Article

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Docosahexaenoic Acid Attenuates Endoplasmic Reticulum Stress-Induced Neuron Apoptosis by Targeting Presenilin 2

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Background: Traumatic neuronal injury (TNI), a type of traumatic brain injury (TBI), features apoptosis of cortical neurons. Knowing that docosahexaenoic acid (DHA) is enriched in the neuronal cell membranes and related to brain function, we aimed to study the mechanism behind the effect of DHA on TNI model neurons.
Methods: Neurons were derived from Sprague-Dawley rats and a TNI model was established by using a rotating scribe injury device. Four experimental groups were set up based on neuronal injury and DHA concentration, namely the Control, TNI, TNI + DHA-25, and TNI + DHA-50 groups. The cell morphology, toxicity and neuron apoptosis were assessed by means of light microscopy, lactate dehydrogenase (LDH) release assessment, and terminal-deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay, respectively. Protein levels of sorcin (SRI), activating transcription factor 6 (ATF6), glucose-regulated protein 78 (GRP78), C/Ebp-homologous protein (CHOP), cleaved-caspase-12 (C-Cas-12) and presenilin 2 (PSEN2) were determined by western blotting. PSEN2 overexpression plasmid and short hairpin RNA against SRI (shSRI) were used for transfection. In the transfection experiment, neurons were assigned into six groups, namely TNI, TNI + DHA-50, short hairpin RNA for negative control (shNC) + Vector, PSEN2, shSRI, and PSEN2 + shSRI groups, and the latter four groups were treated with TNI + DHA. *PSEN2* and *SRI* mRNA levels were measured by quantitative real-time polymerase chain reaction (qRT-PCR). Cytotoxicity-, apoptosis- and endoplasmic reticulum stress (ERS)-related proteins were examined in the neurons.
Results: DHA at a dose of 50 µM attenuated TNI-induced neuronal injury, cell toxicity ($p < 0.001$), cell apoptosis ($p < 0.001$), and levels of ERS-related proteins (ATF6, GRP78, CHOP and C-Cas-12) ($p < 0.001$), PSEN2 and SRI in neurons ($p < 0.001$). Besides, 50 µM of DHA regulated cell toxicity, cell apoptosis and ERS-related proteins by modulating the interaction between PSEN2 and SRI in neurons ($p < 0.05$).
Conclusions: DHA mitigates neuron apoptosis induced by SRI-activated ERS via targeting PSEN2.

Keywords: traumatic brain injury; traumatic neuronal injury; docosahexaenoic acid; neuron apoptosis; presenilin 2

Introduction

Traumatic brain injury (TBI) is a debilitating and fatal brain disease [1], potentially resulting in shock wave injury and non-shock wave injury [2]. Treating TBI faces serious challenges in some cases due to the lack of effective treatment [3]. Recent years have witnessed the emergence of some novel therapies, chiefly including fluid therapy [4], music therapy [5], temperature management [6], and deep brain stimulation [7]. However, TBI patients are still plagued by poor prognosis and high mortality rates. Notably, traumatic neuronal injury (TNI), a type of TBI, can lead to apoptosis in cortical neurons [8]. Recently, the TNI model has gained broader recognition for being widely used in *in vitro* investigations of molecular mechanisms underlying TBI [9]. Owing to the knowledge gap in the molecular mechanism of TBI, no effective therapies have been proposed to mitigate brain injury or promote brain repair. Hence, this lacuna calls for more studies to explore new molecular mechanisms that serve as therapeutic targets of TBI.

In recent years, more and more findings have demonstrated the critical role of endoplasmic reticulum stress (ERS) in the pathogenesis of TBI [10,11]. The endoplasmic reticulum is a vital organelle involved in protein quality control and cell homeostasis [12]. The ERS-related proteins mainly include activating transcription factor 6 (ATF6), glucose-regulated protein 78 (GRP78), C/Ebp-homologous protein (CHOP) and cleaved-caspase-12 (C-Cas-12) [13]. Of note, the sorcin (SRI) level is upregulated in TBI according to the TBI-related dataset (GSE59645). SRI is an early marker of neurodegenerative diseases, which is abundant in Alzheimer's disease, Parkinson's disease and Huntington's disease [14]. SRI, a kind of calcium sensor responsible for maintaining calcium ion storage in the endoplasmic reticulum, plays a role in many calcium-related functions and

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is an early marker of ERS, with its upregulation indicating ERS activation [14,15]. It has been found that SRI silencing can cause a decrease in ATF6 transcription activity [16].

Docosahexaenoic acid (DHA) has been confirmed to serve as an inhibitor of ERS [17]. DHA is enriched in the neuronal cell membranes and is related to brain function [18]. Particularly, DHA protects against neuronal damage in TBI mice [19]. Through SwissTargetPrediction analysis, presenilin 2 (PSEN2) is found to be a possible target molecule of DHA. PSEN2 is one of the three proteins that are dominantly mutated in familial Alzheimer's disease, which is related to brain function networks [20]. An interaction exists between PSEN2 and SRI [21], but the molecular mechanisms involving PSEN2 and SRI behind the TNI-triggered neuronal apoptosis remain elusive. Therefore, our study aimed to explore whether DHA mitigates neuronal apoptosis induced by SRI-activated ERS via targeting PSEN2.

