ER stress mediated-autophagy contributes to neurological dysfunction in traumatic brain injury via the ATF6 UPR signaling pathway

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Abstract. A major public health problem, traumatic brain injury (TBI) can cause severe neurological impairment. Although autophagy is closely associated with the pathogenesis of TBI, the role of autophagy in neurological deficits is unclear. The purpose of the present study was to investigate the molecular mechanisms of endoplasmic reticulum (ER) stress-induced autophagy and its detrimental effects on neurological outcomes following TBI. A rat model of TBI was established by controlled cortical impact. ER stress activation, autophagy induction and autophagic flux dysfunction were examined in the damaged hippocampus post-TBI. Pharmacological inhibition of ER stress significantly blocked post-traumatic autophagy activation, as evidenced by decreased conversion of microtubule-associated protein 1 light chain 3 (LC3)-I to LC3-II and Beclin-1 expression levels in the hippocampus region. Short hairpin RNA-mediated activating transcription factor 6 knockdown significantly prevented ER stress-mediated autophagy stimulation via targeting essential autophagic genes, including autophagy related (ATG)3, ATG9 and ATG12. Furthermore, neurological scores, foot fault test and Morris water maze were used to evaluate the neurological functions of TBI rats. The results revealed that the blockage of ER stress or autophagy attenuated TBI-induced traumatic damage and functional outcomes. In conclusion, these findings provided new insights into the molecular mechanisms of ER stress-induced autophagy and demonstrated its potential role in neurological deficiency following TBI.

Introduction

Traumatic brain injury (TBI) is one of the primary causes of death and disability among individuals aged 1-45 years (1). It is estimated that 69 million people suffer from TBI each year, among these, 81% exhibit mild and 11% exhibit moderate symptoms (2). Furthermore, the incidence rate among males is twice that among females (3). Notably, patients with TBI frequently experience persistent personality changes and severe neurological dysfunction, including cognitive loss, as well as physical and psychological deficiency (4). Research has confirmed that TBI can trigger a series of pathological and physiological reactions, ultimately leading to neurological outcomes (5,6). Moreover, TBI is one of the primary risk factors for a number of neurological diseases, including stroke, epilepsy and neurodegenerative disease (7). Therefore, it is important to understand the molecular mechanisms of post-traumatic neurological dysfunction and to identify novel therapeutic approaches for the treatment of TBI.

Autophagy, a conserved catabolic process, maintains cellular homoeostasis by degeneration of unfolded/misfolded protein and damaged organelles in a lysosome-dependent pathway (8). The presence of autophagosomes and the increase in microtubule-associated protein 1 light chain 3 (LC3)-II expression levels in damaged brain tissue have been observed in specimens from patients with TBI (9) and TBI models (10). These results suggest that the autophagy pathway participates in the pathophysiological process of TBI. However, the precise role of autophagy in TBI-induced histological and neurological outcomes is complicated and remains unknown. It has been reported that persistent activation of autophagy is a protective mechanism for neurological recovery following TBI (11), and the inhibition of TBI-induced autophagy may enhance neuronal apoptosis and microglia activation (12). Conversely, certain studies have reported that autophagy induction post-TBI aggravates brain injury, neuroinflammation, neuronal death and long-term neurological outcomes (13-15). These findings indicate that autophagy induction serves a detrimental role in the pathogenesis of TBI. Previous studies have revealed that the induction of neuronal autophagy in the hippocampal region can lead to behavioral and cognitive impairments following TBI (16,17). However, the upstream regulatory mechanism and the function of autophagy induction remain unclear.

Evidence has revealed that there is a potential interaction between the activation of autophagy and endoplasmic reticulum (ER) stress (18). ER dysfunction, also known as ER stress, is caused by the accumulation of misfolded and unfolded proteins (19). In order to restore ER function and

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cellular homeostasis, the unfolded protein response (UPR) is induced via activation of three ER transmembrane proteins, including ER to nucleus signaling 1 (ERN1), eukaryotic translation initiation factor 2 α kinase 3 (EIF2AK3) and activating transcription factor 6 (ATF6) (20). ER stress and autophagy dysfunction serve significant roles in exacerbating lipid metabolic disorder and steatohepatitis (21). Recent evidence has demonstrated that ER stress contributes to the loss of newborn hippocampal neurons and alteration of dendritic arbors following TBI (22). It has been revealed that impaired ER stress can give rise to brain injury expansion and behavioral and cognitive deficits following juvenile TBI (23). Nevertheless, activation of ER stress-associated ATF6 in post-ischemic neurons has been revealed to significantly decrease infarct volume and neurologic dysfunction within 24 h of a stroke (24). It has been reported that ER stress is closely associated with the occurrence of autophagy in numerous types of disease, including cerebral ischemia, neurodegeneration and brain injury (25,26). The present study aimed to investigate the roles and the molecular mechanisms of ER stress-mediated autophagy in a rat model of TBI and to identify novel therapeutic targets for the treatment of patients with TBI.

Materials and methods

Animals and experimental groups. A total of 160 adult (age, 2-3 months) male Sprague-Dawley rats, (weight, 280-300 g; Beijing Vital River Laboratory Animal Technology Co., Ltd.) were maintained under standard laboratory conditions (12-h light/dark cycle; temperature, 21±1˚C; humidity, 60%) with free access to water and food. Rats were randomly assigned to the following groups: Sham (n=20), TBI (n=30), 3-MA (n=30), 4-phenylbutyric acid (4-PBA; n=30), saline (n=30), lentivirus (LV)-ATF6 short hairpin (sh)RNA (n=10) and LV-scrambled shRNA (n=10). Each experiment was performed ≥3 times.

