Dysfunctional ER-mitochondrion Crosstalk Leads to Subsequent ER Stress-induced Neuronal Apoptosis and Neurological Deficits in an Experimental Rodent Model of Severe Head Injury

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Research

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Abstract

Introduction: Previous studies have shown proper interorganelle communication between the endoplasmic reticulum (ER) and mitochondria (ER-mitochondrion crosstalk) is crucial for cellular homeostasis. Mitochondria-associated membranes (MAMs) provide an excellent platform and play an essential role in different signaling pathways to maintain cellular viability. However, the time course and potential pathological effects of this ER-mitochondrion physical and functional tethering in traumatic brain injury (TBI) remain unknown. We tested the hypothesis that dysfunctional ER-mitochondrion crosstalk at the acute phase of injury results in ER stress-induced neuronal apoptosis and neurological deficits in a mouse model of TBI.

Methods: Male C57BL/6 mice were subjected to severe TBI (sTBI) using a controlled cortical impact (CCI) device. Transmission electron microscopy, western blot, immunofluorescence staining, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL), and MitoSOX assays were used to analyze posttraumatic ER-mitochondrion physical contact, Ca\(^{2+}\) transfer from the ER to mitochondria, mitochondrial reactive oxygen species (ROS) production, the unfolded protein response (UPR), the neuroinflammatory response and ER stress-mediated apoptosis in the perilesional cortex. The brain water content (BWC) and Evans blue dye extravasation were used to assess the blood-brain barrier (BBB) integrity, and a modified neurological severity score (mNSS) to evaluate neurological function in mice.

Results: We showed sTBI led to significant MAM reorganization in the mouse cerebral cortex during the first 24 hr after injury. Enhanced ER-mitochondrion crosstalk peaked at 6 hr post injury and was significantly correlated with increased ER-mitochondrion Ca\(^{2+}\) transfer, mitochondrial ROS overproduction, elevated ER stress and UPR levels, and augmented levels of proinflammatory cytokines. In vivo experimental downregulation of PACS2, a protein essential for ER-mitochondrion tethering, restored mitochondrial Ca\(^{2+}\) homeostasis and alleviated mitochondrial oxidative stress by downregulating the IP\(_3\)R\(_1\)-GRP75-VDAC1 axis, inhibited ER stress and suppressed the inflammatory response through the PERK/eIF2\(\alpha\)/ATF4/CHOP signaling pathway, blocked Caspase 12-dependent ER stress-mediated apoptosis, and reduced BBB permeability, resulting in significantly improved neurological function in mice subjected to sTBI.

Conclusions: These results indicate that dysfunctional ER-mitochondrion crosstalk at the acute stage of injury might be primarily involved in the neuronal apoptosis and neurological deficits following sTBI, and specific modulation of ER-mitochondrion crosstalk might be a novel promising therapeutic strategy for sTBI.

Introduction

Traumatic brain injury (TBI) remains a leading cause of death and disability among children and young adults in China, with an estimated annual cost of $20 billion for medical expenses. Despite extensive
basic and clinical research on TBI during the last 150 years, promising preclinical data have not translated into successful clinical trials, and no effective pharmacological interventions are available to date[1]. TBI mainly consists of primary mechanical insult and multifactorial secondary injury that evolves over hours to days and ultimately leads to neuronal cell death[2]. This secondary injury cascade provides a window of opportunity for therapeutic intervention[3].

Although the molecular mechanism that underlies secondary brain injury is not completely defined, dysfunctional cellular interorganelle communication has emerged as a critical event in alterations following primary insult[4–6]. The endoplasmic reticulum (ER) and mitochondria play essential physiological roles in cells through the control of multiple signal transduction pathways[7]. The ER physically and biochemically interacts with mitochondria via mitochondria-associated ER membranes (MAMs), which serve as an excellent scaffold for crosstalk and allow rapid exchange of biological molecules to maintain cellular viability[8]. MAMs are conserved dynamic structures found across eukaryotic phyla and are characterized by a unique lipid profile and the expression of a specific group of proteins involved in Ca$^{2+}$ signaling, phospholipid biosynthesis, protein folding, membrane tethering, and stress signals transferring[9–11]. Accordingly, MAMs are crucial for not only the efficient transfer of Ca$^{2+}$ from the ER to mitochondria, proper mitochondrial bioenergetics, mitochondrial dynamics, lipid synthesis, and autophagosome assembly but also ER stress, an accumulation of unfolded proteins in the ER lumen, initiating a defensive the unfolded protein response (UPR) to safeguard cellular survival[12]. MAMs were recently shown to not only have numerous physiological and metabolic functions but also to contribute substantially to a variety of pathological conditions, including obesity, insulin resistance, aging, tumorigenesis, axon regeneration, Alzheimer's disease, Parkinson syndrome and other neurodegenerative disorders[13–17]. Although the pathological impacts of ER and mitochondrial dysfunction have primarily been observed and investigated independently in TBI[18–20], the time course and potential pathological effects of ER-mitochondrion physical and functional crosstalk on severe TBI (sTBI) remain poorly understood.

Endoplasmic reticulum (ER) is the largest cellular organelle, where all secreted and membrane proteins are synthesized and properly folded. The accumulation of unfolded and misfolded proteins causes ER stress and induces the unfolded protein response (UPR) to maintain cellular homeostasis[21]. ER stress activates the UPR signaling network and eventually promotes cell death in a wide range of pathologies including cerebral ischemia, traumatic brain injury, Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis [22].

Here, we report that sTBI drives an abnormal acute increase in MAM formation in the mouse cerebral cortex, with the most enhanced ER-mitochondrion crosstalk in neurons occurring at 6 hr post injury as an early event in the course of secondary brain injury development. Such increased ER-mitochondrion tethering correlates with increased Ca$^{2+}$ flux from the ER to mitochondria, increased mitochondrial reactive oxygen species (ROS) production, elevated ER stress levels, increased unfolded protein response (UPR) signaling, and an augmented neuroinflammatory response. Suppression of phosphofurin acidic cluster sorting protein 2 (PACS2), a distinct protein critical for ER-mitochondrion associations, improves
neuronal homeostasis and reduces subsequent ER stress-mediated apoptosis and blood-brain barrier (BBB) leakage, resulting in improved neurological function in mice subjected to sTBI. These results indicate that dysfunctional ER-mitochondrion crosstalk at the acute stage of injury might be primarily involved in the subsequent neuronal apoptosis and neurological deficits following TBI, and specific modulation of ER-mitochondrion crosstalk might be a novel promising therapeutic strategy for sTBI.

Materials And Methods

Animal care and experiments were conducted following ethical approvals provided by the Small Animal Protection Board of Tianjin Medical University.

Animals

Adult male C57BL/6 mice weighing 22 - 25 g (8 - 10 weeks old) at the time of surgery were purchased from the Experimental Animal Laboratories of the Academy of Military Medical Sciences (Beijing, China). All mice were housed individually in a temperature- (20 ± 2 °C) and humidity-controlled (55 ± 5%) vivarium and maintained on a standard 12-hr light/dark cycle (7:00 a.m. to 7:00 p.m) with access to food and water ad libitum. All efforts were made to minimize the number of mice used and their suffering. In all experiments, the data were obtained by investigators blinded to the experimental design.

Experimental design

In the present study, the following separate experiments were conducted:

In Experiment 1, we investigated the dynamic changes in neuronal ER-mitochondrion physical contacts after TBI. Thirty-six mice were randomly assigned to the following six groups: sham, TBI 1 hr, TBI 3 hr, TBI 6 hr, TBI 12 hr, and TBI 24 hr. We used transmission electron microscopy (TEM) to examine the time profile of ER-mitochondrion physical tethering, as described below.

In Experiment 2, we investigated altered MAM-resident protein expression and mitochondrial ROS production after TBI. One hundred eighty mice were randomly allocated to the following six groups: sham, TBI 1 hr, TBI 3 hr, TBI 6 hr, TBI 12 hr, and TBI 24 hr. The ER-mitochondrion tethering proteins phosphofurin acidic cluster sorting protein 2 (PACS2) and mitofusin-2 (MFN2) were analyzed by immunofluorescence staining and western blot. The expression levels of inositol 1,4,5-trisphosphate receptor type 1 (IP$_3$R$_1$), glucose-regulated protein 75 (GRP75), voltage-dependent anion channel 1 (VDAC1), and Sigma 1 receptor (Sigma-1R) were also measured by western blotting. The mitochondrial ROS content was detected by MitoSOX staining as described below.

