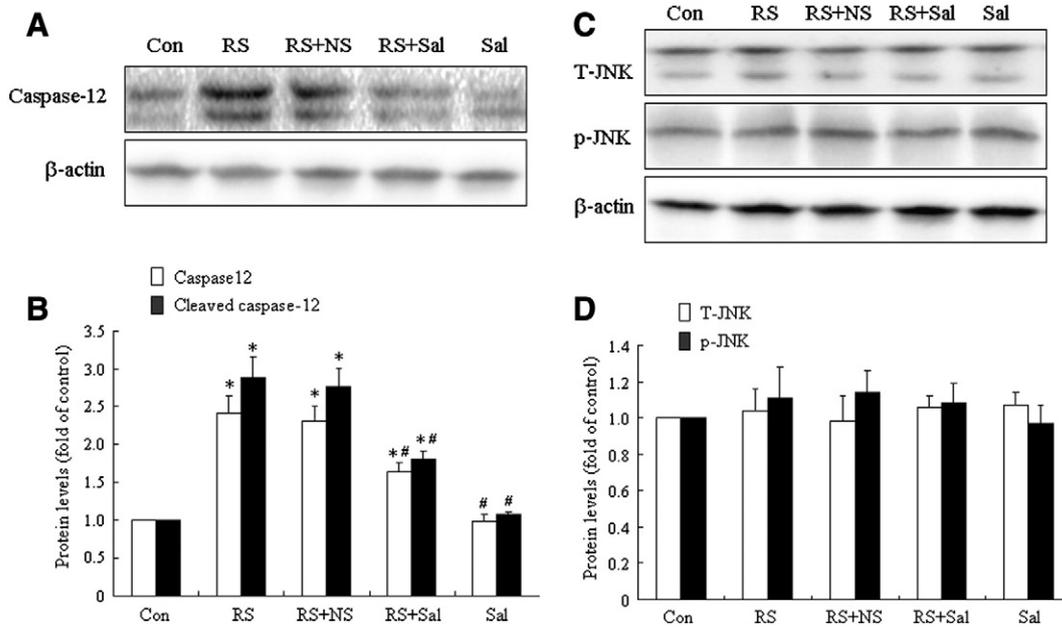
variant (sXBP-1) codes for a transcription factor that induces the expression of ER chaperones [21,22]. In our present study, the protein and mRNA expression levels of sXBP-1 were both increased in restraint stress rats, and salubral administration before restraint stress significantly inhibited the increases ( $F_{(4,59)} = 28.49$ ,  $p < 0.001$ ;  $F_{(4,59)} = 41.16$ ,  $p < 0.001$ , post hoc Duncan's test) (Fig. 3A–C). These data suggested that restraint stress, a common psychological and physical stress model, triggered the UPR in hippocampus in this experimental paradigm.

### 3.3. The endoplasmic reticulum stress-induced apoptotic pathway is activated in hippocampus of restraint stress rats

During prolonged or severe ERS, the cytoprotective UPR can switch to a pro-apoptotic response to initiate cell death [23]. Therefore, we questioned whether the ERS-mediated apoptotic pathway is a component of restraint stress-induced hippocampal cell death. So far, the ERS-mediated apoptotic pathway is only partially characterized, although some ERS-specific components of the pathway have been identified [24]. For instance, the transcription factor CHOP has been shown to play a central role by altering the balance of pro- and anti-apoptotic Bcl-2 proteins to promote apoptosis [25]. In our present study, we examined the protein and mRNA levels of CHOP using Western blot and quantitative RT-PCR analysis. As shown in Fig. 4A–C, restraint stress significantly increased the protein and the mRNA expression levels of CHOP in hippocampus by 224% and 311%, respectively compared to unstressed control, while the increases were effectively inhibited by salubral pretreatment ( $F_{(4,59)} = 27.21$ ,  $p < 0.001$ ;  $F_{(4,59)} = 19.45$ ,  $p < 0.001$ , post hoc Duncan's test).



**Fig. 4.** Effects of restraint stress on the protein and mRNA levels of CHOP. (A) The protein expression of CHOP was detected by Western blot analysis. (B) Protein levels of CHOP. The level of each protein was quantified by densitometric analysis and normalized to the level of  $\beta$ -actin. (C) The relative mRNA levels of CHOP. Relative mRNA expression levels of CHOP were measured by quantitative RT-PCR using the  $\Delta\Delta C_t$  method with the GAPDH mRNA as an internal control. The data are expressed as mean  $\pm$  SEM ( $n = 12$  rats/group). Con, unstressed normal control group; RS, restraint stress group; RS + NS, restraint stress combined with normal saline administration group; RS + Sal, restraint stress combined with salubral administration group; Sal, unstressed with salubral administration group. \*  $p < 0.05$  compared with Con. #  $p < 0.05$  compared with RS.



