Molecular Mechanism of ATF6α/S1P/S2P Signaling Pathway in Hippocampal Neuron Apoptosis in SPS Rats

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Original Research

Keywords: Posttraumatic stress disorder, Single-prolonged stress, Activating transcription factor 6α, Hippocampus

Posted Date: February 25th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-170376/v1

Version of Record: A version of this preprint was published at Journal of Molecular Neuroscience on March 18th, 2021. See the published version at https://doi.org/10.1007/s12031-021-01823-9.

Abstract

Apoptosis of hippocampal neurons is one of the mechanisms of hippocampal atrophy in posttraumatic stress disorder (PTSD), and it is also one of the important reasons of memory disorder in PTSD patients. The endoplasmic reticulum stress (ERS) mediated by activated transcription factor 6α(ATF6α)/site 1 protease (S1P)/S2P is involved in cell apoptosis, but it is not clear whether it is involved in hippocampal neuron apoptosis caused by PTSD. The PTSD rat model was constructed by the single-prolonged stress (SPS) method. The experiment was divided into two parts: (1) Control group, SPS 1d group, SPS 7d group, SPS 14d group. (2) Control group, SPS 7d group, SPS 7d+AEBSF group, control+AEBSF group. 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) is an ATF6α pathway inhibitor. The expression of ATF6α, glucose regulated protein (GRP78), S1P, S2, C/EBP homologous protein (CHOP), caspase-12 protein and mRNA in the hippocampus of PTSD rats were detected by immunohistochemistry, Western blotting and qRT-PCR. The apoptosis of hippocampal neurons was detected by TUNEL staining. In experiment 1, the protein and mRNA expression of ATF6α, GRP78 increased gradually in SPS 1d group and SPS 7d group, but decreased in SPS 14d group(P<0.01). In experiment 2, compared with the control group, the protein and mRNA expression of ATF6α, GRP78, S1P, S2P, CHOP, caspase-12 and apoptosis rate were significantly increased in SPS 7d group(P<0.01). However, the protein and mRNA expression of ATF6α, GRP78, S1P, S2P, CHOP, caspase-12 and apoptosis rate were significantly decreased after AEBSF pretreatment(P<0.01). SPS induces apoptosis of hippocampal neurons by activating ERS mediated by ATF6α, suggesting that ERS-induced apoptosis is involved in the occurrence of PTSD.

Introduction

Posttraumatic stress disorder (PTSD) has experienced sudden huge disasters (earthquake, sea disaster, flood, snow disaster, war), or unusual threats (such as violence), the main manifestations of PTSD were: (1) repeated intrusive traumatic experience reappearance, abnormal memory; (2) persistent avoidance and emotional disorder; (3) increased alertness. In recent years, with the increasing number of sudden disasters at home and abroad, the incidence rate of PTSD has been increasing year by year. It has become a serious psychological obstacle and the first public attention after disasters(Qi et al. 2016; Sin et al. 2017). MRI examination of PTSD patients showed hippocampal shrinkage with abnormal memory function(Apfel et al. 2011; Hines et al. 2014). It has been found that apoptosis of hippocampal neurons may be one of the pathological mechanisms related to PTSD memory impairment(Fan et al. 2011). However, the fine regulation mechanism needs to be further studied.

Excessive endoplasmic reticulum stress (ERS) can initiate apoptosis pathway (Hetz and Saxena 2017; Lee et al. 2018; Sisinni et al. 2019). PTSD usually occurs after suffering from various disasters mentioned above. These disaster stimuli are all super stimulation, which may cause ERS and initiate unfolded protein reaction (UPR), and positively regulate protein folding, promote unfolded protein degradation and restore endoplasm the network environment is stable(Wan and Jiang 2016). When the unfolded protein is excessively deposited, exceeding the processing capacity of UPR, resulting in

excessive ERS, cell apoptosis caused by the endoplasmic reticulum pathway apoptosis mechanism will be initiated(Sharma et al. 2019).

The molecular weight of ATF6α is 90 kDa (p90ATF6α), which belongs to a class of transcription factors containing a transmembrane domain. Under normal conditions, ATF6α mainly exists in the form of zymogen (p90ATF6α) and is tightly bound to Glucose-regulated protein (GRP78). When ERS occurs, ATF6α is separated from GRP78, released from endoplasmic reticulum and hydrolyzed to a fragment of about 50 kDa, namely P50 ATF6α fragment. The p50ATF6α fragment can be further translocated into nucleus and act on expression of ERS-related genes, such as C/EBP-homologous protein (CHOP), etc. (Stauffer et al. 2020). However, when the endoplasmic reticulum homeostasis cannot be restored, the apoptosis process will be initiated and executed by caspase 12 that resides in the endoplasmic reticulum, which is the key signal for ERS to induce apoptosis(Fan et al. 2020). During ERS, the activation of ATF6α can specifically up-regulate the expression of caspase 12, which leads to cell apoptosis(Martelli et al. 2020). Therefore, this study aims to establish a PTSD rat model through single-prolonged stress (SPS), to investigate the effect of ATF6/S1P/S2p signaling pathway on apoptosis of hippocampal neurons, to lay a theoretical foundation for further study of PTSD mechanism.

Materials And Methods

Experimental Animal

200 healthy male Wistar rats (provided by China Medical University), weighing 150-180 g, 6-7 weeks old, given standard food and water, 5 per cage, indoor temperature 22±2℃, humidity is 55±5%. The experimental study will be carried out after one week of adaptive feeding. The litter is changed every 2 days, rats are placed under a 12-hour light/dark cycle (lighting time 8:00-20:00).

PTSD Model preparation and grouping

After being adapted and processed in the laboratory, the experiment consists of two parts: (1) control group, SPS 1d group, SPS 7d group, SPS 14d group, 25 rats in each group. (2) Control group, SPS 7d group, SPS 7d+AEBSF group, control+AEBSF group, 25 animals in each group. 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) is an ATF6 pathway inhibitor. The specific steps of PTSD model preparation are as follows: first restrain the rat for 2 hours, and then immediately compulsively swim for 20 minutes, and then recover for 15 minutes After that, diethyl ether anesthetized the rat to lose consciousness. The rat fed without interference, and had a regular diet. The administration method of AEBSF is stereotactic brain injection: after the rat is anesthetized, fixed on the stereotaxic instrument, locate the hippocampus on both sides, drill the cranium, microinjection, the speed is 0.2μl/min, and the dosage is 10μl (300μM). Leave the needle for 10 minutes after the injection, sew the skin, and keep it warm until it wakes up.

Behavioral Test

In order to evaluate the modeling status of PTSD rats, Morris water maze experiment and open field experiment (Beijing dongle Technology Co., Ltd, China) were carried out. The rat behavior test was performed 7 days after SPS. Carefully observe the general state of each group of rats (including diet and mental state, etc.). The room for behavioral testing is illuminated by indirect white light. The rats are tested at 9: 00-11: 00 in the morning every day. The experimenter is at least 1 meter away from the equipment during the test. The experimental data of each group is recorded, and then the rats are returned to the cage in. At the same time, use 75% alcohol to clean the equipment to avoid residual information (such as feces, urine, and odor) from affecting the subsequent experimental rats.