Materials and Methods

Primary Culture of Cortical Neurons and TNI Model

Eight Sprague-Dawley (SD) pregnant rats (female, 250–280 g) were obtained from Laboratory Animal Center, Nantong University (Nantong, China). Cortical neurons were obtained from Sprague-Dawley rats at embryonic days 16–18 as previously described [22]. Briefly, in aseptic condition, cerebral cortex was obtained from each rat after performing cervical dislocation, followed by dissection and then mincing in Dulbecco's modified eagle medium (DMEM; supplemented with L-glutamine; SH30022.01B; HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS; 10100147; Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C. Tissue digestion was performed in 0.25% trypsin (C0203; Beyotime, Shanghai, China) (37 °C, 15 min). Neurons were resuspended in neurobasal medium (21103049; Thermo Fisher Scientific, Waltham, MA, USA) containing 2% B-27 supplement (17504044; Thermo Fisher Scientific, Waltham, MA, USA) and cultured in Greiner CELLCOAT® dish (poly-D-Lysine-coated; GN664940; Merck, Shanghai, China) (37 °C, 5% CO_2, 95% air), with medium refreshed every two days. After 16 days, cells were collected for further experiments when >95% of cells were confirmed to be neurons. Primary neuronal cells were tested negative for mycoplasma.

A TNI model was established in neurons [22]. A rotating scribe injury device, incorporating a rotating cylinder with 10 holes, 10 steel needles, and a permanent magnet, was applied to establish a neuronal injury model. The cylinder holes were separated at the same interval from the center, with steel needles freely passing through. A magnet under the Greiner CELLCOAT® dish (664950, Greiner Bio-One, Frickenhausen, Germany) was employed to ensure that steel needles could be adsorbed on the cell layer when the cylinder rotated. Ten concentric circular scratches (1.5 mm distance) were made in the cell layer after one turn of the device. The model constructed as described has been widely used in research and is able to recapitulate cellular and morphological features of severe TNI.

Treatments and Grouping

Following incubation, neurons were divided into different groups. In the first part, TNI was induced in neurons using a rotating scribe injury device for 1, 3, 6, 12, 24 and 48 h.

In the second part, the neurons were divided into four groups based on neuronal injury and DHA concentration: Control (normal culture), TNI (12-h treatment with the rotating scribe injury device), TNI + DHA-25 (48-h treatment with 25 µM DHA after TNI modeling), and TNI + DHA-50 (48-h treatment with 50 µM DHA after TNI modeling) [23]. DHA was bought from MedChemExpress (HY-B2167; Shanghai, China). The morphology of neurons was photographed using a light microscope at 100× magnification (LV150; Nikon Inc., Tokyo, Japan).

In the third part, neurons were subjected to transfection with/without pDONR223 vector/PSEN2 overexpression plasmid (YouBio Biology, G166007; Changsha, China) or short hairpin RNA for negative control (shNC)/short hairpin RNA against SRI (shSRI). shSRI (5′-GCCTTATGGTTTCAATGCTGG ATAGAGATATGTCTGGCACG-3′) and shNC (5′-AATTCTCCGAACGTGTCACGT-3′) were ordered from Yunzhoubiota (Guangzhou, China).

In the fourth part, after a 12-h treatment with rotating scribe injury device, six experimental groups of neurons were set up based on DHA treatment and the vectors transfected, namely TNI, TNI + DHA-50, shNC + Vector groups (neurons were treated with 50 µM DHA, and then experienced 48-h co-transfection with shNC and pDONR223 vector), PSEN2 group (neurons were treated with 50 µM DHA, and then transfected with PSEN2 overexpression plasmid for 48 h), shSRI group (neurons were treated with 50 µM DHA, and then transfected with shSRI for 48 h), and PSEN2 + shSRI group (neurons were treated with 50 µM DHA, followed by 48-h co-transfection with PSEN2 overexpression plasmid and shSRI).

Immunofluorescence

The cultured cortical neurons with good growth conditions on the 7th day were removed from the incubator. After aspirating the culture medium, the cultured cell layers were washed with phosphate-buffered saline (PBS) 3 times. Following this, the neurons were subjected to 30-min fixation in 4% paraformaldehyde, PBS washing (3 times), 0.1% Triton-X100 treatment (20 min), PBS washing again (3 times), and sealing using buffer solution with 5% skim milk (30 min). Then, the neurons were incubated with anti-microtubule associated protein 2 (MAP2) antibody (1:1000,

ab183830, Abcam, Cambridge, UK) at 4 °C overnight. After being washed 3 times, the neurons were incubated with FITC-coupled secondary antibody (1:1000, ab150077, Abcam, Cambridge, UK) for 2 h on the second day. The nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI). The cover glass was mounted on the slide with an anti-fluorescence quenching agent prior to observation with a fluorescence microscope.

Transfection

Neurons (5×10^5/well) were cultured to 60%–70% confluence in 6-well plates, and treated according to the grouping. Lipofectamine 3000 reagent (L3000001; Thermo Fisher Scientific, Waltham, MA, USA) was used for transfection. Reduced Serum Medium (11058021; Thermo Fisher Scientific, Waltham, MA, USA) was bought from Thermo Fisher Scientific. Liquid A (5 µg pDONR223 vector/PSEN2 overexpression plasmid/shNC/shSRI + 125 µL reduced serum medium) and liquid B (10 µL Lipofectamine 3000 reagent + 125 µL reduced serum medium) were prepared 5 min in advance, and then fully mixed (15 min). Finally, the mixture of liquid A and B as described above were incubated with neurons (37 °C, 5% CO_2, 48 h).