**Fig. 5.** Effects of restraint stress on the protein levels of caspase-12, T-JNK and p-JNK. (A) The protein expression of caspase-12 was detected by Western blot analysis. (B) Protein levels of caspase-12 and its cleavage. (C) The protein expressions of T-JNK and p-JNK were detected by Western blot analysis. (D) Protein levels of T-JNK and p-JNK. The level of each protein was quantified by densitometric analysis and normalized to the level of  $\beta$ -actin. The data are expressed as mean  $\pm$  SEM ( $n = 12$  rats/group). Con, unstressed normal control group; RS, restraint stress group; RS + NS, restraint stress combined with normal saline administration group; RS + Sal, restraint stress combined with salubribral administration group; Sal, unstressed with salubribral administration group. \*  $p < 0.05$  compared with Con. #  $p < 0.05$  compared with RS.

Another specific component of the pathway is caspase-12, which is identified as the first ER-associated member of the caspase family and is activated by ERS [26]. This novel caspase is regarded as a representative molecule implicated in the cell death-executing mechanisms relevant to ERS [27]. In order to examine the involvement of caspase-12 activation in restraint stress-induced hippocampal apoptosis, it was investigated by Western blot. As shown in Fig. 5A and B, the cleavage of caspase-12 occurred when rats were subjected to restraint stress, and decreased when pretreated salubribral ( $F_{(4,59)} = 18.28$ ,  $p < 0.001$ , post hoc Duncan's test).

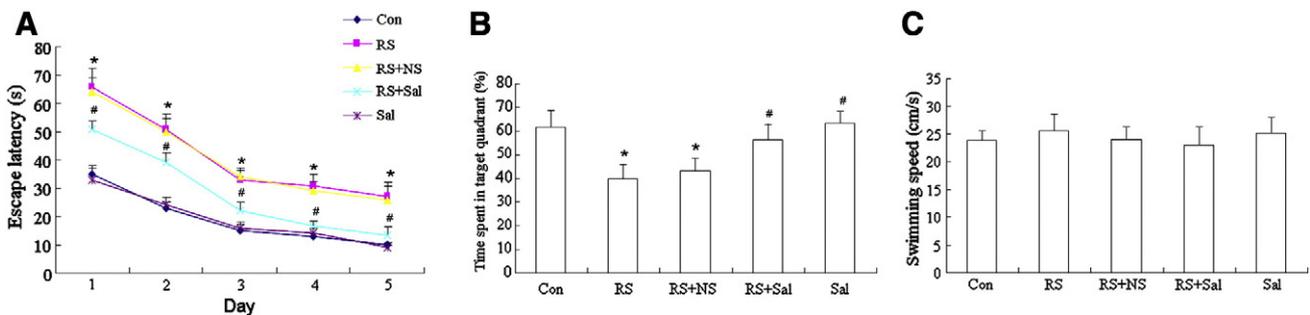
The JNK pathway mediated the third apoptotic pathway. We assessed the total(T)- and phosphorylated(p)-JNK protein expression levels using western blot analysis. Notably, the protein levels of T- and p-JNK were no obvious difference among each group ( $F_{(4,59)} = 2.11$ ,  $p > 0.05$ ;  $F_{(4,59)} = 1.57$ ,  $p > 0.05$ , post hoc Duncan's test) (Fig. 5C, D), which implied that the JNK pathway was not the primary pathway involved in the restraint stress-induced hippocampal apoptosis.

Combined, these results pointed out that not only the protective UPR was prompted by restraint stress, but prolonged stress induced severe

damage that initiated the CHOP and caspase-12 mediated apoptotic pathway.