Water Maze Test

In order to test the learning and spatial memory abilities of rats, Morris water maze experiment was performed on the rats 7 days after SPS. There were 10 rats in the control group and 10 rats in the SPS7d group. The water maze consists of a circular pool, a video analysis system and a computer. The diameter of the ferrous metal circular pool is about 150 cm. Tap water is injected into the pool and the water temperature is controlled at 20-22℃. The experiment process is divided into two parts: positioning navigation and space exploration experiment. (1) Positioning and navigation experiment: each rat enters the water from the four quadrants of the pool in turn towards the pool wall. After the rat climbs on the platform and maintains it for 2 s, the timing is stopped and recorded as the escape latency period. The video chase system records the swimming path of the mouse. After the rats boarded the platform, they were kept on the platform for 15 s, then the rats were wiped dry and placed in a warm environment. On days 1 to 3, the platform is placed about 1 cm above the liquid surface; on days 4 to 5, the platform is placed about 1 cm below the liquid surface. (2) Space exploration experiment: after 24 hours, the platform is removed and the space exploration experiment is carried out. Each mouse enters the water from the quadrant opposite to the quadrant where the original platform is located, and records the swimming speed of the mouse within 60 s and the time spent in the original platform area.

Open Field Experiment

In order to evaluate the autonomous exploration behavior of rats, and evaluate the anxiety and fear states of rats in unfamiliar environments, there are 10 rats in the control group, SPS 7d group of 10 rats. The rat open field reaction box is 30-40 cm high and the bottom side is 100 cm long. The bottom is divided into 25 small squares of 4 cm×4 cm. The inner wall is painted black. A digital video camera is installed 2 m above it. The field of view is required to cover the entire Open field. Before the experiment, the rats were placed in a small square for 20 minutes. In a quiet environment, the rats were placed in the middle of the square. The 5 minutes behavior of the rats, the number of times of entering the central area and the percentage of movement distance in the central area were recorded.

Take material

Five rats in each group were anesthetized with 2% Pentobarbital Sodium (Shanghai Sixin Biotechnology Co., Ltd, China), and 4% paraformaldehyde (PFA) was perfused to the rats to make their limbs stiff,

gradient alcohol dehydration, xylene transparent, routine Paraffin embedded, 5 μm thickness coronal section.

Immunohistochemical Staining

The sections prepared by fixed paraffin embedding were deparaffinized and washed in PBS; 3% H2O2 at room temperature for 12 minutes, PBS washed 3 times, 3 minutes each; 3 % Goat serum (Beijing Zhongshan Jinqiao, China) for 30 min; without washing, mouse anti-ATF6α monoclonal antibody (1: 300), mouse anti-GRP78 monoclonal antibody (1: 200), rabbit anti-CHOP polyclonal antibody (1: 200) (Santa Cruz Biotechnology, USA), and overnight at 4°C. Wash with PBS 3 times, 3 min each time; add biotin secondary antibody (Beijing Zhongshan Jinqiao, China), room temperature 30 min; wash with PBS; add dropwise SABC reagent (Beijing Zhongshan Jinqiao, China), Room temperature 30 min; PBS wash 3 times, 3 min each time; DAB color development (Beijing Zhongshan Jinqiao, China), hematoxylin counterstain, gradient alcohol dehydration, xylene transparent, mounting. Observed and photographed under an optical microscope (Olympus, BX60, Japan), Image Pro Plus software was used to calculate the average positive rate for semi-quantitative analysis.

TUNEL Staining

Sections were deparaffinized, washed with PBS, 4% PFA for 30 min, washed with PBS, and 0.1% Triton X-100 (Boster, China) for 2 min in an ice bath to prepare TUNEL detection solution, TdT Enzyme: TUNEL detection solution (Suzhou Kaiji Biotechnology Co., Ltd, China) was prepared with the volume of fluorescent labeling solution=1:24, washed with PBS, 50 μl TUNEL reaction solution was dropwise added and kept at 37 ℃ for 60 min, washed with PBS, after mounting the slide, take photos with fluorescence microscope, the apoptosis rate = TUNEL cells/total cells × 100%.

Western Blotting for Detecting the Expression of ATF6α, GRP78, S1P, S2P, CHOP, Caspase-12 in the Hippocampus

The hippocampal tissue was isolated by decapitation. After weighing, the lysate was added at a ratio of 1:3, homogenized and ultrasonically crushed, centrifuged for 30 min (12000 rmp/min). Determination of protein concentration by BCA Kit (SuoLaibao Bio, Beijing, China) was 3mg/ml; and each tissue sample was divided into 50 μg protein, 10% SDS-PAGE denaturing gel electrophoresis (Bio-Rad, USA), concentrated gel 90v , 30 min and separation gel 110v, 120 min; transfer to PVDF membrane (Beijing solebao, China) at constant pressure, overnight at 4°C, blocking with 5% BSA (SuoLaibao Bio, Beijing, China) for 2 h; prepare primary antibody incubation solution Mouse anti-ATF6α (1:1000), mouse anti-GRP78 (1:1000), rabbit anti-S1P and S2P polyclonal antibody (Thermo Fisher, USA; 1:500); rabbit anti-CHOP (1:5000), rabbit anti-Caspase-12 polyclonal antibody (Santa Cruz Biotechnology, USA; 1:5000), mouse anti-GAPDH and mouse anti-β-actin monoclonal antibody (Proteintech, China; 1:5000), put the PVDF membrane in the antibody incubation solution, and after 3 times of TBST rinsing, the second antibody (1:5000) was added, incubate at room temperature for 2 hours, rinse 3 times with TBST. ECL Luminometer (Bio-Rad, USA) develops color. Image J software was used to analyze the gray value of the

band, and the ratio of the target band and the internal reference GAPDH/β-actin×100% was used as the relative protein expression.

qRT-PCR

Ten rats in each group were anesthetized and decapitated to separate the hippocampus. The total RNA was extracted with Trizol reagent (SuoLaibao Bio, Beijing, China), quantified with a spectrophotometer (Thermo Fisher, USA). Take 1 μg of RNA and reverse transcription of RNA into cDNA. Conditions: 37°C, 15min, 85°C, 5sec. Perform quantitative PCR detection, Conditions: 95℃, 2min, 95℃, 30s, 60℃, 30s). Collect the fluorescent signal, 40 cycles) to start the reaction. GAPDH was used as internal reference. The sequence of each primer is shown in Table 1.