Controlled cortical impact (CCI) model for TBI. A CCI rat model of TBI was used in the present study according to a previous study (27). Animals were initially anesthetized with isoflurane (5%) in oxygen for 2 min and maintained with isoflurane (3%) in oxygen (0.8 l/min) for the duration of the procedure. Following exposure of the skull, a 6-mm craniotomy was performed lateral to the sagittal suture midway between lambda and bregma. Cortical injury was delivered using an electronic controlled pneumatic impact device (TBI0310; Precision Systems and Instrumentation, LLC) using the following parameters: Strike velocity, 5.0 m/sec; strike depth, 3.0 mm; dwell time, 500 msec. Body temperature was maintained at 37˚C with a thermoregulating heating pad. In the Sham group, rats were subjected to craniotomy, but did not receive CCI treatment.

Drug administration and intracerebroventricular injection of lentiviral vector. Rats were anesthetized with isoflurane (5%) in oxygen for 2 min and maintained with isoflurane (3%) in oxygen (0.8 l/min). In the 3-MA group, a single intracerebroventricular injection of autophagy inhibitor 3-MA (600 nmol, diluted in 0.9% sterile saline to a final volume of 5 μl; Sigma-Aldrich; Merck KGaA) was administered at the onset of brain surgery. Subsequently, 4-PBA (ChemeGen (Shanghai) Biotechnology Co.,Ltd.), a common ER stress inhibitor, was diluted with 0.9% sterile saline to a concentration of 10 mg/ml, and then intraperitoneally injected into rats at a dose of 100 mg/kg immediately post-TBI. A total of 6 μl LV-ATF6 or LV-scramble shRNA ($5x10^9$ TU/ml; Beijing Syngentech Co., Ltd.) was injected into lateral ventricle at 48 h prior to TBI surgery. Transfection efficiencies were analyzed by reverse transcription-quantitative (RT-q)PCR and western blot assay.

Modified neurological severity score (mNSS). Neurological measurement was performed using the mNSS test at 1, 3, 7, 14 and 28 days post-TBI. The mNSS comprises motor, sensory, reflex and beam walking tests (28). Neurological function was graded on a scale from 0 to 18 (normal, 0; maximal deficit, 18).

Foot fault test. In order to evaluate sensorimotor function, the foot fault test was performed before TBI and at 1, 3, 7, 14 and 28 days post-injury. Rats were allowed to walk on a grid. With each weight-bearing step, a paw may fall or slip between the wires; if this occurred, it was recorded as a foot fault (29). A total of 50 steps were recorded for each right forelimb.

Morris water maze (MWM). The ability of spatial learning and memory was assessed using a MWM task. Spatial learning began on days 24-28 post-TBI and consisted of four daily trials. Animals were placed in the pool facing the wall at each of the four potential start locations in a randomized manner and had 90 sec to locate a hidden platform. Rats that failed to find the platform within 90 sec were recorded as having a maximum latency score of 90 sec. Finally, the mean time spent in the target quadrant searching for the missing platform and the percentage of time spent in the correct quadrant were analyzed.

Hematoxylin and eosin (H&E) staining. Animals were perfused intracardially with PBS followed by 4% paraformaldehyde (PFA) in PBS in room temperature for 30 min. Brain tissue was fixed in 4% PFA at 4˚C for 72 h, embedded in paraffin and cut into sections (thickness, 5 μm). The slices underwent xylene dewaxing and alcohol gradient rehydration and were stained with hematoxylin for 10 min followed by eosin for 2 min at room temperature. The sections were observed under a light microscope (Leica Microsystems GmbH; magnification, x400).

RT-qPCR assay. Total RNA was extracted from hippocampus tissue using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and then reverse transcribed to generate cDNA with the Revert Aid™ First Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. The primers were as follows: ATF6 forward, 5'-TCGCCTTTTAGTCCGGTTCTT-3' and reverse, 5'-GGCTCCATAGGTCTGACTCC-3'; autophagy-related (ATG)3 forward, 5'-GAGGCTACCCTAGACACAAGG-3' and reverse, 5'-GGCTGCCGTTGCTCATCATA-3'; ATG9 forward, 5'-AACTTTACGTGGCACGAGGT-3' and reverse, 5'-TGACGACGGACATTCCAAGG-3'; ATG12 forward, 5'-TCCCCGGAACGAGGAACTC-3' and reverse, 5'-TTCGC TCCACAGCCCATTTC-3'; β-actin forward, 5'-CCCATCTA

TGAGGGTTACGC-3' and reverse, 5'-TTTAATGTCACGCAC GATTTC-3'. RT-qPCR was performed using SYBR-Green ER™ qPCR SuperMix for the iCycler (Invitrogen; Thermo Fisher Scientific, Inc.) under the following cycling parameters: 95˚C for 5 min, followed by 40 cycles of 15 sec at 94˚C, 60˚C for 15 sec and 72˚C for 30 sec. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (30). β-actin was used as the endogenous control.