In Experiment 3, we studied ER stress, UPR signaling, and neuroinflammatory response after TBI. Thirty-six mice were randomly allocated into the following six groups: sham, TBI 1 hr, TBI 3 hr, TBI 6 hr, TBI 12 hr and TBI 24 hr. The expression levels of GRP78, phosphorylated protein kinase (PKR)-like ER kinase (p-
PERK), PERK, p-eIF2α, eIF2α, activating transcription factor 4 (ATF4), cleaved interleukin-1β (IL-1β), and tumor necrosis factor alpha (TNFα) were determined by western blot.

In Experiment 4, we examined cleaved Caspase 12-dependent ER stress-mediated apoptosis after TBI. Thirty mice were randomly assigned to the following five groups: sham, TBI 6 hr, TBI 12 hr, TBI 24 hr, and TBI 72 hr. Immunoblot assays were used to detect the expression of cleaved Caspase 12, cleaved Caspase 3, cleaved Poly(ADP-Ribose) Polymerase 1 (PARP1), C/EBP homologous protein (CHOP), B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X (BAX), and cytochrome C (CytC).

In Experiment 5, we studied the effects of Pacs2 silencing on MAM-resident protein expression, ROS production, ER stress and UPR activation, and the neuroinflammatory response at 6 hr after TBI. Forty-eight mice were randomly assigned to the following four groups: sham, TBI, TBI + PACS2 siRNA, and TBI + NC siRNA. The protein expression of PACS2, Mfn2, IP3R1, GRP75, VDAC1, Sigma-1R, GRP78, p-PERK, PERK, p-eIF2α, eIF2α, ATF4, cleaved IL-1β, and TNFα was assayed by immunoblot, and mitochondrial ROS production was detected by MitoSOX staining.

In Experiment 6, we investigated the potential effects of PACS2 knockdown on BBB functions, ER stress-mediated apoptosis, and neurological outcomes. One hundred twenty mice were randomly allocated into the following four groups: sham, TBI, TBI + PACS2 siRNA, and TBI + NC siRNA. Neurological scores were used to evaluate the neurological functions of the mice at preinjury and at postinjury days 1, 3, 5, 7, and 14. At 72 hr post injury, extravasation of Evans blue (EB) and the brain water content (BWC) were measured in all groups. cleaved Caspase 12, cleaved Caspase 3, cleaved PARP1, CHOP, Bcl-2, BAX, and CytC expression levels were also determined by western blot. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay and immunofluorescence staining were performed in each group at 72 hr after injury.

All mice in the sham group were subjected to the same surgical procedure but were not treated with the controlled cortical impact (CCI) device.

**Severe traumatic brain injury model**

CCI-induced brain injury is an extensively characterized and broadly used preclinical model of head injury[21, 23, 24]. In brief, a digital electromagnetic CCI device (eCCI Model 6.3; Custom Design, Richmond, VA, USA) was used to establish the sTBI model in male C57BL/6 mice. The mice were allowed to adapt to their new environment for 1 week before surgery and then anesthetized with 10% chloral hydrate. The depth of anesthesia was assessed by monitoring the pedal withdrawal reflex and respiratory rate. Then, the mice were placed in a stereotaxic apparatus, and the surgical site was clipped and cleaned with Nolvasan scrubs. A 4.0-mm hole was drilled into the right parietal bone to expose the dura. The CCI device subsequently impacted the skull at a depth of 2.5 mm and a velocity of 5 m/s over a period of 200 ms. The incision was closed immediately following injury, and the mice were then placed in heated cages to allow recovery from anesthesia at room temperature (RT). Fig. 1 shows the hematoxylin and eosin
(H&E) staining of the cerebral cortex at 24 hr after CCI, which confirmed the severe injury of the mice used in this study.

**Transmission electron microscopy and MAM quantification**

Tissue (1 mm × 1 mm) samples were obtained from the perilesional cortex of TBI and sham mice and fixed in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M PBS (pH 7.4) overnight at 4°C for 1 hr. The tissues were then washed in 0.1 M phosphate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in the same buffer for 30 min at RT. The specimens were dehydrated through a graded series of ethanol and embedded in a mixture of Epon resin. Serial sections were cut on an ultratome and double stained with uranyl acetate and lead citrate. Finally, the ultrathin sections were examined by TEM at 80 kV.

ER-mitochondrion imaging and quantification were performed according to a previous study[25]. Briefly, 60 images of mice in each experimental group (6 mice from each group) were obtained at ×6,800 magnification, and ImageJ (National Institutes of Health, Bethesda, Maryland, USA) was used to analyze ER-mitochondrion contacts and mitochondrial morphology. We delineated the mitochondria and ER membranes using the free-hand tool. Two independent investigators blinded to the experimental design calculated the ratio of ER adjacent to mitochondria to mitochondrial perimeter, and the total number and area of mitochondria.

**Immunofluorescence and image analysis**

At designated time points, mice were sacrificed with an overdose of 10% chloral hydrate and then immediately perfused through the heart with PBS followed by 4% paraformaldehyde. The brain was rapidly dissected and embedded in OCT medium (Sakura, Oakland, CA, USA). Coronal sections of 8-μm thickness were cut on a cryostat at -20°C and imprinted on poly-L-lysine-coated slides. The sections were stained for NeuN (neuronal marker), glial fibrillary acidic protein (GFAP, astrocyte marker), and PACS2 and Mfn2 (markers of ER-mitochondrion contacts).

In brief, the sections were fixed with 2% paraformaldehyde lysine periodate (PLP), rinsed three times with PBS (pH 7.4), and blocked with 1% normal donkey serum in PBS containing 0.1% Triton X-100 PBST at RT for 1 hr. The sections were then incubated with a rabbit anti-PACS-2 antibody diluted at 1:1000 (Abcam, Cambridge, MA, USA), a rabbit anti-Mfn2 antibody diluted at 1:1000 (Abcam), a mouse anti-NeuN antibody diluted 1:100 (Cell Signaling Technology, Danvers, MA, USA), and a mouse anti-GFAP antibody diluted at 1:1000 (Abcam, Cambridge, MA, USA) in PBST containing 1% normal donkey serum at 4°C overnight followed by extensive washing with PBS. Finally, the sections were incubated with Alexa Fluor-conjugated anti-mouse or anti-rabbit IgG (1:1000, Invitrogen, Grand Island, NY, USA) for 3 hr at RT. The nuclei were counterstained with Hoechst for 5 min.

Images of each section were captured using a fluorescence microscope (Olympus IX81, Tokyo, Japan), and the data were analyzed from 15 randomly selected microscopic fields (five fields per section x three
sections per mouse) with ImageJ (National Institutes of Health, Bethesda, Maryland, USA).

**Western blotting**

Mice were sacrificed by transcardiac perfusion with cold PBS to eliminate the proteins expressed by blood cells at designated time points. Their brain tissues were homogenized in ice-cold RIPA buffer (Beyotime) containing phenylmethylsulfonyl fluoride (PMSF, 1 mM final) for 30 min and then centrifuged for 10 min (12,000 rpm, 4°C). After centrifugation, the supernatants were collected and boiled with 4x sample buffer at 95°C for 10 min. The total protein content was determined by the BCA protein assay kit (Thermo). Proteins (8 μg per lane) and prestained molecular weight markers (Thermo) were separated by SDS/PAGE and transferred to PVDF membranes (Roche, Canada), which were then blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 2 hr at RT. After blocking, the blots were incubated overnight at 4°C with primary antibodies (Table 1), rinsed with TBS, incubated with the appropriate HRP-conjugated secondary IgG for 1 hr at RT and then developed with the ECL system (Millipore, Billerica, MA, USA). Protein expression was quantified by ImageJ (National Institutes of Health, Bethesda, Maryland, USA) according to the mean pixel density of each protein band, and β-actin was employed as a loading control.