Western Blotting

Total proteins were extracted from the cultured neurons using a commercial protein extraction kit (C006225; Sangon Biotech, Shanghai, China). Next, the extracted proteins were subjected to concentration determination using a protein concentration assay kit (P0010; Beyotime, Shanghai, China). The protein samples were denatured at 99 °C in boiling water for 5 min. Following separation (gel reagent, P0012A; Beyotime, Shanghai, China) and transference onto membranes (0.45 µm; YA1711; Solarbio, Beijing, China), blocking was performed using 5% bovine serum albumin (BSA) blocker (37525; Thermo Fisher Scientific, Waltham, MA, USA) at 25 °C for 1 h. Subsequently, the membranes were incubated with primary antibodies at 4 °C overnight and then with secondary antibodies at 25 °C for 1 h. Table 1 provides information on related antibodies. The internal reference used was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Western blot detection and imaging were performed with electro-chemiluminescence reagent (32209; Thermo Fisher Scientific, Waltham, MA, USA) and a gel imaging analysis system (BIS910; Beijing Dongsheng Innovative Biotechnology Co., Ltd., Beijing, China), respectively, followed by the analysis using ImageJ software (version 8.0; National Institutes of Health, Bethesda, MD, USA). Each group consists of 3 samples.

Determination of Lactate Dehydrogenase Release

Lactate dehydrogenase (LDH) release in neurons can reflect cell toxicity. The rat LDH assay kit was procured from Fine Biotech (ER0646; Wuhan, China). The collected cell culture supernatant was centrifuged (20 min, 1000 $\times g$, 4 °C) and tested as per the assay manufacturer's instructions. Stop solution (50 µL) was added into each well and absorbance (450 nm) was measured by a microplate reader (HTS-XT; Bruker Optics, Rheinstetten, Germany). Cytotoxicity was determined using the following formula:

Cytotoxicity (%) = [(Treated sample absorbance – Control sample absorbance)/(Maximum cell enzyme activity absorbance – Control sample absorbance)] × 100

LDH release was determined using the following formula:

LDH release (fold of control) = Cytotoxicity of treated sample/Cytotoxicity of control

TUNEL Staining Assay

Apoptosis of neurons was measured by the one-step terminal-deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) apoptosis assay kit (C1090; red fluorescence; Beyotime, Shanghai, China). After a 24-hour culture in 12-well plates, neurons (1×10^5 cells/well) were treated for 48 h according to the grouping design. Following fixation and washing, neurons were incubated with an enhanced immunostaining permeabilization solution (P0097; Beyotime, Shanghai, China). After washing again, neurons were cultured with 50 µL TUNEL detection solution (5 µL terminal deoxynucleotidyl transferase [TdT] + 45 µL fluorescent labeling solution) and staining by DAPI staining solution (300 µL; C1006; Beyotime, Shanghai, China). Finally, cell apoptosis was imaged with a fluorescence microscope at 200× magnification (Axiovert 200M; ZEISS Inc., Baden-Wurttemberg, Germany). The number of positive cells was analyzed using the ImageJ software. The TUNEL-positive rate was determined as follows:

TUNEL-positive rate (%) = (Number of positive cells/Number of total cells) × 100

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA extracted with a Trizol kit (R0016; Beyotime, Shanghai, China) was reverse-transcribed into cDNA using the reverse transcription reagents (B639278; Sangon Biotech, Shanghai, China). cDNA was then mixed with real-time PCR reagent (FP313-01; Tiangen Biotech, Beijing, China) and amplified for 40 cycles (95 °C for 15 s and 60 °C for 1 min). A qRT-PCR instrument (MJOpticon2, Bio-Rad Inc., Hercules, CA, USA) was applied to detect gene expression. *GAPDH* was employed as the internal reference. Gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method [24]. Sequences of primers used in this study, synthesized by Sangon Biotech (Shanghai, China), are presented in Table 2. Each group contains 3 samples.

Statistical Analysis

All data, expressed as mean ± standard deviation, were analyzed by GraphPad 8.0 software (GraphPad Soft-

Table 1. Antibodies used in this study.

Name	Catalog	Molecular weight (kDa)	Dilution	Manufacturer
SRI	ab71983	22	1:1000	Abcam, Cambridge, UK
ATF6	ab37149	85	1:1000	Abcam, Cambridge, UK
GRP78	ab21685	75	1:1000	Abcam, Cambridge, UK
CHOP	PA071811	31	1:2000	Cusabio, Houston, TX, USA
C-Cas-12	ab62484	46	1:1000	Abcam, Cambridge, UK
PSEN2	ab51249	23	1:20,000	Abcam, Cambridge, UK
GAPDH	ab8245	36	1:5000	Abcam, Cambridge, UK
Goat anti-rabbit	ab97051	—	1:10,000	Abcam, Cambridge, UK
Goat anti-mouse	ab96879	—	1:10,000	Abcam, Cambridge, UK

SRI, sorcin; ATF6, activating transcription factor 6; GRP78, glucose-regulated protein 78; CHOP, C/Ebp-homologous protein; C-Cas-12, cleaved-caspase-12; PSEN2, presenilin 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Primer sequences of related genes.

Genes	Primer sequences (5′→3′)
PSEN2 (forward)	CTCATGGCCCTGGTGTTCAT
PSEN2 (reverse)	CTCTGGATCGTAAGGGAGCTG
SRI (forward)	TGTGCAAAACCAGTGACCCT
SRI (reverse)	CTGTCTCTCCTCCCCTGACA
GAPDH (forward)	CCGCATCTTCTTGTGCAGTG
GAPDH (reverse)	ACCAGCTTCCCATTCTCAGC

ware Inc., San Diego, CA, USA). Differences with $p < 0.05$ were regarded as statistically significant. Multi-group comparisons were accomplished with a one-way analysis of variance (ANOVA). Post-hoc tests were performed using the Tukey test.

Results

DHA Decreased Neuronal Injury, Cell Toxicity, Cell Apoptosis, ERS-Related Protein Levels, PSEN2 Level and SRI Level in TNI-Induced Neurons

To understand the effect of TNI on SRI expression level, we treated neurons using a rotating scribe injury device for 0, 1, 3, 6, 12, 24 and 48 h. Our results demonstrated an initial increase in SRI level and a subsequent decrease after TNI occurrence in neurons (Fig. 1, $p < 0.001$). Particularly, SRI expression peaked at 12 h. With this, TNI modeling was accomplished by treating the cells using a rotating scribe injury device for 12 h, and the TNI model constructed was utilized in subsequent experiments.