Western blotting. Hippocampal specimens were extracted and homogenized in RIPA buffer containing protease/phosphatase inhibitors (Beyotime Institute of Biotechnology). The lysates were centrifuged at 12,000 x g for 15 min at 4˚C. The concentrations of proteins were examined using the BCA assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (30 μg) were loaded into the wells of 10% SDS-PAGE and then transferred to nitrocellulose membranes. Then, 5% non-fat dry milk in TBST containing 0.1% Tween-20 was used for blocking non-specific protein binding for 2 h at room temperature. The following primary antibodies were used: Rabbit polyclonal anti-LC3 (1:500; cat. no. AF1720; Beyotime Institute of Biotechnology), Beclin-1 (1:1,000; cat. no. AF5123; Beyotime Institute of Biotechnology), SQSTM1/p62 (1:400; cat. no. AF5312; Beyotime Institute of Biotechnology), GRP78 (1:500; cat. no. AF0171; Beyotime Institute of Biotechnology) and ATF6 (1:500; cat. no. AF6243; Beyotime Institute of Biotechnology) and monoclonal anti-β-actin (1:5,000; cat. no. 3700T; Cell Signaling Technology, Inc.) at 4˚C overnight. Then, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The bands were visualized by enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc.). The expression levels of proteins were normalized to those of β-actin and quantified using ImageJ software (version 1.46; National Institutes of Health).

Immunofluorescence staining. The brains were removed, fixed in 4% PFA for 24 h at room temperature, immersed in 30% sucrose for 72 h at room temperature, embedded at optimal cutting temperature (Leica Microsystems GmbH) and sectioned (thickness, 10 μm) using a Leica cryostat (Leica Microsystems, Inc.). Sections were treated with 5% donkey serum and 0.1% Triton X-100 for 1 h at room temperature, then incubated with the primary antibodies: NeuN (1:100; cat. no. 94403; Cell Signaling Technology, Inc.) and LC3 (1:100; cat. no. sc-271625; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. Following washing in PBS, sections were incubated with Alexa Fluor 488 donkey anti-mouse IgG (1:200; R37114; Invitrogen; Thermo Fisher Scientific, Inc.) and Alexa Fluor 594 donkey anti-rabbit IgG (1:200; cat. no. R37119; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Subsequently, nuclear staining was performed using DAPI (Beyotime Institute of Biotechnology) for 15 min at room temperature. Images were obtained using a laser scanning confocal microscope (DM 5000B; Leica Microsystems GmbH; magnification, x400).

Statistical analysis. Data are presented as the mean ± SD, and each experiment was repeated three times. Sigma Plot software (version 18.0; IBM Corp.) was used to analyze all data. The unpaired Student's t-test was used for comparisons of two groups. Differences among multiple groups were analyzed by one-way ANOVA with Bonferroni's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Autophagy is initiated and autophagic flux is impaired in the hippocampus post-TBI. In order to study the induction of the autophagic process in the rat hippocampus, western blot analysis was used to detect the levels of endogenous LC3 conversion and Beclin-1. Cytosolic LC3-I, a soluble LC3, is formed by ATG4-mediated cleavage and forms a conjugate with phosphatidylethanolamine to produce the lipid conjugated LC3-II that is anchored in autophagosome membranes (10). Therefore, the ratio of LC3-II to LC3-I is widely recognized and used as a marker for the formation of autophagosomes. Moreover, Beclin-1 is also essential for autophagy initiation (8). TBI increased conversion of LC3-II to LC3-I and Beclin-1 expression levels in the hippocampal tissue of rats in a time-dependent manner (Fig. 1A). These results indicated that the autophagic process was successfully initiated in the damaged region of the hippocampus. Next, the expression levels and localization of LC3 in the hippocampus were observed. LC3 protein was primarily located in the neurons of the hippocampus (Fig. 1B). Next, the changes of autophagic flux were evaluated by analyzing the degradation of SQSTM1/p62 protein, which is a key indicator of autophagic flux (31). Compared with the Sham group, a significant decrease of SQSTM1 was observed in post-traumatic hippocampus tissue, suggesting that autophagic flux was impaired and selective degradation of SQSTM1 by autophagy was inhibited (Fig. 1C). Overall, these data indicated that TBI triggered autophagy initiation, but did not enhance the degradation of autophagic protein.

Activation of ER stress is mediated by ATF6 in the hippocampus post-TBI. In order to investigate whether ER stress was induced in the hippocampus following TBI, the expression levels of ER stress-associated proteins GRP78 and ATF6 were examined. GRP78, an ER chaperone, is important for ER stress signaling, as well as protein quality control and folding (32). Upon ER stress, GRP78 dissociates on unfolded proteins to activate EIF2AK3, ERN1 and ATF6 (33). As anticipated, compared with the Sham group, the protein expression levels of GRP78 were significantly increased in the hippocampus post-TBI in a time-dependent manner (Fig. 2). These results indicated that TBI triggered the activation of ER stress in the rat hippocampus. In addition, following ER stress, precursor ATF6 (90 kDa), which is anchored in the ER membrane, translocates to the Golgi apparatus and is cleaved to produce transcriptionally active ATF6 (50 kDa) (34). Western blot results revealed that transcriptionally active ATF6 was upregulated in the hippocampus tissue, consistent with the expression pattern of GRP78. Conversely, 90 kDa precursor ATF6 was significantly downregulated in a time-dependent manner. Collectively, the data indicated that TBI may induce ER stress via the activation of the ATF6 UFP pathway.