**Mitochondrial reactive oxygen species content**

MitoSOX-based assays were used to detect mitochondrial ROS production according to the manufacturer’s instructions (Thermo Fisher, Waltham, USA) and previous studies [26-28]. Perilesional cortex tissues were dispersed into a single-cell suspension and washed with 10 mM PBS twice. A 5 mM MitoSOX™ reagent stock solution in HBSS/Ca/Mg buffer was diluted to a 5 μM MitoSOX reagent working solution, in which the cells were incubated for 10 min at 37°C. Fluorescence signals were read and independently and blindly quantified by ImageJ. All experimental procedures were performed in the dark.

**siRNA transfection**

siRNA transfection was performed in vivo according to the method of Zhao L. et al. [29]. To knockdown Pacs2, a total of 1.32 μg/5 μL PACS-2 siRNA (Sangon Biotech, Shanghai, China) or NC siRNA (Sangon Biotech, Shanghai, China) was diluted with an equal volume of Entranster™-in vivo transfection reagent (Engreen, Beijing, China). The solution was mixed gently and injected intracerebroventricularly (i.c.v) into mice 48 hr prior to TBI. In terms of the i.c.v injection[30], a 1-mm cranial burr hole was drilled into the skull, and a 30-gauge needle on a Hamilton syringe was implanted into the lateral ventricle using the following stereotactic coordinates: 1.5 mm posterior to bregma, 1.0 mm right lateral to the midline, 2 mm in depth, with an injection speed of 1μL/min (total volume=5μL). The transfection rate is approximately 85 ± 5%.

**Modified neurological severity scores**
The modified neurological severity score (mNSS) was used to evaluate neurological function, as described previously[31]. Neurological assessments were performed at baseline before the injury and at post injury days 1, 3, 5, 7, and 14 using the mNSS. The assessments included motor, sensory, reflex, and balance tests. These scores were used 1) to ensure the relative uniformity in injury severity and 2) to compare neurological impairments among mice receiving different treatments. The tests were performed by two independent observers who were blinded to the experimental conditions and treatments.

**Brain water content**

Hemispheric cerebral edema was determined by measuring the BWC, as previously described[31]. Mice were sacrificed with an overdose of chloral hydrate at 72 hr post injury. Their brains were promptly removed, and the hemispheres were immediately weighed (wet weight) and then placed in an incubator at 100°C for 24 hr. The samples were weighed again to determine the dry weight, and the BWC was calculated as follows: (wet weight - dry weight)/wet weight x 100%.

**Evans blue dye extravasation**

The BBB permeability of the cerebral hemispheres was assessed by measuring the extravasation of EB dye 72 hr after TBI. EB dye injected intravenously binds instantaneously to albumin and other plasma proteins and serves as a marker for plasma exudation. In brief, EB (2% in PBS, Sigma) was injected slowly through the jugular vein (4 ml/kg) and allowed to circulate for 1.5 hr. Then, mice were sacrificed and transcardially perfused with PBS followed by 0.9% saline. The hemispheres were removed, frozen in -55°C isopentane and freeze-dried. The freeze-dried specimens were homogenized in formamide (1:20) and incubated at 60°C overnight, and the homogenates were then centrifuged at 14000 rpm for 30 min to collect the supernatant. The EB content in the supernatant was determined spectrophotometrically at OD 620 nm (Thermo Scientific). The tissue EB concentration was quantified using a standard linear curve and expressed as micrograms per gram of brain tissue.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling assay**

The TUNEL assay is a well-defined method for detecting apoptotic DNA fragmentation in a cell[32]. We used the In Situ Cell Death Detection Kit, POD (Roche, Germany) to detect apoptosis in the perilesional cortex of the mouse brain at 72 hr after TBI. According to the manufacturer's instructions, the sections were fixed in acetone for 8 min at 4°C. After being rinsed with PBS, the brain sections were treated with 3% BSA for 30 min at 37°C and then incubated with the TUNEL reaction mixture in the dark for 90 min at 37°C. The nuclei were counterstained with Hoechst for 5 min. Images of each section were captured using a fluorescence microscope (Olympus IX81, Tokyo, Japan), and the data were analyzed from 15 randomly selected microscopic fields (five fields per section x three sections per mouse) with ImageJ (National Institutes of Health, Bethesda, Maryland, USA).

**Data analysis**
The data are presented as the mean ± standard deviation (SD) and were analyzed using Prism 8.3.1 (GraphPad Software, San Diego, CA). Parameters were compared by ANOVA followed by Tukey's multiple comparisons test. Pearson's correlation coefficients (r) were calculated to assess the strength of relationships. P values < 0.05 were considered statistically significant.

Results

Dynamic changes in neuronal ER-mitochondrion contacts in the mouse cerebral cortex after TBI

The ER and mitochondria interact to form dynamic contact sites, which are responsible for the integration of several cellular functions, including Ca\(^{2+}\) homeostasis, oxidative stress, ER stress, neuroinflammation, and survival[15, 33]. For this reason, we speculated that ER-mitochondrion contacts induce characteristic changes in cerebral cortex neurons to orient different signaling pathways for effective crosstalk and cellular viability after sTBI.

To investigate ER-mitochondrion physical interactions and mitochondrial morphology, we first used TEM to examine neurons in the mouse perilesional cortex at preinjury and at 1, 3, 6, 12, and 24 hr post injury. The neuronal cell bodies were distinguished from glial cell bodies using previously described morphologic criteria[20, 34]. We observed that prior to injury, mitochondria exhibited a moderately dense matrix and a normal cristae architecture (Fig. 2a, g, m). Significant structural changes and a reduced number of neuronal mitochondria were observed immediately after injury, as expected (Fig. 2b-f, n-r). Some mitochondria exhibited disorganized morphologies with low to high extents of swelling and an electron-lucent matrix. At 6 hr post injury, neuronal mitochondria showed severe swelling and inner membrane-associated dense granular inclusions, which is a distinguishing mitochondrial feature of calcium overload and irreversible injury, along with a markedly high degree of apposition to the ER (Fig. 2d, j, p). Detailed quantitative analysis of cerebral sections from each time point demonstrated that the proportion of the ER in close contact with mitochondria to the mitochondrial perimeter began to increase at 1 hr, peaked at 6 hr, and returned to slightly above baseline at 24 hr (Fig. 2B). The neuronal mitochondrial number was significantly reduced after injury (Fig. 2C), but the mitochondrial area was significantly elevated at 6 hr (Fig. 2D), indicating mitochondrial morphological changes and swelling.

Together, these results suggest a high amount of neuronal MAM formation at the acute phase of sTBI than in the phase prior to injury. This ER-mitochondrion interface might recruit and orient various signaling proteins needed to regulate productive crosstalk and cellular homeostasis.

Altered expression of MAM-resident proteins and mitochondrial oxidative stress in the mouse cerebral cortex after TBI

Given that more ER-mitochondrion physical contacts were observed at the acute phase of TBI than in the phase prior to injury and that the MAM subdomain is enriched for specific proteins, such as the ER-mitochondrion tether proteins PACS2 and Mfn2, we next examined the expression of these proteins in the mouse perilesional cortex before injury and at 1, 3, 6, 12, and 24 hr after injury. The protein levels of
PACS2 and Mfn2 increased rapidly after injury, peaking at 6 hr, declining gradually, and returning to the baseline level by 24 hr (Fig. 3B, C). These findings were further substantiated by double immunofluorescence labeling of PACS2 with NeuN (a neuronal marker) and GFAP (an astrocyte marker) in the ipsilateral cerebral cortex, and the ER was more frequently attached to the neuronal mitochondria at 6 hr following TBI than at the other time points (Fig. 3A).

It is well known that within the ER-mitochondrion tethering complex, IP$_3$R$_1$ located on the ER membrane is physically and functionally linked to the VDAC located on the mitochondrial outer membrane via chaperone GRP75. Sigma-1R is a Ca$^{2+}$-sensitive and ligand-operated receptor chaperone that regulates ER-mitochondrion interorganelle Ca$^{2+}$ signaling. Because any disruption of mitochondrial Ca$^{2+}$ homeostasis can contribute to increased mitochondrial ROS production and oxidative stress, we explored the possible functional consequence of the increased ER-mitochondrion connectivity in sTBI. The levels of IP$_3$R$_1$, GRP75, VDAC1, and Sigma-1R were markedly elevated and the highest amounts of mitochondrial ROS production were observed at 6 hr after injury, when MAM formation peaked simultaneously (Fig. 4A-C). Altogether, these results not only further support our morphological and microscopic observations of a significant number of ER-mitochondrion contacts in the acute stage of sTBI but also suggest that numerous anchoring proteins and functional components involved in Ca$^{2+}$ transport are expressed at these sites, and the crosstalk between ER and mitochondria is associated with mitochondrial oxidative stress.