### 3.4. Inhibition of endoplasmic reticulum stress alleviates the cognitive impairments induced by restraint stress

In order to evaluate if endoplasmic reticulum stress is involved in cognitive impairments induced by restraint stress, the Morris water maze test was carried out. As shown in Fig. 6A, all animals showed a progressive decline in the escape latency with training. Compared with the unstressed controls, restraint stress rats exhibited significantly prolonged escape latency during all sessions. However, the poor performance was mitigated by pretreatment with salubribral ( $F_{(24,275)} = 6.41$ ,  $p < 0.001$ , post hoc Duncan's test). In the probe trial, the restraint stress rats spent significantly less time in the target quadrant than the control rats, while pretreatment with salubribral significantly improved the performance ( $F_{(4,59)} = 5.88$ ,  $p < 0.001$ , post hoc Duncan's test) and showed no difference from the control group (RS + Sal vs. con,  $p > 0.05$ ; Sal vs. con,  $p > 0.05$ ) (Fig. 6B). However, for swimming



**Fig. 6.** Effects of restraint stress and salubribral pretreatment on the performance of Morris water maze in rats. (A) Latency to reach escape platform (s). (B) Time spent in target quadrant (%). (C) Swimming speed (cm/s). The data are expressed as mean  $\pm$  SEM ( $n = 12$  rats/group). Con, unstressed normal control group; RS, restraint stress group; RS + NS, restraint stress combined with normal saline administration group; RS + Sal, restraint stress combined with salubribral administration group; Sal, unstressed with salubribral administration group. \*  $p < 0.05$  compared with Con. #  $p < 0.05$  compared with RS.

speed, no significant differences were observed among the five groups ( $F_{(4,59)} = 1.34, p > 0.05$ , post hoc Duncan's test) (Fig. 6C). In addition, salubrinal per se had no significant effects on cognition.

#### 4. Discussion

In the present study, we determined the role of ERS in the modulation of restraint-induced hippocampal apoptosis and cognitive impairments. We demonstrated that RS initiated hippocampal apoptosis and cognitive impairments, which was effectively inhibited by salubrinal pretreatment. As the marker of ERS and the target molecule of UPR, the expressions of GRP78 and sXBP-1 were markedly increased in RS rats. Moreover, the CHOP and caspase-12 mediated apoptotic pathway crucially contributed to hippocampal cell death induced by ERS.

Stress is an unavoidable life experience that can disturb body homeostasis and affect different organs and systems, including the central nervous system (CNS) [28]. Prolonged stress is known to be a critical risk factor for the onset of several neuropsychiatric disorders, such as anxiety, fear, major depression, even some neurodegenerative diseases [29]. Animal models provide one of the most efficient ways to identify and study the underlying neurobiological mechanisms of these neuropsychiatric disorders under the stress responses, such as electrical shock, restraint, forced swimming, social defeat and so on. In the present study, we prepared the rat restraint stress model for 21 consecutive days and investigated the effects of stress on cognitive function.

The hippocampus, as part of the limbic system, is involved in both emotional and cognitive functions, and is one of the brain structures that have been extensively studied with regard to the actions of stress. Recent imaging studies in humans revealed that the hippocampus undergoes selective volume reduction in stress-related neuropsychiatric disorders such as recurrent depressive illness [30]. Several animal experiments have demonstrated that repeated stress affects neuronal function in the hippocampus. For example, Watanabe et al. revealed that repeated or chronic stress causes dendritic atrophy in CA3 pyramidal neurons of the rodent hippocampus [31]. The animals subjected to 6 h of physical restraint each day for 21 days showed the hippocampus-dependent learning and memory impairments. Moreover, behavioral deficits in animal models of chronic stress have been associated with loss of hippocampal neurons [6]. However, so far, the molecular and cellular mechanisms of cognitive function changes caused by chronic stress are to a large extent unknown.

Apoptosis is a conserved active cellular mechanism functioning in many important biological processes. In the nervous system, various deleterious conditions could trigger the apoptosis response in neurons and glial cells. Some recent studies indicate that apoptosis may be one of the most important neuropathological mechanisms for cell or hippocampal atrophy induced by stress [32]. In the predatory stress animal model, Nissl staining supports that long-lasting stress may induce the neuronal cell loss by apoptosis in hippocampus [33]. Heine et al. also indicate that chronic stress may suppress proliferation and facilitate apoptosis in the rat dentate gyrus at the same time [34]. In line with those of previous studies, in our present experiment, Annexin V and PI staining for flow cytometry analysis showed that the percentage of hippocampal cells including early and late apoptotic cells was significantly increased when rats were subjected to restraint stress, from 1.61% in unstressed control to 14.32%. Western blot analysis revealed that in restraint stress rat hippocampus, the protein levels of cleaved caspase-3 and the ratio of Bax and Bcl-2 were significantly up-regulated as compared with unstressed controls. All these data confirmed that exposure of hippocampus to restraint stress induced apoptotic cell death. However, some researchers didn't get similar results in the hippocampus of stressed animals [35,36]. Taking regard of the reasons for this inconsistency, methodological differences may be important, such as duration of chronic stress, recovery time after stress, stress procedure and detection method.