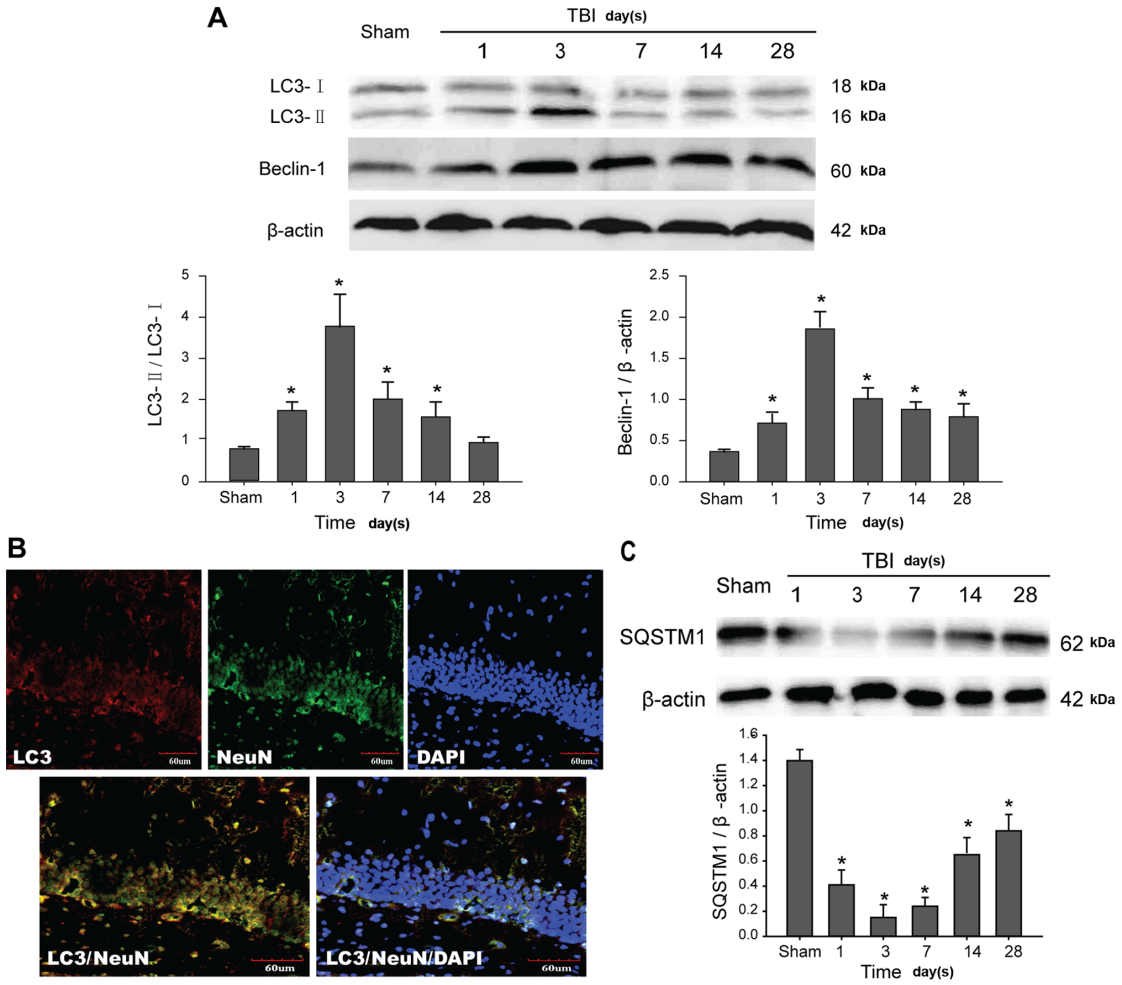

Figure 1. TBI-induced autophagy initiation and autophagic flux dysfunction in the hippocampus. (A) Representative bands and statistical analysis of western blots of protein expression levels of autophagy markers, such as LC3 and Beclin-1. (B) Autophagy marker LC3 was co-transfected into hippocampal neurons at 3 days post-TBI. Red, LC3; green, neuronal marker NeuN staining. Scale bar, 60 μm. (C) Representative bands and statistical analysis of western blots of protein expression levels of autophagic flux marker SQSTM1 in the hippocampus. Data are presented as the mean ± SD. *P<0.05 vs. Sham. TBI, traumatic brain injury; LC3, microtubule-associated protein 1 light chain 3.

Autophagy is partially induced by activation of ER stress following TBI. In order to elucidate the association between ER stress pathways and autophagy activation, ER stress inhibitor 4-PBA was used to investigate whether the occurrence of autophagy was caused by ER stress activation. Administration of 4-PBA significantly decreased the protein expression levels of ER stress markers in the rat hippocampus at 3 days post-injury (Fig. 3). Notably, TBI-induced autophagy was significantly prevented in rats pretreated with 4-PBA, as evidenced by the downregulation of Beclin-1 and upregulation of SQSTM1. These results indicated that ER stress was a positive regulator of autophagy induction in the rat model of TBI.

ER stress induces autophagy initiation via activation of the ATF6 UPR pathway. Next, the effects of ATF6 knockdown on the expression levels of autophagic makers at 3 days post-TBI were investigated. Firstly, ATF6 expression in the brain tissues was silenced using lentiviral-based shRNA. As expected, the stable decrease in ATF6 mRNA and protein in the hippocampus were mediated by ATF6 shRNA treatment (Fig. 4A). Furthermore, RT-qPCR results demonstrated that ATF6 knockdown significantly decreased mRNA levels of autophagy-associated genes, including ATG3, ATG9 and ATG12, leading to inactivation of autophagy (Fig. 4B). Additionally, western blot results revealed that silencing ATF6 alleviated ER stress-induced autophagy, as indicated by a significant decrease in conversion of LC3-I to LC3-II as well as Beclin-1 expression levels (Fig. 4C). These findings indicated that the ATF6 pathway was involved in ER stress-induced autophagy via regulating downstream autophagic genes.