**Alteration of ER stress, UPR signaling and neuroinflammatory responses in the mouse cerebral cortex after TBI**

MAMs are crucial for not only the efficient transfer of Ca$^{2+}$ from the ER to mitochondria, proper mitochondrial bioenergetics, mitochondrial dynamics, lipid synthesis, and autophagosome assembly but also ER stress, the UPR, and neuroinflammatory responses[12, 35–37]. MAMs have been considered a critical hub to transfer stress signals from the ER to mitochondria, most notably under the condition of loss of ER proteostasis, by engaging the UPR[38]. Given that RNA-dependent PERK, a key ER stress sensor, is located in the ER membrane within the ER-mitochondrion tethering complex[39, 40], we assumed that altered crosstalk between neuronal ER and mitochondria affects ER stress, the PERK branch of the UPR and neuroinflammation. We analyzed the protein expression profiles of ER stress-related molecules and proinflammatory cytokines in the pericontusional cortex at different time points up to 24 hr after TBI. We showed that the expression of the 78 kDa glucose-regulated protein (GRP78), also referred to as BiP, a classical marker of ER stress, was significantly elevated at 3 hr and peaked at 6 hr following TBI (Fig. 5D, $P < 0.05$), while the levels of p-PERK/PERK, p-eIF2$\alpha$/eIF2$\alpha$, and ATF4 exhibited comparable dynamic changes with peak expression at 6 hr (Fig. 5A-C, $P < 0.05$). The production of the procytokines cleaved IL-1$\beta$ and TNF$\alpha$ also peaked at 6 hr (Fig. 5E-F, $P < 0.05$). Furthermore, Pearson's correlation analysis revealed that PACS2 expression was significantly positively correlated with the levels of Mfn2, IP$_3$R$_1$, VDAC1, Sigma-1R, GRP75, ROS, GRP78, p-PERK/PERK, p-eIF2$\alpha$/eIF2$\alpha$, ATF4, cleaved IL-1$\beta$, and TNF$\alpha$ within 24 hr of injury (Fig. 6, $P < 0.05$).
These data, together with the altered ER-mitochondrion connection, suggest that the enhanced ER and mitochondria coupling in the acute stage of TBI is associated with increases in the Ca\(^{2+}\) flux from the ER to mitochondria, ROS production, ER stress and UPR signaling as well as with an augmented neuroinflammatory response.

**Caspase 12-dependent ER stress-mediated apoptosis following increased ER-mitochondrion coupling in the mouse cerebral cortex**

ER stress-induced apoptosis involves triggering the PERK-eIF2\(\alpha\)-ATF4 branch of the UPR, upregulating CHOP expression, and activating Caspase 12, a regulator specific to ER stress-induced apoptosis[21, 41, 42]. To explore the possible pathological consequence of increased ER-mitochondrion crosstalk on subsequent ER stress-mediated apoptosis, we assessed the protein expression of cleaved Caspase-12, cleaved Caspase-3, cleaved PARP1, CHOP, Bcl-2, Bax and Cytc at different time points from 6 to 72 hr post injury. As expected, the levels of cleaved Caspase-12, cleaved Caspase-3, cleaved PARP1, CHOP, and Cytc began to rise immediately after the enhancement of ER-mitochondrion crosstalk at 6 hr, reached significance at 12 hr, and peaked at 72 hr after injury, when the Bcl-2/Bax ratio decreased significantly to its lowest level (Fig. 7). Taken together, our data demonstrated that enhanced ER-mitochondrion communication preceded ER stress-induced apoptosis, suggesting that ER-mitochondrion crosstalk may play an essential role in the secondary injury cascade after TBI.

**Silencing Pacs2 alleviated ER-mitochondrion Ca\(^{2+}\) transfer and mitochondrial oxidative stress, reduced ER stress and UPR activation, and suppressed neuroinflammatory responses**

If upregulated MAM functioning in neurons is related to ER-mitochondrion Ca\(^{2+}\) transfer and mitochondrial oxidative stress, ER stress and UPR activation, and neuroinflammatory responses in the acute stage of stTBI, then decreased contacts between the two organelles should have the opposite effects. For this reason, to explore the mechanistic relationship among ER-mitochondrion crosstalk, cellular homeostasis and neuroinflammation, we suppressed the ER-mitochondrion physical connection by silencing cerebral PACS2 expression. PACS2 siRNA treatment significantly reduced the expression levels of ER-mitochondrion tethers PACS2 and Mfn2 by approximately 50% at 6 hr post injury (Fig. 8A-B). Silencing Pacs2 resulted in a modest but significant reduction in IP\(_3\)R\(_1\), GRP75, VDAC1, Sigma-1R expression along with a significant reduction in ROS production at 6 hr after injury (Fig. 8C-E). In addition, the expression levels of GRP78, p-PERK/PERK, p-eIF2\(\alpha\)/eIF2\(\alpha\), ATF4, cleaved IL-1\(\beta\), and TNF\(\alpha\), which were significantly elevated in TBI mice at 6 hr post injury, were also significantly reduced in mice treated with PACS2 siRNA (Fig. 8F-K). These results demonstrate that reducing ER-mitochondrion crosstalk at the acute stage of TBI abolishes ER-mitochondrion Ca\(^{2+}\) transfer and mitochondrial oxidative stress, ER stress and UPR activation, and neuroinflammatory responses.

**Knocking down Pacs2 reduced TBI-associated BBB permeability, reversed ER stress-mediated apoptosis, and improved neurological function**
In an attempt to further mechanistically link ER-mitochondrion coupling to secondary brain injury and neurological deficits after TBI, we attempted to mitigate BBB leakage, alleviate ER stress-mediated apoptosis, and improve neurological function by diminishing ER-mitochondrion crosstalk. Knocking down PACS2 led to significant reductions in EB extravasation and the BWC at 72 hr post injury compared to the injury control group (Fig. 9A-C). The neurological scores of mice preconditioned with PACS2 siRNA were significantly lower than those of injury control mice during a 14-day follow-up period (Fig. 9C). PACS2 siRNA successfully reduced the protein expression of cleaved Caspase 12, CHOP and Cytc and increased the Bcl-2/Bax ratio (Fig. 9G-J). More importantly, these phenomena were accompanied by a significant reduction in the percentage of TUNEL-positive neuronal cells in the perilesional cortex at postinjury day 3 compared to the injury control (Fig. 9E-F). These results demonstrated that reducing ER-mitochondrion crosstalk at the acute stage of TBI improved cerebrovascular function, increased cellular viability, and ameliorated neurological deficits. In total, this loss-of-function approach supports the concept that targeting the structural components of MAMs may be an effective strategy to improve neurological outcomes in the context of sTBI.

Discussion

In the present study, we investigated changes in acute ER-mitochondrion crosstalk in the cerebral cortex and mechanistically linked such changes to subsequent ER stress-mediated neuronal apoptosis and neurological deficits after sTBI. Indeed, we demonstrated the following: (1) sTBI drove an abnormal acute increase in MAM formation in the cerebral cortex, with the most enhanced ER-mitochondrion tethering occurring in neurons at 6 hr post injury as an early event in the pathological course of secondary brain injury development. (2) Such increased ER-mitochondrion coupling was significantly positively correlated with increases in the elevated Ca\(^{2+}\) flux from the ER to mitochondria, mitochondrial oxidative stress and ROS production, ER stress and UPR signaling as well as with an augmented neuroinflammatory response. (3) This strengthened ER-mitochondrion crosstalk at the acute phase of injury was followed by BBB leakage and Caspase 12-dependent ER stress-mediated neuronal apoptosis. (4) More importantly, diminishing the early ER-mitochondrion connection led to reduced Ca\(^{2+}\) accumulation, oxidative stress and ROS production in mitochondria and to alleviated ER stress, UPR signaling and neuroinflammation, in turn leading to restoration of the impaired BBB permeability, Caspase 12-dependent ER stress-induced neuronal apoptosis, and neurological function. These results indicate that dysfunction in acute ER-mitochondrion crosstalk might be primarily involved in the subsequent neuronal apoptosis and neurological deficits following sTBI, and specific modulation of acute ER-mitochondrion crosstalk might be a novel promising therapeutic strategy for patients with TBI.