The signature protein of cortical neurons was MAP2, which is stained green in immunofluorescence staining. Nuclei were stained blue by DAPI in immunofluorescence staining. More than 95% of the cells were cortical neurons (Fig. 2A). Furthermore, to dissect the role of DHA in TNI model neurons, we treated neurons with DHA (25 or 50 μM) after TNI modeling. Our data revealed that the damaged synapses in neurons became shorter, accompanied by cell body atrophy, in the TNI group. However, the cell morphology was comparatively improved in the TNI + DHA

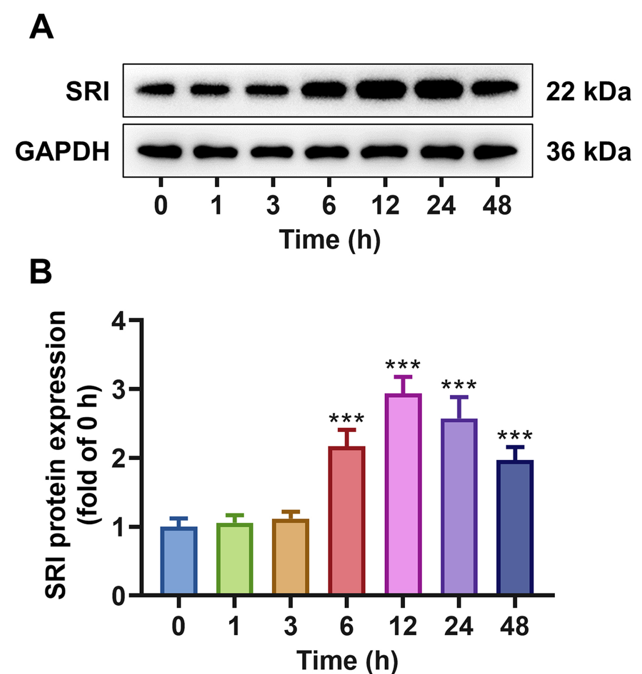

Fig. 1. SRI level was initially increased and then decreased after TNI modeling in neurons. (A) Western blot results of SRI protein level, with GAPDH being the internal reference. (B) Quantitative measurement of SRI protein level. $^{***}p < 0.001$ vs. 0 h group. n = 3 in each group. TNI, traumatic neuronal injury; SRI, sorcin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