Pharmacological inhibition of ER stress or autophagy improves TBI and neurological deficiency. The involvement ER stress-mediated autophagy in post-traumatic histological and neurological impairment was investigated. Compared with the Sham group, a greater number of damaged neurons were observed in the cortex and hippocampus CA1 region of rats with TBI (Fig. 5). However, compared with the TBI and saline groups, 4-PBA or 3-MA treatment was revealed to decrease neuronal death and loss following TBI. Results revealed that treatment with 4-PBA or 3-MA significantly improved sensorimotor functional recovery, as indicated by decreased mNSS (Fig. 6A) and frequency of forelimb foot fault occurrence (Fig. 6D). Moreover, 4-PBA or 3-MA significantly improved

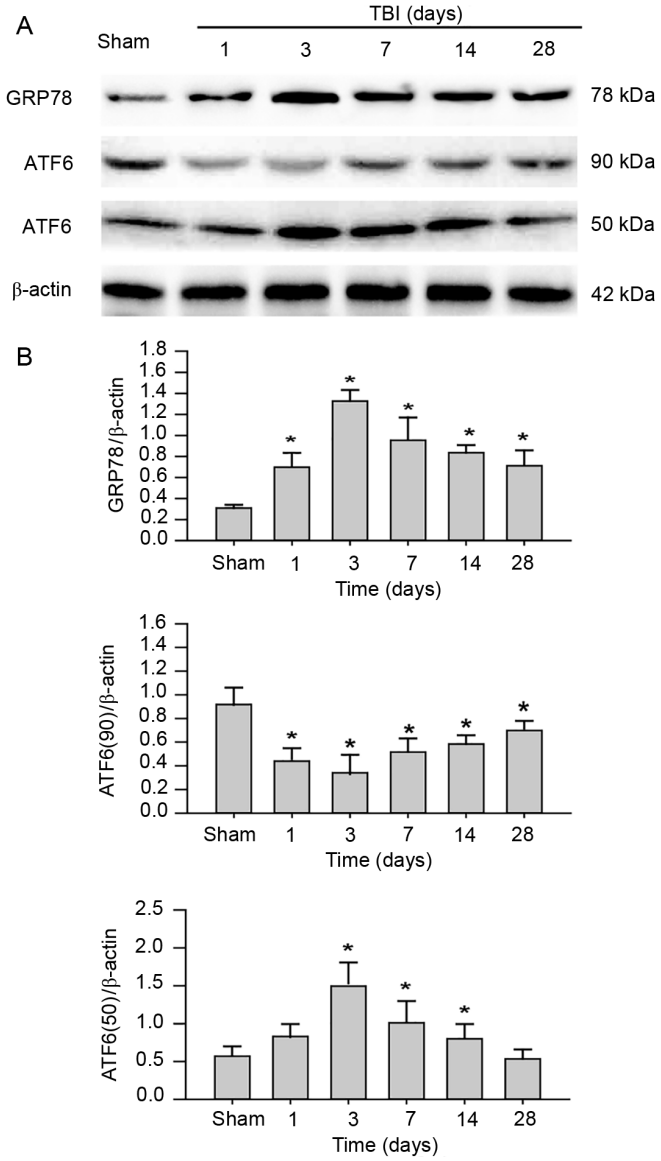

Figure 2. TBI-mediated time-dependent activation of ER stress in the hippocampus. (A) Representative bands and (B) statistical analysis of western blotting of the expression levels of ER stress markers, such as GRP78 and 90 and 50 kDa ATF6. Data are presented as the mean ± SD. *P<0.05 vs. Sham. TBI, traumatic brain injury; ER, endoplasmic reticulum; ATF, activating transcription factor.