Increasing evidence suggests that the dysfunctional ER-mitochondrion crosstalk occurs in a variety of neurological diseases. Mutations in presenilins upregulate MAM function and increase ER-mitochondrion communication in patients with both the familial and sporadic forms of Alzheimer's disease (AD), indicating that AD is fundamentally a disorder of ER-mitochondrion communication\[37, 43\]. Furthermore, dysfunctional MAM signaling impairs neuronal calcium homeostasis, mitochondrial dynamics, ER
function, and autophagy, eventually leading to axonal degeneration in amyotrophic lateral sclerosis (ALS) and hereditary motor and sensory neuropathy (HMSN) [7]. The loss and impairment of Sigma-1R, a MAM protein, leads to axonal and motor neuron degeneration by affecting calcium homeostasis, ER stress, mitochondrial dynamics and transport [44], and mutation of Mfn2 alters the interplay between ER and mitochondria, contributing to the development of Charcot-Marie-Tooth type 2A (CMT2A), a dominant axonal form of peripheral neuropathy [45]. Research and clinical interest have been increasingly focused on understanding the critical role of ER-mitochondrion crosstalk in the pathological course of sTBI [46–48]. To the best of our knowledge, no data on the spectrum of ER-mitochondrion interactions in TBI are available to date. Because MAM dysfunction might be the common denominator underlying disease development, we propose that increased MAM activity and ER-mitochondrion communication lie at the heart of TBI pathogenesis. In support of this view, we note that both the physical connection and the functional crosstalk between these two organelles increase rapidly after injury, peaking at 6 hr post injury. The early onset of enhanced MAM functioning and ER-mitochondrion communication are the initial events in the pathological course of sTBI, leading to disruption of mitochondrial homeostasis and subsequent neuronal apoptosis and neurological deficits.

Mitochondria are in close proximity to the ER and form an elaborate platform to ensure precise modulation of pathophysiological Ca\(^{2+}\) signal transfer from the ER to mitochondria [7, 49]. It has been shown that ER-resident IP\(_3\)Rs physically associate with the cytosolic fractions of the mitochondrial chaperone GRP75 and the VDAC1 of the outer mitochondrial membrane (OMM) to form a Ca\(^{2+}\)-transfer complex. This multiprotein structure is crucial for effective IP\(_3\)-dependent ER-mitochondrion Ca\(^{2+}\) coupling [50]. Three IP3R isoforms were shown to be explicitly engaged in Ca\(^{2+}\) shuttling from the ER to mitochondria, and IP\(_3\)R\(_1\) was predominantly enriched at MAMs and more efficient at sustaining Ca\(^{2+}\) delivery to mitochondria from store-operated Ca\(^{2+}\) entry (SOCE), a central mechanism in cellular calcium signaling and in maintaining cellular calcium balance [51, 52]. Sigma-1R is a chaperone protein residing at MAMs, where it interacts with several partners, such as IP\(_3\)Rs, regulating the Ca\(^{2+}\) exchange between the ER and mitochondria. Consistent with this, we showed that acute MAM formation at 6 hr post injury led to increased activity of the IP\(_3\)R\(_1\)-GRP75-VDAC1 complex and the Sigma-1R chaperone as well as to excessive mitochondrial ROS production in the cerebral cortex. Inhibition of acute MAM formation restored mitochondrial calcium homeostasis and oxidative stress.

It has been recognized that ROS overproduction causes oxidative damage to essential cellular components, including neurons, astrocytes, and vascular elements, and is the primary cause of secondary brain injury after TBI [19, 53]. Mitochondria not only initiate the generation of such ROS but also enhance cellular and mitochondrial dysfunction by becoming targets of their damaging products through a vicious cycle of oxidative toxicity [54]. Excessive ROS is believed to be a major stimulus that triggers ROS-dependent ER stress, which occurs when the capacity of the ER to fold proteins becomes saturated under oxidative stress [11, 55–57]. ER stress activates a signaling network called the UPR to alleviate this stress and restore ER homeostasis, promoting cell survival and adaptation [58]. Here, we indeed found that TBI induced ROS production followed by UPR signaling. However, suppression of acute
MAM formation alleviated ROS-dependent ER stress and reduced BBB leakage, which is also supported by our previous study on subarachnoid hemorrhage (SAH) that found that ER stress inhibitors attenuated acute brain injury by rescuing cerebrovascular dysfunction[41]. Recent reports note that inhibiting ER stress restores the dysfunction of retinal endothelial cells by decreasing NO production and downregulating the expression of ICAM-1, NOS, NF-κB, and VEGF[59] and improves endothelium-dependent vasorelaxation through restoration of endothelial ER calcium homeostasis and Sirt1 activation [60] [61]. Together, these results suggest that acute MAM formation is the primary pathological cascade element of secondary brain injury following TBI.

The UPR is mediated through three ER-transmembrane effector proteins, PERK, inositol-requiring enzyme 1 (IRE1), and ATF4. PERK is uniquely enriched at MAMs [42] and has been considered not only a central regulator of ER stress but also a major transcription factor pathway to convey apoptosis after ROS-based ER stress[39]. Under ER stress, PERK dissociates from GRP78 and undergoes oligomerization and autophosphorylation, which leads to phosphorylation of the eukaryotic initiation factor eIF2α. The downstream phosphorylation of eIF2 leads to increased expression of ATF4 and translocation into the nucleus, where it binds to the unfolded protein response element (UPRE), resulting in transcriptional modification of CHOP, a proapoptotic gene transcription factor that initiates inflammation as well as programmed cell death [8, 40, 62, 63]. Caspase 12 is one of 14 cysteine protease family members that is localized to the ER and activated by ER stress. Caspase 12 has been demonstrated to mediate ER-specific apoptosis and neuroinflammation[64–67] and is a regulator specific to ER stress-induced apoptosis but is not involved in non-ER stress-induced cell death[67]. Significant upregulation of caspase-12 expression was observed after experimental SAH or cerebral ischemia[41, 68, 69]. In the present study, we certainly demonstrated that TBI-induced MAM formation led to the activation of the PERK/eIF2α/ATF4/CHOP signaling pathway, procytokine release, and Caspase 12-dependent ER stress-mediated neuronal apoptosis. Interfering with the crosstalk between ER and mitochondria at the early stage of secondary brain injury successfully suppressed this PERK-dependent branch of the UPR to reduce Caspase 12 expression, increase the Bcl-1/Bax ratio, promote neuronal survival and improve neurological function. Together with our previous study on SAH [41], this observation validates a causal role of Caspase 12-dependent ER stress in the induction of neuronal apoptosis following TBI.

**Conclusions**

In conclusion, dysfunctional ER-mitochondrion crosstalk at the acute stage of injury might be primarily involved in the neuronal apoptosis and neurological deficits following sTBI, and specific modulation of ER-mitochondrion crosstalk might be a novel promising therapeutic strategy for patients with TBI.

**Abbreviations**

ER: endoplasmic reticulum; MAMs: mitochondria-associated membranes; TBI: traumatic brain injury; CCI: controlled cortical impact; TUNEL: terminal deoxynucleotidyl transferase ‐mediated dUTP nick end labeling; UPR: unfolded protein response; BWC: brain water content; BBB: blood-brain barrier; mNSS:
modified neurological severity score; ROS: reactive oxygen species; PACS2: phosphofurin acidic cluster sorting protein 2; TEM: transmission electron microscopy; MFN2: mitofusin-2; IP₃R₁: inositol 1,4,5-trisphosphate receptor type 1; GRP75: glucose-regulated protein 75; VDAC1: voltage-dependent anion channel 1; Sigma-1R: Sigma 1 receptor; VDAC1: voltage-dependent anion channel 1; PERK: phosphorylated protein kinase-like ER kinase; eIF2α: eukaryotic initiation factor 2; ATF4: activating transcription factor 4; IL-1β: interleukin-1β; TNFα: tumor necrosis factor alpha; CHOP: C/EBP homologous protein; Bcl-2: B-cell lymphoma 2; BAX: Bcl-2-associated X; Cytc: cytochrome C; GRP78: glucose-regulated protein; UPRE: unfolded protein response element;

**Declarations**

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**Author’s contributions**

X.C. conceived and designed the study. L.M., G.G., X.G., M.S., Y.C., and developed the methodology and performed the experiments. X.C., W.Y. and J.Z. interpreted the results. G.G., X.G., and M.S. performed the data analysis and prepared the figures. X.C., L.M., G.G., and X.G. wrote the manuscript. X.C., W.Y., and J.Z. reviewed and revised the manuscript and supervised the study. All authors read and approved the manuscript.