Table 1 Primer sequences

Name	Sense primer	Antisense primer	Product(bp)
ATF6α	5′-CAGTCTCCCCTTTCCTTGTATG-3′	5′-GCTTCTGGTGCTGCTTTGTAG-3′	513
GRP78	5′-GACCCTGACTCGGGCTAAAT-3′	5′-TGGACAGCGGCACCATATG-3′	243
CHOP	5′-ACTTTGGCATGGTGGAAGGG-3′	5′-ACTTGGCAGGTTTCTCCAGG-3′	354
S1P	5′-GGCAATGATGGACCTCTCTAT-3′	5′-CTGGAAGAAAAGCGAGCG-3′	30
S2P	5′-TAATCGTGCCTTTTACAGTTGGG-3′	5′- CATGGCAATCACGCCAAACA-3′	30
Caspase-12	5′-GCACATTCCTGGTCTTTATGTCCC-3′	5′-TTCCTCATCTGTATCAGCAGTGGC-3′	242
GAPDH	5′-TCCTGCACCACCAACTGCTTAG-3′	5′-AGTGGCAGTGATGGCATGGACT-3′	102

Statistical analysis

Statistical analysis was performed using SPSS 22.0 statistical software. Measurement data were expressed as mean ± SEM. One-way analysis of variance was used for comparison between groups, and LSD-*t* test was used for multiple comparisons between groups. $P<0.05$ indicates that the difference is statistically significant.

Results

Behavioral Verification of Rat Model of PTSD

The results of the Morries water maze positioning navigation experiment showed that the average escape latency of rats in the SPS group was significantly higher than that in the control group on the 1st to 5th

day after SPS(P<0.01, Fig. 1a, b). The analysis results of the space exploration experiment showed that the percentage of time spent in the target quadrant in the SPS group (13.01±0.88) was significantly lower than that of the control group (22.43±0.76) (P<0.01, Fig. 1c). In the open field experiment, the number of times the rats in the SPS group entered the central area (3.73±1.16) and the percentage of the central area movement distance (8.27±0.66) were significantly lower than those in the control group (6.87±0.99, 13.29±1.43) (P<0.01, Fig . 1d, e, f).

Changes in Apoptosis of Hippocampal Neurons of SPS Rats

After TUNEL staining, the cell nucleus stained red under the fluorescence microscope is the apoptosis-positive cell. In the control group, a relatively small amount of positive apoptosis cells were seen. The number of apoptosis-positive cells increased after SPS for 1 day. The number of apoptotic positive cells in the SPS 7d group was significantly higher than that in the control group, and the number of apoptotic positive cells reached a peak 14d after SPS (Fig. 2a). The difference in the number of apoptotic positive cells between SPS groups and the control group is shown in (Fig. 2b).

Changes in ATF6α Expression in the Hippocampus of SPS Rats

The positive expression of ATF6 α was brown yellow granules (Fig. 3a). Compared with the control group, the expression of SPS 1d and SPS 7d ATF6α increased significantly, and the expression of SPS 14d ATF6α decreased significantly (P<0.01, Fig. 3c).

When endoplasmic reticulum stress occurs, ATF6α can be hydrolyzed twice and release a 50kDa fragment, namely p50 ATF6α. In order to understand whether SPS induced the activation of ATF6α, Western blotting showed that the expression of P90 ATF6 α was consistent with that of immunohistochemistry; at the same time, ATF6α appeared in the area of about 50 kDa The active fragment p50 ATF6α has a band and its changing trend is consistent with p90 ATF6α (P<0.01, Fig. 3b, e, f).

qRT-PCR: ATF6α mRNA in SPS 1d, 7d, and 14d groups was 1.18±0.03, 1.54±0.04, and 1.07±0.02 times, respectively, compared to the control group. After SPS, the level of ATF6α mRNA in rat hippocampus gradually increased, and decreased on the 14th day (P<0.01, Fig. 3d).

Changes in GRP78 Expression in the Hippocampus of SPS Rats

The positive expression of GRP78 was mainly distributed in the periplasm of neurons in the hippocampus, and the positive signal was brown-yellow particles (Fig. 4a). The statistical analysis results showed that the positive expression of GRP78 increased on the 1st day after SPS; the positive expression of GRP78 increased significantly on the 7th day after SPS, and the positive expression of GRP78 decreased significantly on the 14th day (P<0.01, Fig. 4c).

Western blotting detected the expression of GRP78 in the hippocampus of each group, and a single band could be seen at 78 kDa. The expression of GRP78 protein gradually increased after 1d of SPS, reached a

peak at 7d, and then decreased. (P<0.01, Fig. 4b, e).

qRT-PCR results showed that the expression of GRP78 mRNA in SPS 1d, 7d, and 14d groups was 1.21±0.02, 1.64±0.06, and 1.43±0.05 times of the change in SPS 1d, 7d, and 14d groups, respectively. That is, after SPS, GRP78 mRNA level gradually increased, reached a peak at 7d, and then decreased. (P<0.01, Fig. 4d).

Effect of AEBSF on ATF6α Pathway in Hippocampus of SPS Rats

Western blotting detected the expression of ATF6α protein in the hippocampus. The results showed that after pretreatment of SPS 7d rats with AEBSF, the expression of p90 ATF6α and p50 ATF6α was significantly reduced (P<0.01, Fig. 5a, b, c), and the two proteases S1P and S2P that cleave ATF6α at the same time also decreased(P<0.01, Fig.5e, f, g). However, after pretreatment with AEBSF in normal rats, it has no significant effect on the ATF6α pathway(P0.05).

qRT-PCR : the expression of ATF6α mRNA in the SPS 7d, SPS 7d+AEBSF and control+AEBSF groups was 1.55±0.02, 1.21±0.03, and 1.08±0.02 times, respectively, which was greater than that of SPS 7d. After pretreatment with AEBSF, the expression of ATF6α mRNA was significantly reduced in rats (P<0.01, Fig. 5d). The folds of S1P mRNA expression in SPS 7d, SPS 7d+AEBSF and control+AEBSF groups relative to the control group were 4.22±0.08, 1.54±0.04, and 0.99±0.02, respectively. That is, after pretreatment of SPS 7d rats with AEBSF, The expression of S1P mRNA was significantly reduced (P<0.01, Fig. 5h). The folds of S2P mRNA expression in SPS 7d, SPS 7d+AEBSF and control+AEBSF groups relative to the control group were 5.15±0.08, 2.57±0.05 and 1.19±0.03 times respectively. That is, after pretreatment with AEBSF on SPS 7d rats, S2P mRNA expression was significantly reduced (P<0.01, Fig. 5i). After pretreatment with AEBSF in normal rats, there was no significant effect on the expression of ATF6α, S1P and S2P mRNA(P0.05).

Effects of AEBSF on the Expression of CHOP and Caspase-12 in Hippocampus of SPS Rats

The positive expression of CHOP brown-yellow particles (Fig. 6a). The results of statistical analysis showed that the expression of CHOP was significantly reduced after pretreatment with AEBSF on SPS 7d rats (P<0.01, Fig. 6a, c). After pretreatment with AEBSF in normal rats, it had no significant effect on CHOP expression(P0.05).