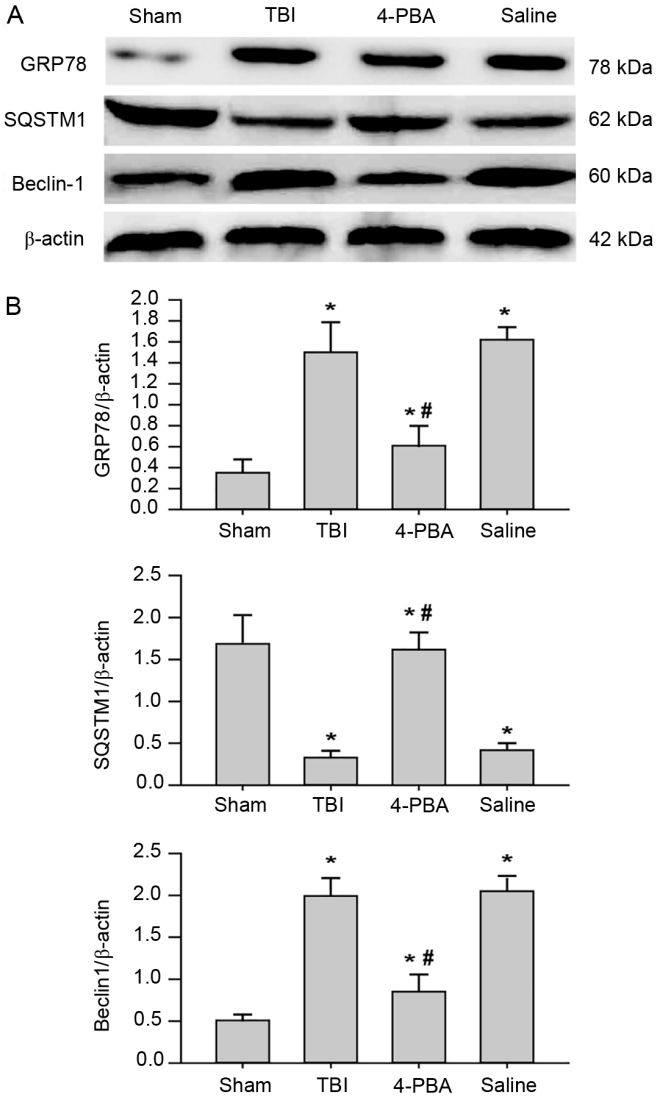

Figure 3. Activation of autophagy is induced by ER stress following TBI. (A) Representative bands and (B) statistical analysis of western blotting revealing that the ER stress inhibitor 4-PBA significantly reversed SQSTM1 and Beclin-1 protein expression levels in the hippocampus at 3 days post-injury. Data are presented as the mean ± SD. *P<0.05 vs. Sham. #P<0.05 vs. TBI. ER, endoplasmic reticulum; TBI, traumatic brain injury; 4-PBA, 4-phenylbutyric acid.

cognitive deficits post-TBI, as evidenced by significantly shortened latency to find the hidden platform (Fig. 6B) and increased percentage of time in the correct quadrant (Fig. 6C). Collectively, these results indicated that suppression of ER stress or autophagy promoted the recovery of neurological dysfunction following TBI.

Discussion

A number of animal models have been developed to mimic human TBI, including CCI, lateral fluid percussion injury, as well as Marmarou's and Feeney's weight-drop model, among them, the CCI model is widely used to study TBI due to the precise control of cortical depth penetration, dwell time and speed of impact (35). A previous study demonstrated that the rat CCI model replicates clinical TBI pathophysiology and neurobehavioral impairment (27). In the present study, relevant phenotypes were demonstrated by brain injury and neuronal loss in the cortex and hippocampus region of TBI rats, accompanied by behavioral and cognitive deficits. Autophagy serves a crucial role in neurobehavioral and cognitive deficiency induced by TBI (36). Although autophagy is usually considered to be an essential process for the clearance of aggregated toxic proteins and damaged organelles, abnormal autophagy has been implicated in the development of TBI (37). In the present study, the activation of autophagy was confirmed in the hippocampus, which was reflected by the increased ratio of LC3-II to LC3-I and Beclin-1 expression levels, in line with previous studies (38,39). However, an increased ratio of LC3-II to LC3-I may result from autophagy activation or autophagic flux defects; therefore, it is essential to determine whether the accumulation of LC3-II anchored in autophagosome is due to the increased upstream activation of autophagy or a blockade