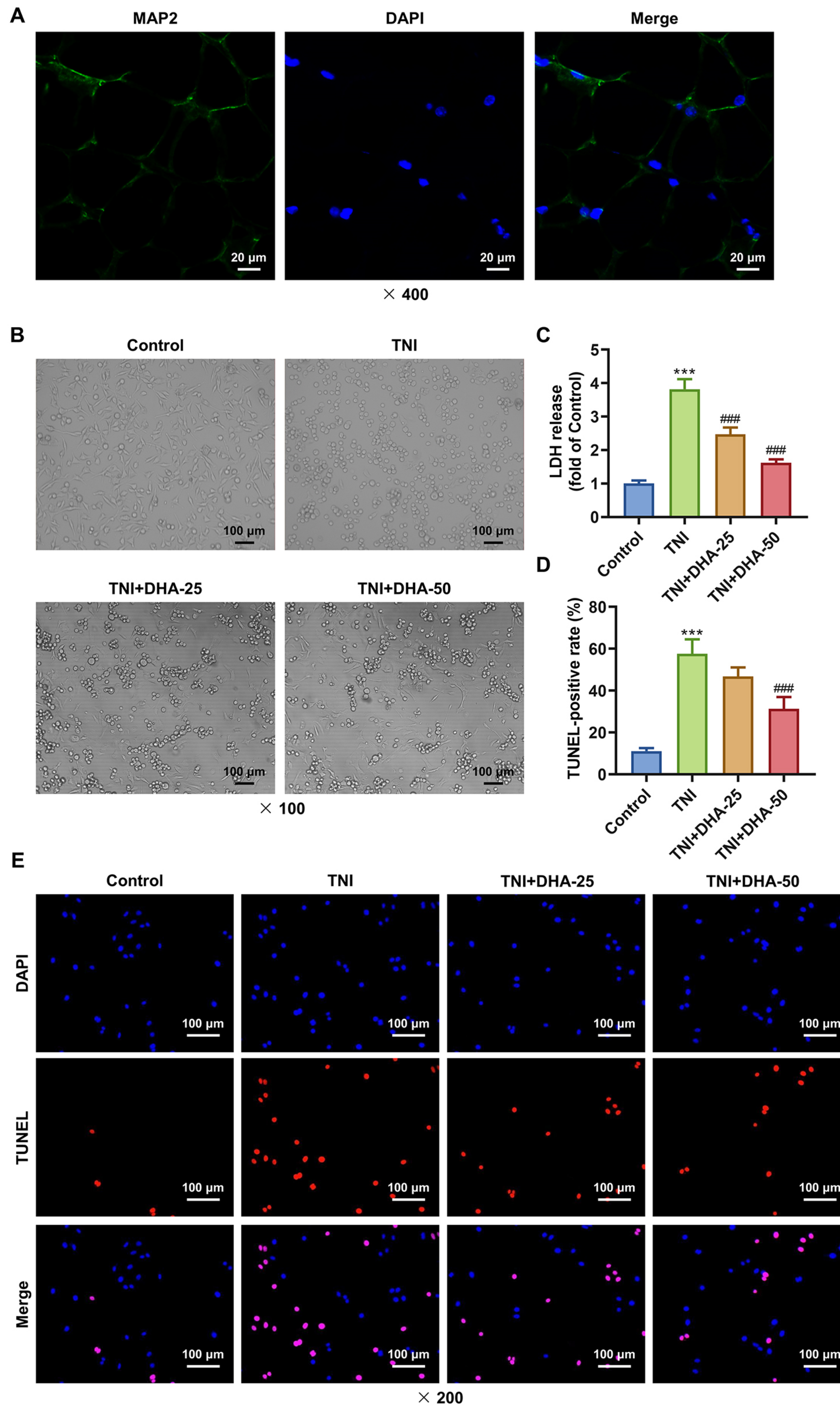

Fig. 2. DHA dose-dependently decreased neuronal injury, cell toxicity and apoptosis in TNI model neurons. (A) Identification of primary neurons. Scale bar: 20 μm; magnification: 400×. (B) Observation of neuron morphology using a light microscope. Scale bar: 100 μm; magnification: 100×. (C) Release of LDH, as a measure of cell toxicity, in neurons. (D,E) Apoptosis of neurons as determined by TUNEL assay. Scale bar: 100 μm; Magnification 200×. n = 3 in each group. $^{***}p < 0.001$ vs. Control group; $^{\#\#\#}p < 0.001$ vs. TNI group. DHA, docosahexaenoic acid; LDH, lactate dehydrogenase; TUNEL, terminal-deoxynucleotidyl transferase-mediated nick end labeling; DAPI, 4′, 6-diamidino-2-phenylindole; MAP2, microtubule associated protein.

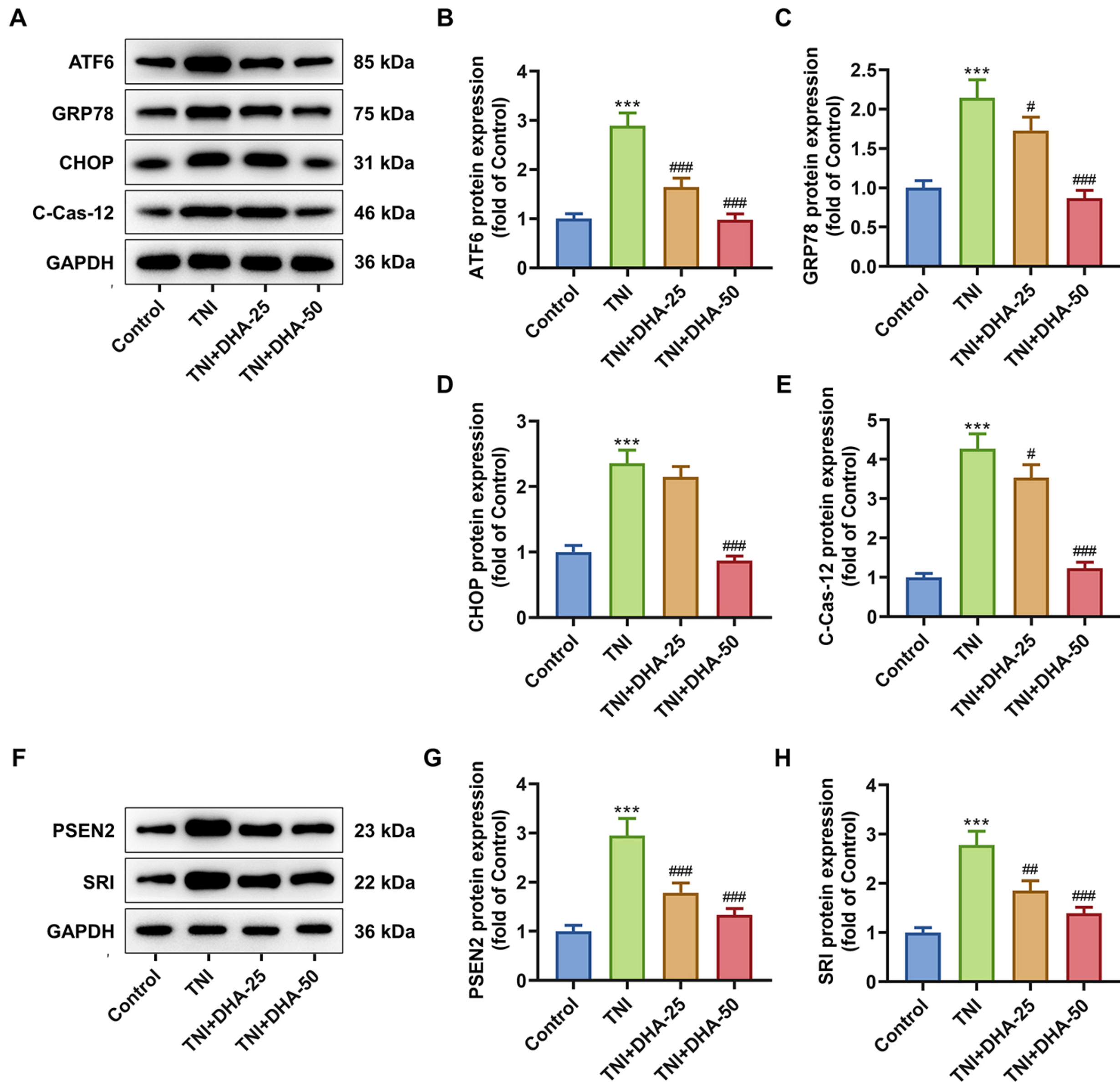

Fig. 3. DHA dose-dependently diminished levels of ERS-related proteins, PSEN2 and SRI in TNI model neurons. (A,F) Western blots of ATF6, GRP78 CHOP, C-Cas-12, PSEN2, and SRI, with GAPDH as the internal reference. Quantitation of protein expression of ATF6 (B), GRP78 (C), CHOP (D), C-Cas-12 (E), PSEN2 (G) and SRI (H). n = 3 in each group. $^{***}p < 0.001$ vs. Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. TNI group. ATF6, activating transcription factor 6; GRP78, glucose-regulated protein 78; CHOP, C/Ebp-homologous protein; C-Cas-12, cleaved-caspase-12; PSEN2, presenilin 2; ERS, endoplasmic reticulum stress.