Western blotting: the expression of CHOP and caspase-12 was significantly reduced after pretreatment with AEBSF in SPS 7d rats. After pretreatment with AEBSF in normal rats, there was no significant effect on the expression of CHOP and caspase-12 (P0.05).

qRT-PCR : the expression of CHOP mRNA in the SPS 7d, SPS 7d+AEBSF and control+AEBSF groups was 6.17±0.05, 4.43±0.06, and 1.05±0.03 times, respectively, which was greater than that of the SPS 7d. After pretreatment of rats with AEBSF, the expression of CHOP mRNA was significantly reduced (P<0.01, Fig. 6d). The expression of caspase-12 mRNA in SPS 7d, SPS 7d+AEBSF and control+AEBSF groups was 18.57±0.87, 12.19±0.27, and 1.17±0.02 times, respectively, that is, AEBSF pretreatment was given to SPS

7d rats Later, the expression of caspase-12 mRNA was significantly reduced (P<0.01, Fig. 6e). After pretreatment with AEBSF in normal rats, it had no significant effect on the expression of CHOP and caspase-12 mRNA (P>0.05).

Effects of AEBSF on Apoptosis in Hippocampus Neuronal of SPS Rats

The results of TUNEL staining showed that fewer apoptosis-positive cells were seen in the control group. The number of apoptosis-positive cells was significantly increased after SPS for 7 days. After pretreatment with AEBSF for SPS rats for 7 days, apoptosis was significantly reduced. The results of statistical analysis showed that after pretreatment with AEBSF for SPS 7d rats, the apoptosis rate was significantly reduced (P<0.01, Fig. 7a, b), while after pretreatment with AEBSF for normal rats, there was no significant effect on the apoptosis rate(P>0.05).

Discussion

PTSD refers to a psychological and physiological abnormal response to severe stressors such as war. Its core clinical symptoms include repeated recurrence of traumatic experiences, increased alertness, continuous avoidance, and negative cognitive and emotional negative. change. Although the understanding of the molecular mechanism of PTSD has made great progress over the years, the exact pathogenesis is still unclear. MRI examination of PTSD patients showed that the hippocampus volume decreased(Milani et al. 2017; Moyer 2016; Nisar et al. 2020), animal experiments also found that the hippocampus of PTSD was closely related to the memory loss after a long delay (Joshi et al. 2020; Shan et al. 2020). The above results show that the abnormality of hippocampus were the important pathogenesis of PTSD. Therefore, in this study, we focus on testing the hippocampus of rats to further understand the pathogenesis of PTSD. SPS is a model widely adopted by PSTD(Lisieski et al. 2018; Yamamoto et al. 2009). In this experiment, we observed the SPS group showed PTSD-like symptoms, such as loss of appetite, irritability, and irritability. The reason is that it may be due to the changes in the behavior and cognitive function of the rats caused by the stress stimulation, which affects the feeding behavior of the rats and the mental abnormal behavior. Morries water maze showed that the average escape latency of the SPS group was significantly higher, and the percentage of time spent in the target quadrant was significantly lower than that of the control group, suggested that SPS damaged the memory of rats. The open field experiment showed that the number of rats entering the central area and the percentage of movement distance in the central area decreased significantly in SPS group. The above results showed that the rats after SPS showed a strong similarity with PTSD patients, which laid a foundation for the further development of this study.

Apoptosis of Hippocampal Neurons in SPS Rats

Apoptosis process involves the activation, expression and regulation of multiple genes(Xu et al. 2019). PTSD and apoptosis of hippocampal neurons influence each other (Chen et al. 2020). In this study, TUNEL staining showed that the apoptosis rate of hippocampus in PTSD rats was significantly increased, and the number of apoptotic cells gradually increased with time, reaching a peak at 14 days

after SPS, which may be caused by hippocampus One of the reasons for the decrease in size and function. In the previous research of our group, we proved that SPS induced ATF6α-dependent ERS and mPFC neuronal apoptosis(Yu et al. 2014). Since ERS induces cell apoptosis, in this article we hypothesize that ERS and endoplasmic reticulum-related apoptosis will participate in the SPS-induced apoptosis molecules in PTSD-like rat hippocampal neurons mechanism.

Abnormal Expression of ATF6α in Hippocampal Neurons in SPS Rats

ATF6α is a typical ERS sensor(Kim et al. 2019; Oka et al. 2019). This study found that, the positive expression of ATF6α increased on the 1st day after SPS, and the positive expression of ATF6α increased significantly on the 7th day after SPS, while the positive expression of ATF6α increased significantly on the 14th day. The positive expression was significantly reduced. When ERS occurs, ATF6α can be hydrolyzed twice and release a 50kDa fragment, namely p50 ATF6α. In order to understand whether SPS induced ATF6α activation, this study further used Western blotting to detect the expression of ATF6α protein in hippocampal neurons. p90 ATF6α was expressed abundantly in normal rats. The expression increased significantly after SPS 7d, and decreased significantly on the 14th day. At the same time, in the region of about 50 kDa, a band of the active fragment of ATF6α, p50 ATF6α, appeared and the trend was consistent with that of p90 ATF6α.The change trend of ATF6α mRNA was consistent with Western blotting. Therefore, SPS induced the activation of ATF6α. According to reports(Yu et al. 2020), activation of ATF6α-related ERS can cause hepatocyte apoptosis. Our group also found that SPS-induced ATF6α-dependent ERS is involved in the apoptosis of mPFC neurons induced by PTSD(Yu et al. 2014). In this study, after ATF6α was activated, hippocampal neuronal apoptosis increased significantly. However, when SPS stimulated for 14 days, the expression of ATF6α was reduced, but the apoptosis was not significantly reduced. This suggests that other mechanisms may be involved in the apoptosis of hippocampal neurons induced by SPS.

Abnormal Expression of GRP78 in Hippocampal Neurons in SPS Rats

GRP78 is closely related to ERS-UPR(Feng et al. 2019; Ibrahim et al. 2019). Under non-stress conditions, GRP78 combines with three endoplasmic reticulum transmembrane proteins including ATF6α to assist in the proper folding and transport of proteins. When ERS occurs, GRP78 dissociates and initiates UPR signaling pathways to relieve stress and restore the homeostasis of the endoplasmic reticulum(Cassimeris et al. 2019). However, if the stimulation intensity is too high and the duration is too long, and UPR cannot effectively alleviate ERS, the UPR signaling pathway will eventually upregulate CHOP and other ERS-specific transcription factors, and initiate cell apoptosis. Eliminate damaged cells(Ardic et al. 2019; Lu et al. 2017). In this experiment, GRP78 expression gradually increased after SPS and decreased on day 14. This indicates that the accumulation of GRP78 in hippocampal neurons is a response to SPS. These experimental data further indicate that SPS induces neuronal chaperone GRP78 in the hippocampus to respond to UPR. In the early stage of UPR, cells tried to enhance the endoplasmic reticulum processing protein ability to restore the homeostasis of the cell environment by increasing the

level of GRP78. However, as time went on, UPR weakened, so there was a relative decrease in GRP78 expression on the 14th day after SPS.