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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.

**Ethics approval and consent to participate**

Animal care and all experiments were conducted following ethical approvals provided by the Small Animal Protection Board of Tianjin Medical University.

**Consent for publication**
Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Menon DK, Maas Al. Traumatic brain injury in 2014: Progress, failures and new approaches for TBI research. Nature reviews Neurology. 2015;11(2):71–2.

2. Algattas H, Huang JH. Traumatic Brain Injury pathophysiology and treatments: early, intermediate, and late phases post-injury. Int J Mol Sci. 2013;15(1):309–41.

3. Maas AIR, Menon DK, Adelson PD, Andelic N, Bell MJ, Belli A, Bragge P, Brazinova A, Buki A, Chesnut RM, et al. Traumatic brain injury: integrated approaches to improve prevention, clinical care, and research. Lancet neurology. 2017;16(12):987–1048.

4. Camara AK, Lesnefsky EJ, Stowe DF. Potential therapeutic benefits of strategies directed to mitochondria. Antioxid Redox Signal. 2010;13(3):279–347.

5. Bartok A, Weaver D, Golenar T, Nichtova Z, Katona M, Bansaghi S, Alzayady KJ, Thomas VK, Ando H, Mikoshiba K, et al. IP3 receptor isoforms differently regulate ER-mitochondrial contacts and local calcium transfer. Nat Commun. 2019;10(1):3726.

6. Madreiter-Sokolowski CT, Ramadani-Muja J, Ziomek G, Burgstaller S, Bischof H, Koshenov Z, Gottschalk B, Malli R, Graier WF. Tracking intra- and inter-organelle signaling of mitochondria. FEBS J. 2019;286(22):4378–401.

7. Bernard-Marissal N, Chrast R, Schneider BL. Endoplasmic reticulum and mitochondria in diseases of motor and sensory neurons: a broken relationship? Cell Death Dis. 2018;9(3):333.

8. Malli R, Graier WF. IRE1alpha modulates ER and mitochondria crosstalk. Nat Cell Biol. 2019;21(6):667–8.

9. Moshkforoush A, Ashenagar B, Tsoukias NM, Alevriadou BR. Modeling the role of endoplasmic reticulum-mitochondria microdomains in calcium dynamics. Sci Rep. 2019;9(1):17072.

10. Missiroli S, Patergnani S, Caroccia N, Pedriali G, Perrone M, Previati M, Wieckowski MR, Giorgi C. Mitochondria-associated membranes (MAMs) and inflammation. Cell Death Dis. 2018;9(3):329.
11. van Vliet AR, Agostinis P. Mitochondria-Associated Membranes and ER Stress. Curr Top Microbiol Immunol. 2018;414:73–102.

12. Schonthal AH: Endoplasmic reticulum stress: its role in disease and novel prospects for therapy. Scientifica (Cairo) 2012, 2012:857516.

13. Veeresh P, Kaur H, Sarmah D, Mounica L, Verma G, Kotian V, Kesarwani R, Kalia K, Borah A, Wang X, et al. Endoplasmic reticulum-mitochondria crosstalk: from junction to function across neurological disorders. Ann N Y Acad Sci. 2019;1457(1):41–60.

14. Filadi R, Theurey P, Pizzo P. The endoplasmic reticulum-mitochondria coupling in health and disease: Molecules, functions and significance. Cell Calcium. 2017;62:1–15.

15. Rowland AA, Voeltz GK. Endoplasmic reticulum-mitochondria contacts: function of the junction. Nature reviews Molecular cell biology. 2012;13(10):607–25.

16. Ren H, Zhai W, Lu X, Wang G. The Cross-Links of Endoplasmic Reticulum Stress, Autophagy, and Neurodegeneration in Parkinson's Disease. Front Aging Neurosci. 2021;13:691881.

17. Barazzuol L, Giamogante F, Brini M, Cali T. PINK1/Parkin Mediated Mitophagy, Ca(2+) Signalling, and ER-Mitochondria Contacts in Parkinson's Disease. Int J Mol Sci 2020, 21(5).

18. Truettner JS, Hu B, Alonso OF, Bramlett HM, Kokame K, Dietrich WD. Subcellular stress response after traumatic brain injury. J Neurotrauma. 2007;24(4):599–612.

19. Khatri N, Thakur M, Pareek V, Kumar S, Sharma S, Datusalia AK. Oxidative Stress: Major Threat in Traumatic Brain Injury. CNS Neurol Disord Drug Targets. 2018;17(9):689–95.

20. Balan IS, Saladino AJ, Aarabi B, Castellani RJ, Wade C, Stein DM, Eisenberg HM, Chen HH, Fiskum G. Cellular alterations in human traumatic brain injury: changes in mitochondrial morphology reflect regional levels of injury severity. J Neurotrauma. 2013;30(5):367–81.

21. Sun D, Gu G, Wang J, Chai Y, Fan Y, Yang M, Xu X, Gao W, Li F, Yin D, et al. Administration of Tauroursodeoxycholic Acid Attenuates Early Brain Injury via Akt Pathway Activation. Frontiers in cellular neuroscience. 2017;11:193.

22. Chadwick SR, Lajoie P. Endoplasmic Reticulum Stress Coping Mechanisms and Lifespan Regulation in Health and Diseases. Front Cell Dev Biol. 2019;7:84.

23. Xu X, Yin D, Ren H, Gao W, Li F, Sun D, Wu Y, Zhou S, Lyu L, Yang M, et al. Selective NLRP3 inflammasome inhibitor reduces neuroinflammation and improves long-term neurological outcomes in a murine model of traumatic brain injury. Neurobiol Dis. 2018;117:15–27.

24. Washington PM, Forcelli PA, Wilkins T, Zapple DN, Parsadanian M, Burns MP. The effect of injury severity on behavior: a phenotypic study of cognitive and emotional deficits after mild, moderate, and severe controlled cortical impact injury in mice. J Neurotrauma. 2012;29(13):2283–96.

25. Arruda AP, Pers BM, Parlakgul G, Guney E, Inouye K, Hotamisligil GS. Chronic enrichment of hepatic endoplasmic reticulum-mitochondria contact leads to mitochondrial dysfunction in obesity. Nat Med. 2014;20(12):1427–35.
26. Zhu Y, Wang H, Fang J, Dai W, Zhou J, Wang X, Zhou M. SS-31 Provides Neuroprotection by Reversing Mitochondrial Dysfunction after Traumatic Brain Injury. Oxid Med Cell Longev. 2018;2018:4783602.

27. Kauffman ME, Kauffman MK, Traore K, Zhu H, Trush MA, Jia Z, Li YR. MitoSOX-Based Flow Cytometry for Detecting Mitochondrial ROS. React Oxyg Species (Apex). 2016;2(5):361–70.

28. Wang Q, Zou MH. Measurement of Reactive Oxygen Species (ROS) and Mitochondrial ROS in AMPK Knockout Mice Blood Vessels. Methods Mol Biol. 2018;1732:507–17.

29. Zhao L, Lu T, Gao L, Fu X, Zhu S, Hou Y. Enriched endoplasmic reticulum-mitochondria interactions result in mitochondrial dysfunction and apoptosis in oocytes from obese mice. J Anim Sci Biotechnol. 2017;8:62.

30. Sundaresan P, Hunter WD, Martuza RL, Rabkin SD. Attenuated, replication-competent herpes simplex virus type 1 mutant G207: safety evaluation in mice. J Virol. 2000;74(8):3832–41.

31. Chen X, Zhao Z, Chai Y, Luo L, Jiang R, Dong J, Zhang J. Stress-dose hydrocortisone reduces critical illness-related corticosteroid insufficiency associated with severe traumatic brain injury in rats. Crit Care. 2013;17(5):R241.