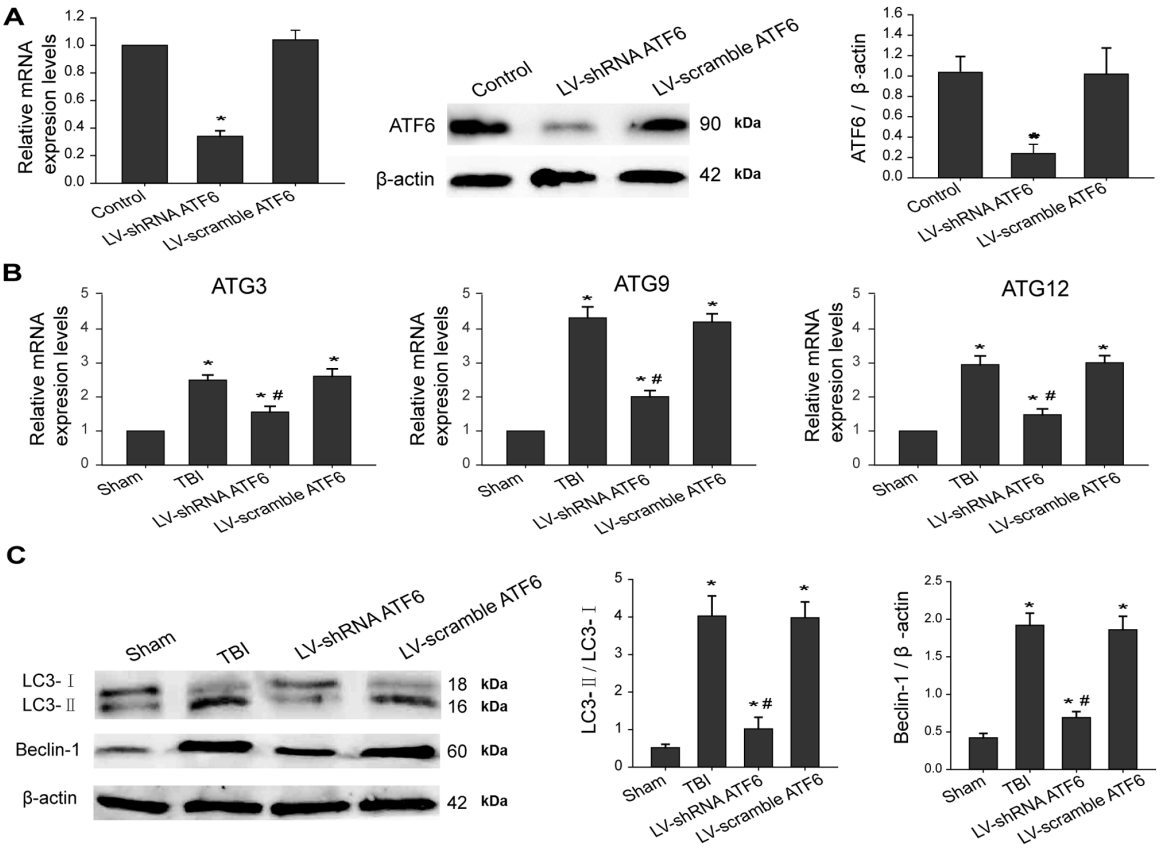

Figure 4. ATF6 URP pathway involvement in endoplasmic reticulum stress-induced autophagy activation following TBI. (A) mRNA and protein expression levels of ATFA6 were detected by reverse transcription-quantitative PCR and western blot assay 48 h after administration of lentiviral vector. Effects of silencing ATF6 on (B) transcriptional levels of autophagic genes ATG3, ATG9 and ATG12 and (C) LC3 turnover and Beclin-1 expression levels in the hippocampus at 3 days post-TBI. Data are presented as the mean ± SD. *P<0.05 vs. Sham. #P<0.05 vs. TBI. ATF, activating transcription factor; TBI, traumatic brain injury; ATG, autophagy-related; LC3, microtubule-associated protein 1 light chain 3; sh, short hairpin.

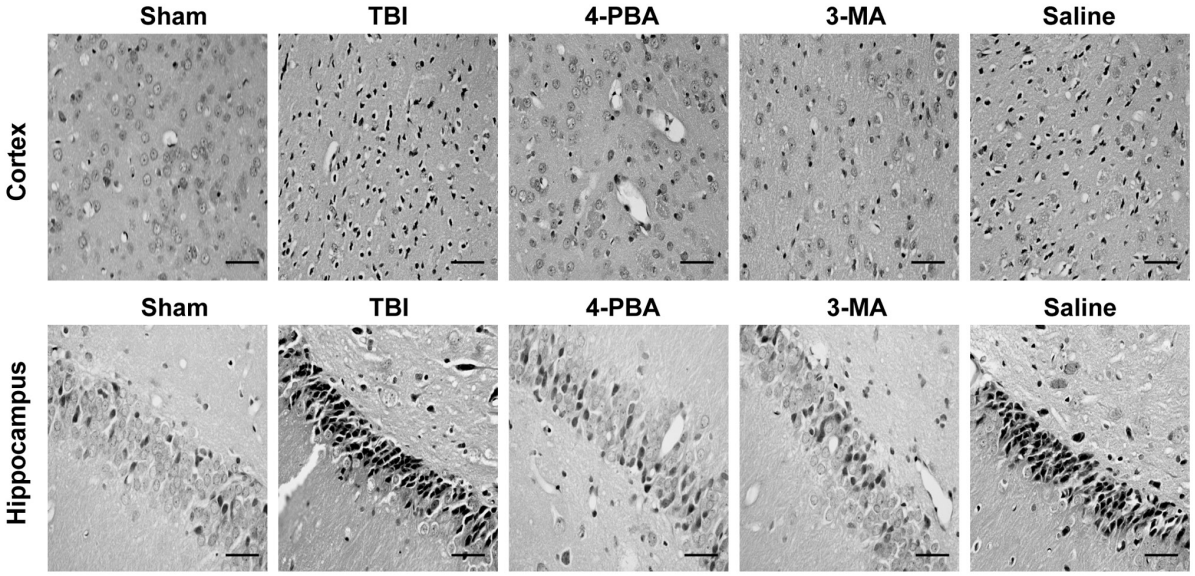

Figure 5. Inhibition of endoplasmic reticulum stress or autophagy improves TBI. Alterations of morphological structure in the cortex and hippocampal CA1 region were assessed via hematoxylin and eosin staining at 3 days post-injury. Scale bar, 20 μm. TBI, traumatic brain injury; 4-PBA, 4-phenylbutyric acid.

of autophagosome-lysosomal fusion. Therefore, autophagic flux was measured via the preferential degradation of SQSTM1 following TBI. SQSTM1, a ubiquitin-binding protein, directly binds to LC3 protein and contributes to its selective degradation by autophagy (40). The present data revealed that the protein expression levels of SQSTM1 were decreased in the post-traumatic hippocampus, suggesting defective autophagic flux following TBI. Notably, these results revealed that TBI