Inhibition Effect of AEBSF on ATF6α/S1P/S2P Pathway in Hippocampal of SPS Rats

Based on the above results, we speculate that SPS may induce ATF6α activation and UPR enhancement, which in turn induces apoptosis of hippocampal neurons. But when SPS 14 days, UPR weakened and the number of apoptosis-positive cells still increased, indicating that other mechanisms may mediate neuronal apoptosis. To observe ATF6α activation inducing SPS to stimulate hippocampal neuron apoptosis, we added ATF6α signaling pathway inhibitors to SPS7d and the control group. It has been reported in the literature that AEBSF can inhibit nuclear transcription of ATF6, prevent ERS-induced ATF6α cleavage, and thus inhibit the transcription of downstream genes of ATF6(Okada et al. 2003). After pretreatment of SPS7d rats with AEBSF, the expressions of p90 ATF6α and p50 ATF6α were significantly reduced. At the same time, the two site proteases S1P and S2P that cut ATF6α also decreased. However, after pretreatment with AEBSF in normal rats, it has no significant effect on the ATF6α pathway. This suggests that AEBSF can significantly inhibit the activation of ATF6α pathway.

Inhibition of ATF6α/S1P/S2P pathway will down-regulate the expression of CHOP and caspase-12

Under normal conditions, the binding of ATF6 to GRP78 was stable. When ERS occurs, ATF6 is separated from GRP78, released from the endoplasmic reticulum, and hydrolyzed into the active fragment p50ATF6α and then exert its own activity(La et al. 2017). After entering the nucleus, it participates in the transcription and expression of ERS-related genes, such as the expression of apoptosis-related cellular molecule CHOP(Xu et al. 2018b). CHOP is an important intermediate molecule linking ERS and apoptosis. Under normal circumstances, CHOP is mainly present in the cytoplasm and the content is very low (Xu et al. 2018a). In severe or prolonged ERS, the activation of three transmembrane proteins on the endoplasmic reticulum membrane can induce the upregulation of CHOP transcription. At present, the continuous up-regulation of CHOP is not conducive to cell survival in the disorder of protein balance, and is the main way to induce apoptosis in UPR(Chen et al. 2019; Lei et al. 2017). On the one hand, CHOP overexpression can promote apoptosis by activating apoptosis-responsive proteins such as ERO1, GADD34 and death receptor DR5. On the other hand, CHOP may participate in cell death by regulating genes that control apoptosis, such as CHOP overexpression. Can significantly inhibit the transcriptional expression of the pro-cell survival gene BCL2(Moriguchi et al. 2019; Poone et al. 2015). In this study, CHOP expression in hippocampal neurons continued to increase after SPS; and CHOP mRNA expression continued to increase. These results indicate that CHOP in hippocampal neurons of PTSD rats was induced to be up-regulated after SPS, and neuronal apoptosis changed. After pretreatment with AEBSF, the expression of CHOP was significantly reduced, and the rate of cell apoptosis was significantly reduced. The endoplasmic reticulum apoptosis pathway mainly induces cell apoptosis by activating caspase-12, CHOP and other apoptotic signaling molecules(Chung et al. 2018). Studies have found that caspase-12-deficient mice can resist apoptosis caused by ERS, while other death stimuli can still induce apoptosis, indicating that caspase-12 is closely related to endoplasmic reticulum pathway apoptosis.

Caspase-12 is considered to be a key molecule that mediates apoptosis in the endoplasmic reticulum pathway(Song et al. 2018). Caspase-12 up-regulates caspase-9 and then activates Caspase-3, leading to apoptosis (Gao et al. 2017). We showed that after SPS7d, the expression of caspase-12 in the hippocampus of rats was significantly increased. After pretreatment with AEBSF, caspase- The expression of 12 was significantly reduced, and the apoptosis rate was significantly reduced. This is consistent with the results of TUNEL staining, suggesting that CHOP and caspase-12 are involved in the regulation of hippocampal neuronal apoptosis in PTSD rats.

In summary, SPS induced apoptosis of hippocampal neurons by activating the ATF6α-mediated ERS response, suggesting that ERS-induced apoptosis is involved in the occurrence of PTSD. However, since the activation of caspase-12 can also occur through the mitochondrial apoptosis pathway, it is necessary to determine whether the mitochondrial apoptosis pathway plays a key role in the induction of hippocampal neuron apoptosis after SPS, which is also one of the limitations of this experiment. In addition, the exact mechanism of SPS-induced ERS and ERS-related apoptosis remains to be further elucidated.

Declarations

o Ethics approval and consent to participate: The animal experiments were performed according to protocols approved by the Ethical Committee of Animal Research at the China Medical University, and all efforts were made to minimize the number of animals used and their suffering. All study participants provided informed consent, and the study design was approved by the appropriate ethics review board.

o Consent for publication: Not applicable.

o Availability of data and materials: All data generated or analysed during this study are included in this published article.

o Competing interests: The authors declare that they have no competing interests.

o Funding: This work was supported by grants from the China National Natural Science Foundation (81571324) and the Science and Technology Project of Liao Ning Province, China (2017225011).

o Authors' contributions: Yanhao Xu and Yuxiu Shi, contributed to the conception of the study and helped perform the analysis with constructive discussion. Liang han, performed the experiment; contributed significantly to analysis and manuscript preparation; performed the data analyses and wrote the manuscript.

o Acknowledgements: The authors are grateful to all staff members of the China Medical University Experiment Center for their technical support.