group (Fig. 2B). Moreover, the release of LDH was higher in the TNI group than in the Control group (Fig. 2C, $p < 0.001$), causing cell toxicity in neurons. Cell apoptosis was significantly more pronounced in TNI model neurons relative to non-TNI model neurons (Fig. 2D,E, $p < 0.001$). However, in the DHA-treated TNI model neurons, cell toxicity (Fig. 2C, $p < 0.001$) and apoptosis (Fig. 2D,E, $p < 0.001$) were dose-dependently attenuated by DHA. These data revealed that DHA could mitigate neuronal injury, cell toxicity, and cell apoptosis in TNI model neurons in a dose-dependent fashion.

In addition, levels of ATF6, GRP78, CHOP and C-Cas-12—the ERS-related proteins—were higher in the TNI group than in the Control group (Fig. 3A–E, $p < 0.001$), which were then dose-dependently decreased by DHA (Fig. 3A–E, $p < 0.05$). More interestingly, upregulation of PSEN2 and SRI was observed in TNI model neurons, when compared to non-TNI model neurons (Fig. 3F–H, $p < 0.001$). Collectively, DHA dose-dependently reduced PSEN2 and SRI levels in TNI model neurons (Fig. 3F–H, $p < 0.01$).

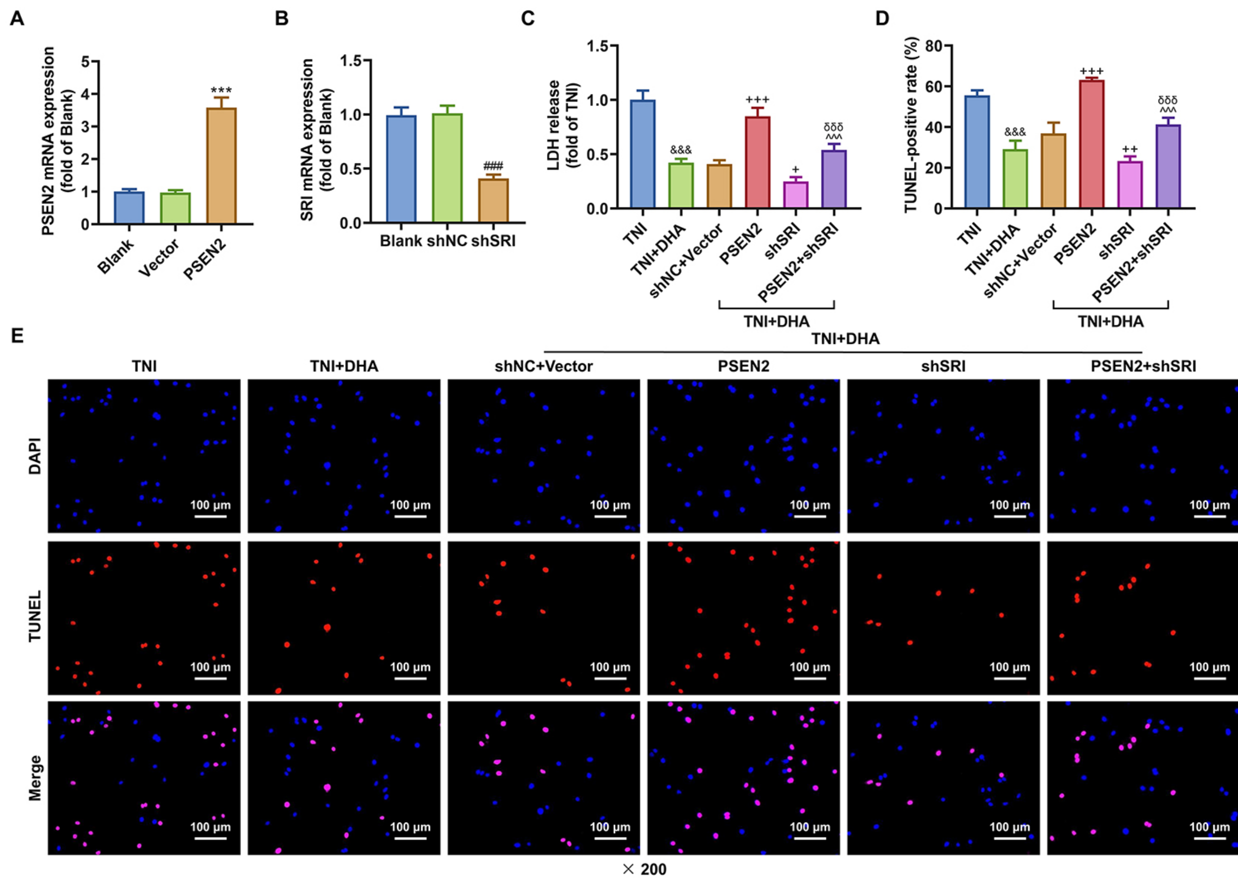

Fig. 4. DHA regulated cell toxicity and apoptosis by modulating the interaction between PSEN2 and SRI in neurons. (A,B) mRNA expression of *PSEN2* and *SRI* mRNA expressions in neurons measured by qRT-PCR assay, with GADPH as an internal reference. (C) LDH release, as a measure of cell toxicity, in neurons. (D,E) Apoptosis of neurons as determined using TUNEL assay. Scale bar: 100 µm; magnification: 200×. $^{***}p < 0.001$ vs. Vector group; $^{\#\#\#}p < 0.001$ vs. shNC group; $^{\&\&\&}p < 0.001$ vs. TNI group; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. shNC + Vector group; $^{\delta\delta\delta}p < 0.001$ vs. PSEN2 group; $^{\wedge\wedge\wedge}p < 0.001$ vs. shSRI group. n = 3 in each group. shNC, short hairpin RNA for negative control; shSRI, short hairpin RNA against sorcin; qRT-PCR, quantitative real-time polymerase chain reaction.