32. Chen X, Zhang KL, Yang SY, Dong JF, Zhang JN. Glucocorticoids aggravate retrograde memory deficiency associated with traumatic brain injury in rats. J Neurotrauma. 2009;26(2):253–60.

33. Phillips M, Voeltz G. Structure and function of ER membrane contact sites with other organelles. Nat Rev Mol Cell Biol. 2016;17(2):69–82.

34. Ong WY, Garey LJ. Ultrastructural characteristics of human adult and infant cerebral cortical neurons. J Anat. 1991;175:79–104.

35. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. Nature reviews Immunology. 2013;13(6):397–411.

36. Nakka VP, Prakash-Babu P, Vemuganti R. Crosstalk Between Endoplasmic Reticulum Stress, Oxidative Stress, and Autophagy: Potential Therapeutic Targets for Acute CNS Injuries. Molecular neurobiology 2014.

37. Schon EA, Area-Gomez E. Mitochondria-associated ER membranes in Alzheimer disease. Mol Cell Neurosci. 2013;55:26–36.

38. Lan B, He Y, Sun H, Zheng X, Gao Y, Li N. The roles of mitochondria-associated membranes in mitochondrial quality control under endoplasmic reticulum stress. Life Sci. 2019;231:116587.

39. Liu ZW, Zhu HT, Chen KL, Dong X, Wei J, Qiu C, Xue JH. Protein kinase RNA-like endoplasmic reticulum kinase (PERK) signaling pathway plays a major role in reactive oxygen species (ROS)-mediated endoplasmic reticulum stress-induced apoptosis in diabetic cardiomyopathy. Cardiovasc Diabetol. 2013;12:158.

40. Amen OM, Sarker SD, Ghildyal R, Arya A. Endoplasmic Reticulum Stress Activates Unfolded Protein Response Signaling and Mediates Inflammation, Obesity, and Cardiac Dysfunction: Therapeutic and Molecular Approach. Front Pharmacol. 2019;10:977.
41. Chen X, Wang J, Gao X, Wu Y, Gu G, Shi M, Chai Y, Yue S, Zhang J. Tauroursodeoxycholic acid prevents ER stress-induced apoptosis and improves cerebral and vascular function in mice subjected to subarachnoid hemorrhage. Brain Res. 2020;1727:146566.

42. Sun D, Chen X, Gu G, Wang J, Zhang J. Potential Roles of Mitochondria-Associated ER Membranes (MAMs) in Traumatic Brain Injury. Cell Mol Neurobiol. 2017;37(8):1349–57.

43. Area-Gomez E, Del Carmen Lara Castillo M, Tambini MD, Guardia-Laguarta C, de Groof AJ, Madra M, Ikenouchi J, Umeda M, Bird TD, Sturley SL, et al. Upregulated function of mitochondria-associated ER membranes in Alzheimer disease. EMBO J. 2012;31(21):4106–23.

44. Bernard-Marissal N, Medard JJ, Azzedine H, Chrast R. Dysfunction in endoplasmic reticulum-mitochondria crosstalk underlies SIGMAR1 loss of function mediated motor neuron degeneration. Brain. 2015;138(Pt 4):875–90.

45. Bernard-Marissal N, van Hameren G, Juneja M, Pellegrino C, Louhivuori L, Bartsaghi L, Rochat C, El Mansour O, Medard JJ, Croisier M, et al. Altered interplay between endoplasmic reticulum and mitochondria in Charcot-Marie-Tooth type 2A neuropathy. Proc Natl Acad Sci U S A. 2019;116(6):2328–37.

46. Logsdon AF, Turner RC, Lucke-Wold BP, Robson MJ, Naser ZJ, Smith KE, Matsumoto RR, Huber JD, Rosen CL. Altering endoplasmic reticulum stress in a model of blast-induced traumatic brain injury controls cellular fate and ameliorates neuropsychiatric symptoms. Frontiers in cellular neuroscience. 2014;8:421.

47. Rubovitch V, Barak S, Rachmany L, Goldstein RB, Zilberstein Y, Pick CG. The Neuroprotective Effect of Salubrinal in a Mouse Model of Traumatic Brain Injury. Neuromolecular medicine 2015.

48. Kaur P, Sharma S. Recent Advances in Pathophysiology of Traumatic Brain Injury. Curr Neuropharmacol. 2018;16(8):1224–38.

49. Patergnani S, Suski JM, Agnoletto C, Bononi A, Bonora M, De Marchi E, Giorgi C, Marchi S, Missiroli S, Poletti F, et al. Calcium signaling around Mitochondria Associated Membranes (MAMs). Cell Commun Signal. 2011;9:19.

50. Filadi R, Pizzo P. ER-mitochondria tethering and Ca(2+) crosstalk: The IP3R team takes the field. Cell Calcium. 2019;84:102101.

51. Bollimuntha S, Pani B, Singh BB. Neurological and Motor Disorders: Neuronal Store-Operated Ca(2+) Signaling: An Overview and Its Function. Adv Exp Med Biol. 2017;993:535–56.

52. Secondo A, Bagetta G, Amantea D. On the Role of Store-Operated Calcium Entry in Acute and Chronic Neurodegenerative Diseases. Front Mol Neurosci. 2018;11:87.

53. Hill RL, Singh IN, Wang JA, Hall ED. Time courses of post-injury mitochondrial oxidative damage and respiratory dysfunction and neuronal cytoskeletal degradation in a rat model of focal traumatic brain injury. Neurochem Int. 2017;111:45–56.

54. Radi R, Cassina A, Hodara R, Quijano C, Castro L. Peroxynitrite reactions and formation in mitochondria. Free Radic Biol Med. 2002;33(11):1451–64.

55. Hetz C. The biological meaning of the UPR. Nature reviews Molecular cell biology. 2013;14(7):404.
56. Senft D, Ronai ZA. **UPR, autophagy, and mitochondria crosstalk underlies the ER stress response.** *Trends in biochemical sciences* 2015.

57. Begum G, Harvey L, Dixon CE, Sun D. ER stress and effects of DHA as an ER stress inhibitor. *Translational stroke research.* 2013;4(6):635–42.

58. Oslowski CM, Urano F. Measuring ER stress and the unfolded protein response using mammalian tissue culture system. *Methods Enzymol.* 2011;490:71–92.

59. Wang CF, Yuan JR, Qin D, Gu JF, Zhao BJ, Zhang L, Zhao D, Chen J, Hou XF, Yang N, et al. Protection of tauroursodeoxycholic acid on high glucose-induced human retinal microvascular endothelial cells dysfunction and streptozotocin-induced diabetic retinopathy rats. *J Ethnopharmacol.* 2016;185:162–70.

60. Kassan M, Vikram A, Li Q, Kim YR, Kumar S, Gabani M, Liu J, Jacobs JS, Irani K: **MicroRNA-204 promotes vascular endoplasmic reticulum stress and endothelial dysfunction by targeting Sirtuin1.** *Sci Rep* 2017, 7(1):9308.

61. Han S, Bal NB, Sadi G, Usanmaz SE, Tuglu MM, Uludag MO, Demirel-Yilmaz E. Inhibition of endoplasmic reticulum stress protected DOCA-salt hypertension-induced vascular dysfunction. *Vascul Pharmacol.* 2019;113:38–46.

62. Moltedo O, Remondelli P, Amodio G. The Mitochondria-Endoplasmic Reticulum Contacts and Their Critical Role in Aging and Age-Associated Diseases. *Front Cell Dev Biol.* 2019;7:172.

63. Gwangwa MV, Joubert AM, Visagie MH. Crosstalk between the Warburg effect, redox regulation and autophagy induction in tumourigenesis. *Cell Mol Biol Lett.* 2018;23:20.

64. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature.* 2000;403(6765):98–103.

65. Salvamoser R, Brinkmann K, O’Reilly LA, Whitehead L, Strasser A, Herold MJ. Characterisation of mice lacking the inflammatory caspases-1/11/12 reveals no contribution of caspase-12 to cell death and sepsis. *Cell Death Differ.* 2019;26(6):1124–37.

66. Roy S, Sharom JR, Houde C, Loisel TP, Vaillancourt JP, Shao W, Saleh M, Nicholson DW. Confinement of caspase-12 proteolytic activity to autoprocessing. *Proc Natl Acad Sci U S A.* 2008;105(11):4133–8.