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Figures

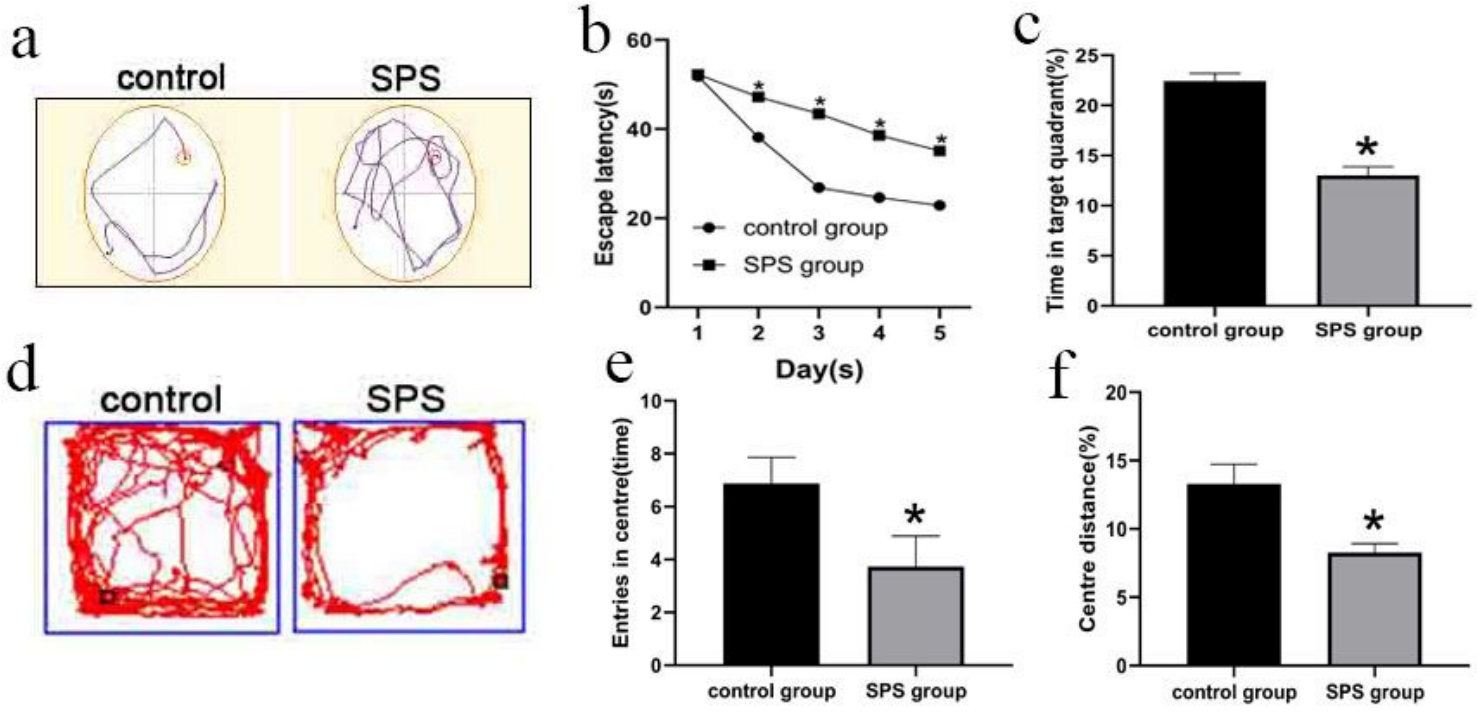

Figure 1

Behavior test results of PTSD rats (a, b, c: water maze test; d, e, f: open field test). a: The trajectory of the rat. b: The escape latency of rats on different test days. c: The percentage of time the rat stays in the target quadrant. d: The trajectory diagram of the rat. e: The number of times the rat entered the central area. f: The percentage of the distance the rat moved into the central area. *P <0.01 vs control rats.

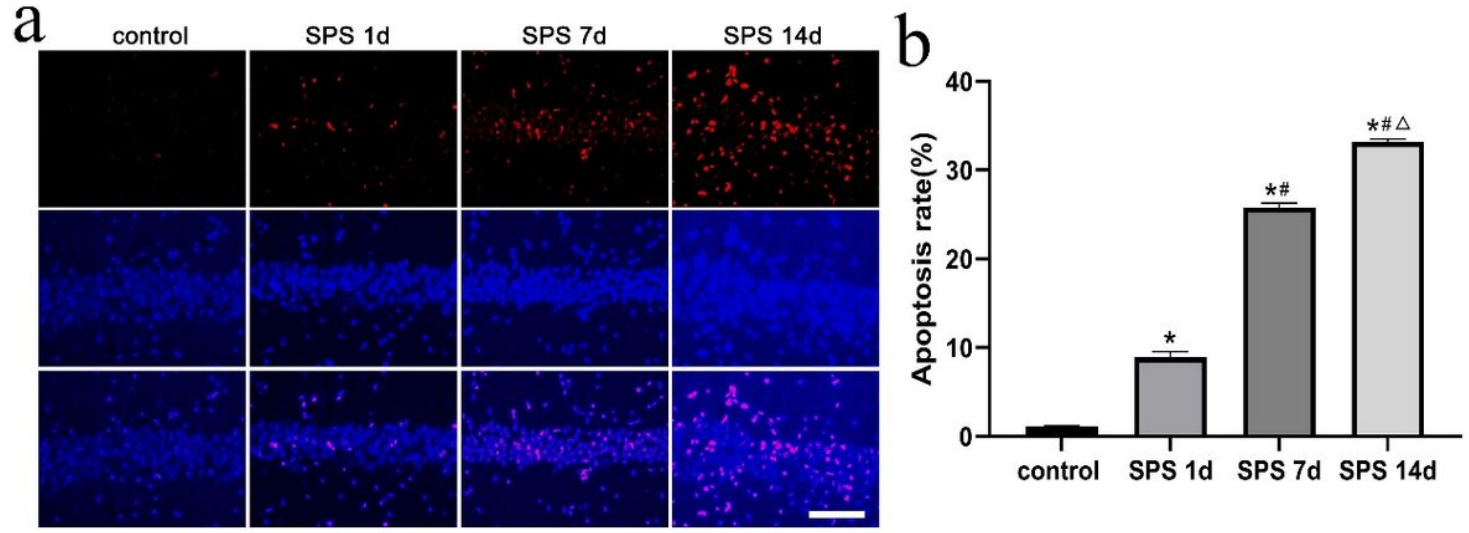

Figure 2

Apoptosis detected by TUNEL assays. The Representative positive cells were shown (Fig. 2a, red, magnification×400). Fig. 2b shows quantification of apoptosis cells. *P <0.01 vs control group, #P <0.01 vs SPS 1d group, △P <0.01 vs SPS 7d group.