Three signaling pathways trigger apoptosis: the death receptor pathway, mitochondrial pathway and ERS-induced apoptosis. Considering

that ERS plays a crucial role in the development of several neurodegenerative diseases, we investigated possible signaling pathways for ERS-initiated apoptosis. ERS is triggered by perturbations in ER homeostasis, causing dissociation of GRP78 from the three major ERS sensors PERK, ATF6 and IRE1 and a resultant launching of the UPR [14]. In order to confirm the effect of restraint stress on rat hippocampus, we examined the expression of GRP78, which serves as a good marker for ERS. Our studies showed that the protein levels of GRP78 were significantly increased in restraint stress rat hippocampus using western blot analysis. Moreover, qRT-PCR analysis revealed that restraint stress increased the mRNA expression of GRP78 by 221%, compared to the unstressed control. In UPR, IRE1 is activated and serves as endoribonuclease which removes 26 ribonucleotides from the X-box binding protein-1 (XBP-1) mRNA, induced by cleaves ATF6, into XBP-1 spliced form (sXBP-1) mRNA to generate a more potent transcription factor sXBP-1 [37]. Therefore, sXBP-1 is a key transcriptional regulator in UPR to activate genes involved in protein folding and degradation to restore ER function. In our present study, the protein and mRNA expression levels of sXBP-1 were both increased in restraint stress rats. The small molecule salubrinal is a selective eIF2 $\alpha$  dephosphorylation inhibitor. Enhanced eIF2 $\alpha$  phosphorylation attenuates translation initiation of most messenger RNAs (mRNAs) and reduces protein synthesis, which allows the cells to restore protein folding capacity and recover from ER stress [18]. In the present study, salubrinal efficiently decreased the protein or mRNA expression levels of GRP78 and sXBP-1, indicating that restraint stress, a common psychological and physical stress model, triggered the UPR in hippocampus in this experimental paradigm.

CHOP, also known as growth-arrest- and DNA-damage-inducible gene 153 (GADD153), was originally identified in response to DNA damage. Normally, CHOP is ubiquitously expressed at very low levels [38]. However, it is robustly expressed in a wide variety of cells by perturbations that induce stress, and CHOP<sup>-/-</sup> cells are resistant to ERS-mediated apoptosis [39]. During ERS, all three arms of the UPR induce transcription of CHOP [40]. The second apoptotic pathway mediated by ERS is caspase-12, which is localized on the cytoplasmic side of ER and is activated only by ERS but not by other apoptotic signals [41]. When activated to cleaved forms, it can directly process caspase-9, which in turn activates caspase-3, thus leading to cell death [42]. Nakagawa and Yuan have revealed that cells lacking caspase-12 are resistant against degeneration induced by amyloid  $\beta$  peptide and by tunicamycin causing ERS [27]. However, because the human gene shows large deletions of caspase-12, caspase-4 has been proposed to fulfill the function of caspase-12 [43]. The third is the JNK pathway. Active IRE1 has been shown to recruit tumor necrosis factor receptor-associated factor 2 (TRAF2). The IRE1-TRAF2 complex formed during ERS can recruit the apoptosis-signal-regulating kinase (ASK1) to activate c-Jun N-terminal kinase (JNK) pathway to promote apoptosis. Activation of JNK has also been reported in response to ER stress and was shown to be IRE1- and TRAF2-dependent [44]. In the present study, we found that in restraint stress rat hippocampus, the protein and mRNA expression levels of CHOP were significantly up-regulated, and caspase-12 was activated and cleaved, which suggested that these two apoptotic pathways crucially contributed to restraint stress-induced hippocampal cell death. However, we found no changes in protein level of phosphorylated JNK, implying that the JNK pathway was not the primary pathway involved in hippocampal apoptosis.

To our knowledge, our data provide important views in deeply understanding the roles of ERS and hippocampal apoptosis in restraint stress-induced cognitive impairments. However, we acknowledge that the restraint stress model employed in this study is only an experimental animal model and the differences between them and human are enormous and complex. Therefore, future studies are needed to validate current findings with more complex systems such as organotypic cultures, even human when stress.

In conclusion, the current study suggested that exposure to restraint stress facilitated hippocampal apoptosis and caused cognitive

impairments. Moreover, the related mechanisms under these actions might be associated with the ERS in hippocampus, and amelioration of ERS could be a novel strategy to prevent and treat impaired cognitive function induced by restraint stress.

### Conflict of interest statement

The authors have no financial conflict of interest.

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