DHA Regulated Cell Toxicity, Cell Apoptosis and ERS-Related Proteins by Modulating the Interaction Between PSEN2 and SRI in Neurons

To investigate the roles of PSEN2 and SRI in DHA-exposed TNI model neurons, we overexpressed PSEN2 and silenced SRI in the cells and confirmed their expression by means of qRT-PCR (Fig. 4A,B, $p < 0.001$). A dose of 50 µM DHA, which was determined to yield the most obvious effect, was applied in further experiments.

Our research results confirmed that the DHA plays a role in the regulation of cell toxicity, apoptosis, and expression of ATF6, GRP78, CHOP and C-Cas-12 in TNI model neurons, which can be offset by PSEN2 overexpression, evidenced by the increased LDH release (Fig. 4C, $p < 0.001$), enhanced cell apoptosis (Fig. 4D,E, $p < 0.001$), and up-regulated ATF6, GRP78, CHOP and C-Cas-12 expression (Fig. 5, $p < 0.001$). Besides, the cell toxicity (Fig. 4C, $p < 0.05$), apoptosis (Fig. 4D,E, $p < 0.01$), and expression of ATF6, GRP78, CHOP and C-Cas-12 (Fig. 5, $p < 0.05$) in DHA-treated TNI model neurons were dramatically reduced by shSRI. These results offered evidence that PSEN2 and SRI may play vital roles in DHA-exposed TNI model neurons.

Discussion

TBI represents a therapeutically challenging brain disease in the world [25]. In particular, TNI is a type of TBI that can lead to neuron apoptosis [26]. DHA has been proven to possess neuroprotective effects on TBI [27]. On this basis, this study was devoted to investigating whether there exists a mechanism underlying the DHA-regulated modulation of neuron apoptosis. As expected, our results demonstrated that DHA reduced neuron apoptosis induced by ERS via the PSEN2/SRI signaling pathway.

Reportedly, mechanical force alone could cause neurite injury in cultured neurons, which is a contributor to chronic TBI [28]. Interestingly, DHA could dose-

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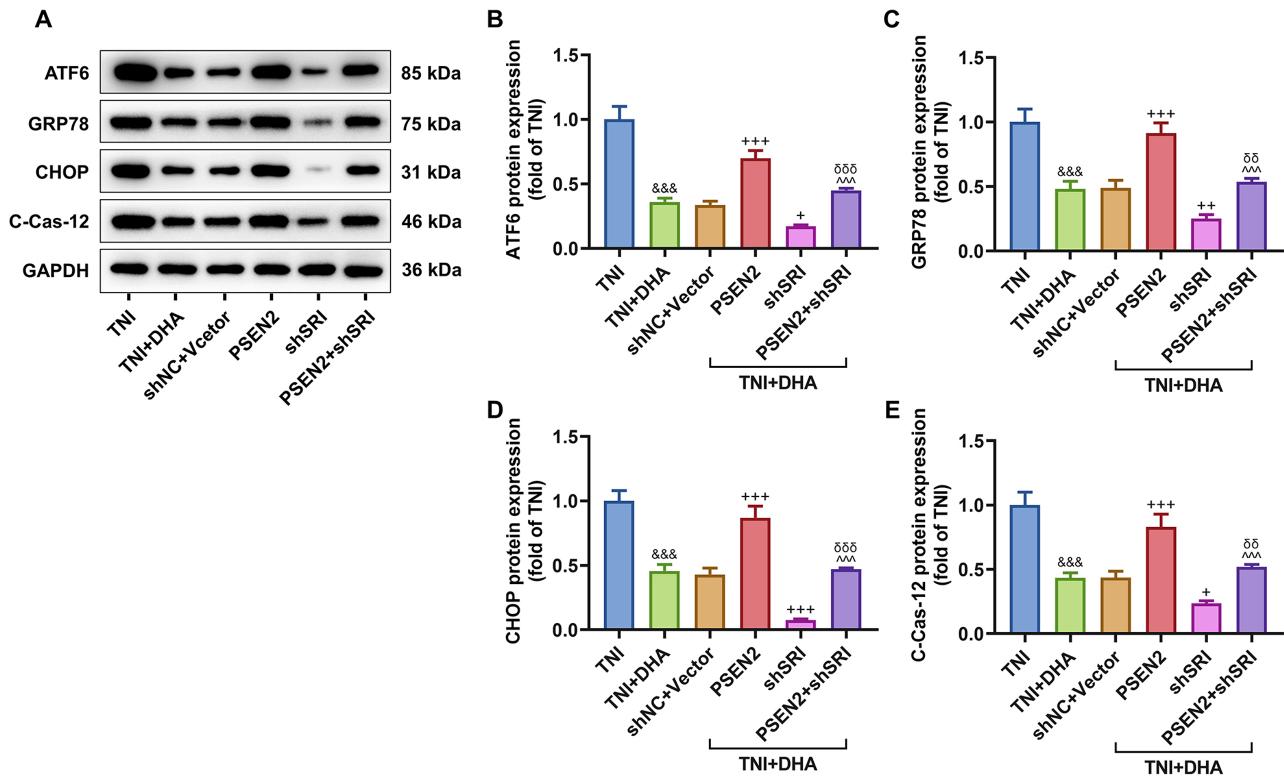