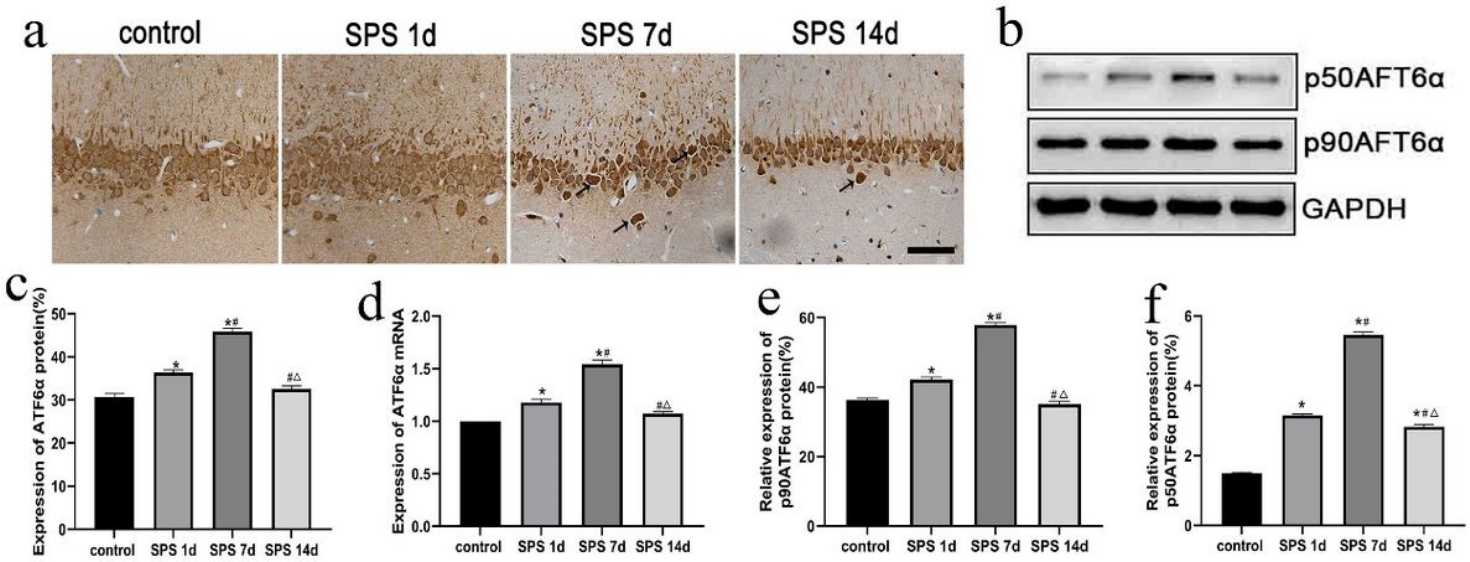

Figure 3

The expression of ATF6α in hippocampus. Fig. 3a, 3c shows the positive expression of ATF6α in rat hippocampus by immunohistochemical staining (×400). Fig. 3b, 3e, 3f shows the relative expression of p90ATF6α and p50ATF6α by Western blotting. Fig. 3d shows the expression of ATF6α mRNA by qRT-PCR. *P <0.01 vs control group, #P <0.01 vs SPS 1d group, △P <0.01 vs SPS 7d group.

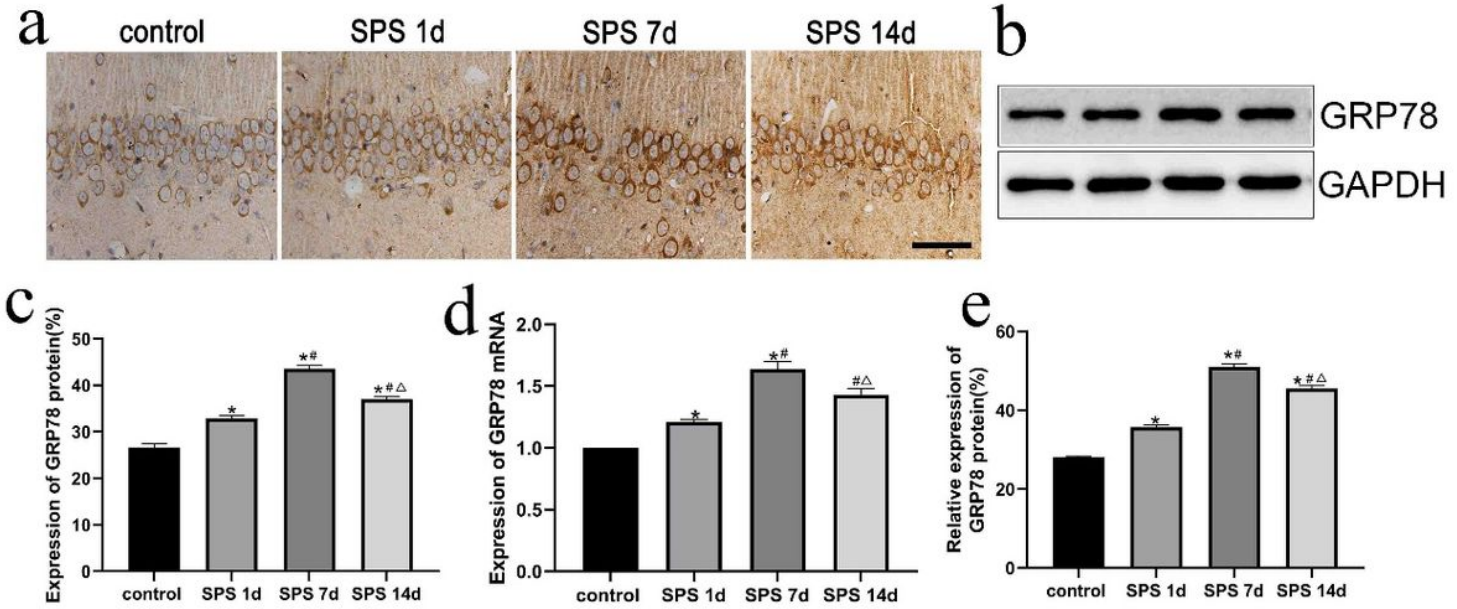

Figure 4

Detection results of ATF6α expression in hippocampus of rats in each group. Fig. 4a, 4c shows the positive expression of ATF6α in rat hippocampus by immunohistochemical staining (×400). Fig. 4b, 4e, 4f shows the relative expression of p90ATF6α and p50ATF6α by Western blotting. Fig. 4d shows the expression of ATF6α mRNA by RT-qPCR. *P <0.01 vs control group, #P <0.01 vs SPS 1d group, △P <0.01 vs SPS 7d group.