67. Garcia de la Cadena S, Massieu L: **Caspases and their role in inflammation and ischemic neuronal death.** *Focus on caspase-12. Apoptosis* 2016, 21(7):763–777.

68. Li H, Yu JS, Zhang HS, Yang YQ, Huang LT, Zhang DD, Hang CH. Increased Expression of Caspase-12 After Experimental Subarachnoid Hemorrhage. *Neurochem Res.* 2016;41(12):3407–16.

69. Zhang H, Song LC, Jia CH, Lu YL. Effects of ATP sensitive potassium channel opener on the mRNA and protein expressions of caspase-12 after cerebral ischemia-reperfusion in rats. *Neurosci Bull.* 2008;24(1):7–12.

**Tables**
| Antibody                  | Vendor | Dilution | Molecular weight (kDa) |
|--------------------------|--------|----------|-----------------------|
| GRP78                    | Abcam  | 1:1000   | 75                    |
| p-PERK (Thr981)          | Santa  | 1:250    | 170                   |
| PERK                     | CST    | 1:500    | 140                   |
| p-eIF2α (Ser51)          | CST    | 1:1000   | 36                    |
| eIF2α                    | CST    | 1:1000   | 38                    |
| ATF4                     | CST    | 1:1000   | 49                    |
| CHOP                     | CST    | 1:1000   | 27                    |
| Cleaved Caspase-12       | Abcam  | 1:1000   | 38                    |
| Bcl-2                    | Santa  | 1:1000   | 28                    |
| Bax                      | CST    | 1:1000   | 20                    |
| Cytc                     | CST    | 1:1000   | 15                    |
| PACS-2                   | Abcom  | 1:1000   | 100                   |
| MFN-2                    | Abcom  | 1:1000   | 86                    |
| IP3R1                    | CST    | 1:1000   | 240                   |
| GRP75                    | Abcom  | 1:1000   | 75                    |
| VDAC1                    | Abcom  | 1:1000   | 57                    |
| Cleaved IL-1β            | CST    | 1:1000   | 18                    |
| TNFα                     | Abcom  | 1:1000   | 17                    |
| β-actin                  | CST    | 1:1000   | 45                    |
| Peroxidase-Conjugated anti-Rabbit IgG (H+L) | ZSGB-BIO | 1:5000 |
| Peroxidase-Conjugated anti-Mouse IgG (H+L) | ZSGB-BIO | 1:5000 |

**Figures**
Figure 1

H&E-stained coronal sections in the cerebral cortex 24 hr after controlled cortical impact (CCI). This photomicrograph confirms the severe injury of mice used in this study.
Figure 2

TBI induces MAM formation and mitochondrial morphological changes in the mouse cerebral cortex. (A) Representative TEM images of neurons in the mouse ipsilateral cortex before injury and at 1, 3, 6, 12, and 24 hr after TBI. (g-l) Schematic diagram illustrating MAM formation and mitochondria in images (a-f). Images (m-f) are magnifications of the boxed areas in images (a-f). (B) Quantification of the ER length adjacent to mitochondria, number of mitochondria (C), and area of mitochondria (D). All graphs represent the mean ± SD, n = 6. * P < 0.05 compared to sham, # P < 0.05 compared to 1 hr, ψ P < 0.05 compared to 3 hr, δ P < 0.05 compared to 12 hr, ω P < 0.05 compared to 24 hr.
Figure 3

TBI drives the dynamic expression of ER-mitochondrion tethering proteins in the mouse cerebral cortex after TBI. (A) Representative photographs of immunofluorescence staining for NeuN (green), GFAP (green), and PACS2 (red) in the pericontusional cortex at 6 hr post injury. (B-C) Representative western blot bands and densitometric quantification of PACS2 and Mfn2. All graphs represent the mean ± SD, n =
6. * P < 0.05 compared to sham, # P < 0.05 compared to 1 hr, ψ P < 0.05 compared to 3 hr, δ P < 0.05 compared to 12 hr, ω P < 0.05 compared to 24 hr.

**Figure 4**

TBI alters the expression of MAM-resident Ca2+ regulatory proteins and mitochondrial ROS production in the mouse cerebral cortex after TBI. (A-B) Representative western blot bands and ImageJ-based quantification of the protein expression levels of IP3R1, GRP75, VDAC1 and Sigma-1R along with mitochondrial ROS production after TBI (C). All the results are expressed as the mean ± SD, n = 6. * P < 0.05 compared to sham, # P < 0.05 compared to 1 hr, ψ P < 0.05 compared to 3 hr, δ P < 0.05 compared to 12 hr, ω P < 0.05 compared to 24 hr.
Figure 5

TBI results in ER stress, UPR activation and neuroinflammatory responses in the cerebral cortex after TBI. (A-D) Representative western blot bands and statistical analyses of proteins involved in the activation of the PERK/elf2α/ATF4 UPR branch. (E-F) Western blot and quantification analyses of the procytokines cleaved IL-1β and TNFα. All the results are expressed as the mean ± SD, n = 6. * P < 0.05 compared to sham, # P < 0.05 compared to 1 hr, ψ P < 0.05 compared to 3 hr, δ P < 0.05 compared to 12 hr, ω P < 0.05 compared to 24 hr.
Figure 6

Correlation among MAM formation, ER-mitochondrion Ca2+ transfer, oxidative stress, UPR signaling, and inflammatory responses in the acute phase of severe TBI. Pearson's correlation analysis revealed that PACS2 expression was significantly positively correlated with the levels of Mfn2, IP3R1, VDAC1, Sigma-1R, GRP75, ROS, GRP78, p-PERK/PERK, p-eIF2α/eIF2α, ATF4, cleaved IL-1β, and TNFα within 24 hr of injury.
Figure 7

Caspase 12-dependent ER stress-mediated apoptosis occurred after the enhancement of ER-mitochondrion crosstalk in the mouse cerebral cortex after TBI. Western blot and quantification analyses of the expression levels of CHOP (A), cleaved Caspase-12 (B), cleaved Caspase-3 (C), cleaved PARP1 (D), Bcl-2/Bax (E) and Cytc (F) immediately after the enhancement of ER-mitochondrion crosstalk at 6 hr following TBI. All graphs represent the mean ± SD, n = 6. *p < 0.05 compared to sham, #p < 0.05 compared to 6 hr, ψp < 0.05 compared to 12 hr, δp < 0.05 compared to 24 hr.
Experimental suppression of MAM formation reduced ER-mitochondrion Ca2+ transfer and mitochondrial oxidative stress, diminished ER stress and UPR activation, and suppressed neuroinflammatory responses. Western blot and quantification analyses showing the expression levels of the ER-mitochondrion tethers PACS2 (A) and Mfn2 (B) following PACS2 siRNA treatment at 6 hr post TBI. (C) Quantification of mitochondrial ROS production by the MitoSOX assay. (D-K) Representative western blot bands and
ImageJ-based densitometric quantification of the indicated proteins involved in ER stress, UPR activation, and neuroinflammation. All graphs represent the mean ± SD, n = 6. *p < 0.05 compared to sham, #p < 0.05 compared to TBI, ψp < 0.05 compared to siRNA-NC.

Figure 9

Experimental knockdown of MAM formation improved cerebrovascular function, increased cellular viability, and ameliorated neurological deficits. (A) Representative photographs of the brains from the
sham, TBI, siPACS2, and siRNA-NC groups following Evans blue (EB) dye injection at 72 hr post injury. Summary data present the EB extravasation (B) and brain water content (C) in cerebral hemispheres after PACS2 siRNA treatment at 72 hr after TBI. (D) The total modified neurological severity scores of TBI mice during a 14-day follow-up period. (E) Representative photographs of the TUNEL staining of neurons in the pericontusional cortex at 72 hr post injury after PACS2 siRNA transfection. (F) Quantification of neuronal apoptosis in the pericontusional cortex at 72 hr post injury after PACS2 siRNA treatment. (G-J) Western blot and quantification analyses of the expression levels of cleaved Caspase-12, CHOP, Bcl-2/Bax and Cytc in the different treatment groups at 72 hr after injury. All graphs represent the mean ± SD, n = 6. *p < 0.05 compared to sham, #p < 0.05 compared to TBI, ψp < 0.05 compared to siRNA-NC.