Fig. 5. DHA regulated ERS-related proteins by modulating the interaction between PSEN2 and SRI in neurons. (A) Western blot of ATF6, GRP78, CHOP and C-Cas-12, with GAPDH as the internal reference. Quantitation of protein expression of ATF6 (B), GRP78 (C), CHOP (D), and C-Cas-12 (E). n = 3 in each group. $^{\&\&\&}p < 0.001$ vs. TNI group; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. shNC + Vector group; $^{\delta\delta}p < 0.01$, $^{\delta\delta\delta}p < 0.001$ vs. PSEN2 group; $^{\wedge\wedge\wedge}p < 0.001$ vs. shSRI group.

dependently promote the density and size of neurites in cultured neurons [29], signifying that DHA may protect against neuronal injury, which is consistent with our research that DHA dose-dependently mitigated neuronal injury. Previous studies have shown enhanced LDH release and neuron apoptosis in the TNI model [30,31], a positive correlation of LDH release with cell toxicity [32], and the abundant release of LDH resulting in cell toxicity and then neuron apoptosis. Furthermore, ERS and ERS-related neuron apoptosis can be induced in mice with experimentally induced TBI [33]. The levels of ATF6, GRP78, CHOP and C-Cas-12—the ERS-related proteins—are upregulated after the activation of ERS [13]. More importantly, the expression of ATF6, GRP78, CHOP and Cas-12 has been shown to increase in TBI model mice [34]. These investigations indicated that ERS can lead to neuron apoptosis, accompanied by upregulated ERS-related proteins. It has been demonstrated that DHA represses LDH release and cell apoptosis induced by hypoxia/reoxygenation injury in human cerebrovascular pericytes [35]. DHA is an inhibitor of ERS and inactivates CHOP protein in chronic traumatic encephalopathy [36]. It has been documented that GRP78 is a molecular target for DHA [37]. These discoveries confirmed that DHA can prevent cell apoptosis, probably by inhibiting the activation of ERS-related proteins. Accordingly, DHA has been postulated for its role in regulating cell toxicity, apoptosis and ERS-related proteins in neurons. Our data revealed that DHA dose-dependently decreased cell toxicity, apoptosis and ERS-related proteins in TNI model neurons.

Previous studies have illustrated that the TNI model created with primary cultured cortical neurons is a typical and reliable model to explore the injury mechanism of TBI *in vitro* [22,38]. In addition, TBI model rats feature upregulated expression of SRI level. It has been suggested that TNI may affect SRI levels in neurons. Our results revealed that TNI model neurons experience an initial surge of SRI level and a subsequent reduction post-modeling. Our study also confirmed that DHA dose-dependently abrogated TNI-induced upregulation of SRI in neurons. Previous reports have demonstrated that the activation of SRI will lead to ERS, which then promotes neuron apoptosis [15,33]. Accordingly, in the present study, we investigated whether and how SRI impacts LDH release and ERS-related protein expression, and found that SRI silencing could markedly strengthen the effect of DHA on LDH release, neuron apoptosis and ERS-related protein expression in TNI model neurons.

A previous study showed that PSEN2 protein expression in mice can be suppressed via a DHA diet [39]. Being

one of the risky genes for brain diseases [40], PSEN2 expression was reported to spike in TBI model mice [41]. The overexpression of PSEN1 exon 9 deletion mutant has also been found to promote LDH release and apoptosis in neuronal cells [42]. PSEN1 is expressed in the ER of neurons, which regulates calcium ion signaling [43]. Both PSEN2 and PSEN1 are PSEN-related genes, and share similar functions [44]. Herein, PSEN2 overexpression attenuated the influence of DHA on LDH release and cell apoptosis in TNI model neurons. These findings corroborated the potential of DHA in abrogating TBI-induced elevation of PSEN2 expression in neurons. Besides, our study showed that PSEN2 overexpression reversed the effect of DHA on ERS-related proteins in TNI model neurons.

In addition, an interaction between PSEN2 and SRI was verified in the current study. Combined with the above-mentioned findings, our results confirmed that the protective effects of DHA on TNI model neurons can be realized by regulating the interaction between PSEN2 and SRI. In addition, the influences of PSEN2 overexpression and SRI silencing on the TNI model and DHA-treated neurons can be offset by the co-transfection of PSEN2 and shSRI. These results indicated that DHA regulates cell toxicity, apoptosis and ERS-related protein expression by modulating the interaction between PSEN2 and SRI in TNI model neurons. To refine these findings, animal experiments are warranted in further studies.

Conclusions

In summary, our study reveals the molecular mechanism underlying the protective effect of DHA on TNI-induced neuron apoptosis. Our results demonstrate that DHA reduces neuron apoptosis induced by SRI-activated ERS via targeting PSEN2, lending credence to the potential value of PSEN2 and SRI as key molecular targets for treating TBI.

Availability of Data and Materials

The analyzed data sets generated during the study are available from the corresponding author upon reasonable request.

Author Contributions

Substantial contributions to conception and design: BJM and HBY. Data acquisition, data analysis and interpretation: GQM, WLH and WZ. Drafting the article or critically revising it for important intellectual content: All authors. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental operations on rats were performed in accordance with the China Council on Animal Care and Use guidelines and were approved by the Ethics Committee of Nantong University for Experimental Animals Welfare (No. S20231220-099).

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Conflict of Interest

The authors declare no conflict of interest.

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