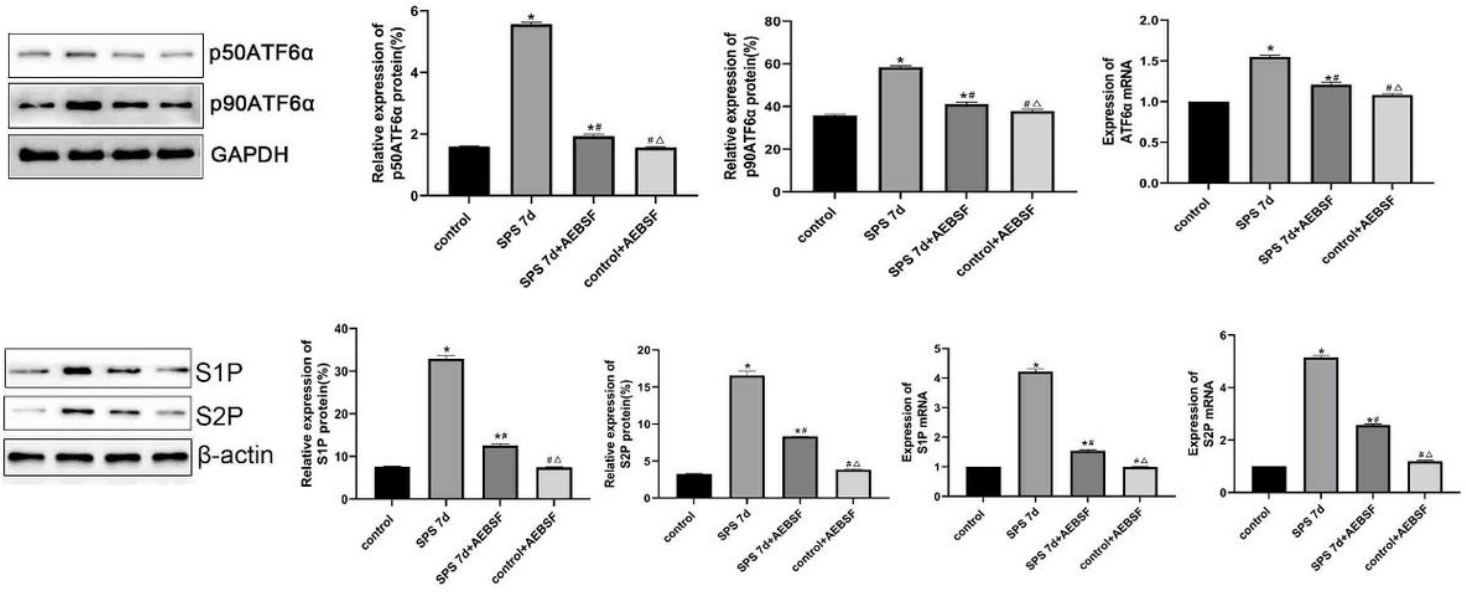

Figure 5

Effect of AEBSF on ATF6α pathway in hippocampus of rats after SPS. Fig. 5a, b, c, e, f, g shows the expression of ATF6α and S1P and S2P in rat hippocampus by Western blotting. Fig. 5d, h, i shows the expression of ATF6α and S1P and S2P mRNA by qRT-PCR. *P <0.01 vs control group, #P <0.01 vs SPS 7d group, △P <0.01 vs SPS 7d+AEBSF group.

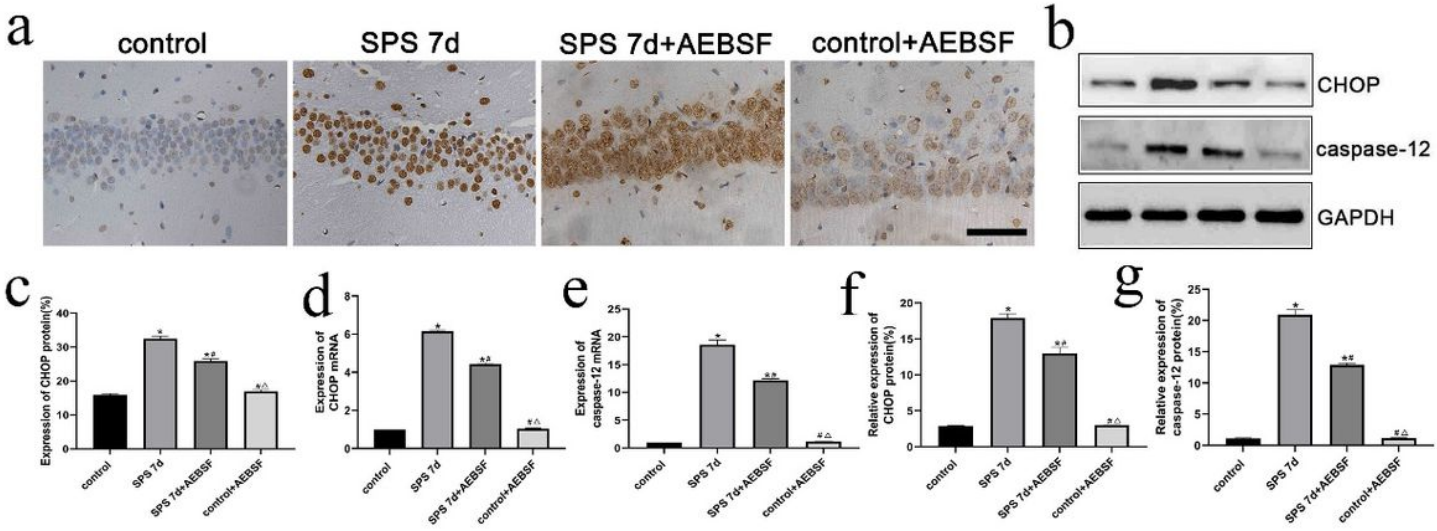

Figure 6

Detection results of CHOP and caspase-12 expression in hippocampus of rats. Fig. 6A, 6C shows the positive expression of CHOP in rat hippocampus by immunohistochemical staining (×400).

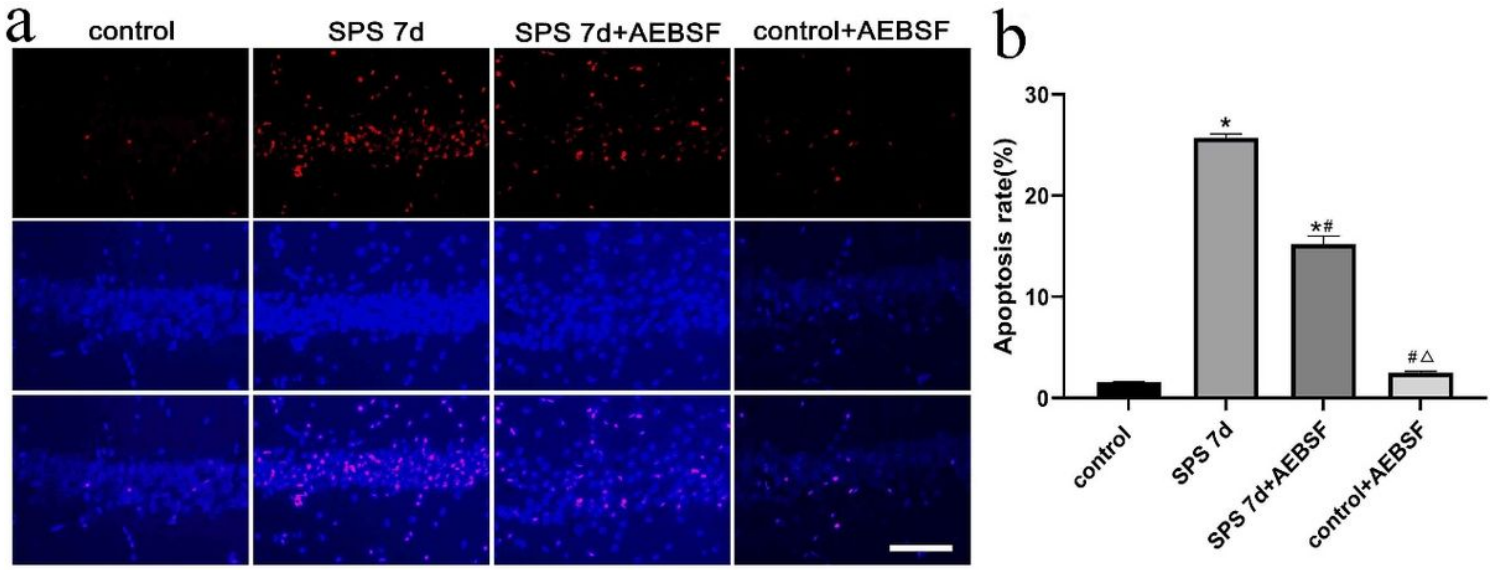

Figure 7

Apoptosis detected by TUNEL assays. The Representative positive cells were shown (Fig. 7a, red, ×400). Fig. 7b shows quantification of apoptosis cells. *P <0.01 vs control group, #P <0.01 vs SPS 7d group, △P <0.01 vs SPS 7d + AEBSF group.