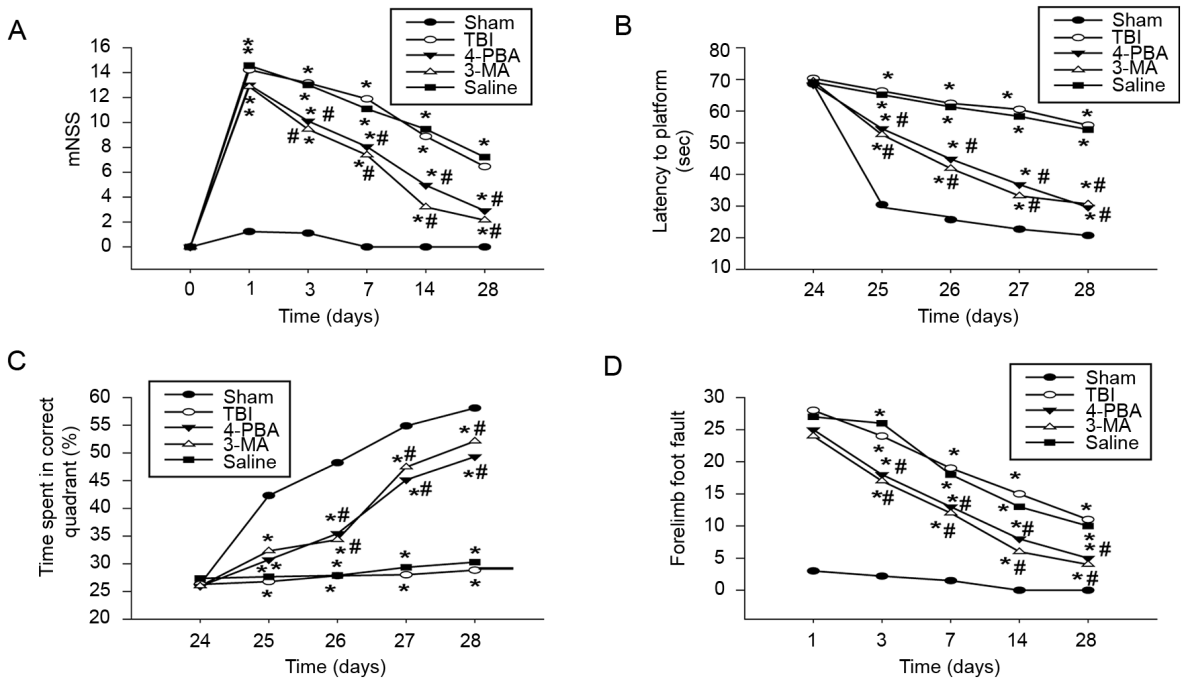

Figure 6. Inhibition of endoplasmic reticulum stress or autophagy improves TBI-induced neurological outcomes. (A) Neurological dysfunction was tested by mNSS at 1, 3, 7, 14 and 28 days post-TBI. Rat spatial learning and memory function were observed by Morris water maze at 24-28 days post-TBI and assessed by (B) latency to locate the platform and (C) percentage time spent in the correct quadrant. (D) Motor functional recovery was evaluated by right forelimb foot fault test at 1, 3, 7, 14 and 28 days post-TBI. Data are presented as the mean ± SD. *P<0.05 vs. Sham. #P<0.05 vs. TBI. TBI, traumatic brain injury; mNSS, modified neurological severity score; 4-PBA, 4-phenylbutyric acid.

triggered autophagy initiation and suppressed autophagosome clearance, leading to autophagic flux impairment. However, the beneficial or detrimental role of TBI-induced autophagy is controversial. The results of the present study suggested that pharmacological inhibition of autophagy significantly ameliorated brain damage, neurological function score and behavioral and cognitive impairment. Therefore, it was speculated that TBI-induced autophagy served a detrimental role in neurological dysfunction and that targeting autophagy may represent a promising approach for the treatment of TBI.

Furthermore, accumulating evidence has demonstrated that ER stress can cause apoptosis, neuronal injury, neuroinflammation, microglia or macrophage activation and neurological outcomes following TBI (41,42). However, the mechanisms by which ER stress mediates autophagy have not been fully characterized in the rat model of TBI to date. In the present study, activation of ER stress was verified in the post-traumatic hippocampus by confirming upregulation of protein levels of ER stress-associated makers. In order to elucidate the molecular mechanism underlying upstream activation of autophagy, rats were pretreated with ER stress inhibitor before TBI induction. The present results revealed that the suppression of ER stress significantly decreased the conversion of LC3-I to LC3-II and Beclin-1 expression levels, as well as neurological dysfunction, indicating that TBI-induced autophagy activation was mediated by ER stress.

It is recognized that the ER stress sensors of ATF6 are crucial for autophagy induction in numerous types of disease including neurodegeneration, stroke, metabolic diseases and cancer (24,43,44). ATF6, an ER stress-induced transcription factor, regulates the expression levels of ER-associated proteins, such as GRP78, DNA damage inducible transcript 3 and X-box binding protein 1 (45). In the present study, it was revealed that increased levels ER stress-associated markers were accompanied by an increase in 50 kDa transcriptionally active ATF6. Therefore, it was concluded that TBI induced the degradation of 90 kDa ATF6 precursor and generated a 50-kDa cleavage product, known as ATF. Concurrently, ATF6 knockdown significantly decreased the conversion of LC3-I to LC3-II and Beclin-1 expression levels, suggesting that ER stress-induced autophagy was regulated by the ATF6 UPR pathway. Next, the molecular mechanisms by which ATF6 pathway modulated autophagy activation were further investigated. A previous study revealed that depletion of ATF6 decreased transcription of ATG3 and Beclin-1 in Japanese encephalitis virus-infected cells (46). Another study indicated that hepatic conditional knockout of ATF6 inhibited autophagy induction by targeting the mTOR pathway (47). It has been reported that ER stress triggers autophagy via ATF6/death-associated protein kinase 1-mediated ATG9 trafficking and Beclin-1 phosphorylation (48). Additionally, it has been revealed that LC3 and ATG12 are transcriptionally upregulated by the ATF6 UPR pathway (49). Accordingly, it was hypothesized that knockdown of ATF6 suppressed the transcriptional levels of key autophagic genes, including ATG3, ATG9 and ATG12 post-TBI. To sum up, these findings indicated that the ATF6 pathway was involved in ER stress-induced autophagy via transcriptionally activating crucial autophagic genes, suggesting inhibition of ATF6 could be a potential therapeutic target for TBI.

In conclusion, ER stress and autophagy were activated in the hippocampus following TBI. Activation of autophagy induced

by ER stress may contribute to neurological dysfunction following TBI. Furthermore, it was confirmed that ER stress caused autophagy activation via ATF6 UPR-mediated transcriptional activation of autophagic genes.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XX designed the research and revised the manuscript. DYW performed the experiments and prepared the manuscript. MYH, JP, YHG and YZ analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Tangshan Gongren Hospital (approval no. 2018550). All procedures were performed in accordance with the Institutional Animal Care and Use Committee of Tangshan Gongren Hospital and complied with the Declaration of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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