ISRIB improves white matter injury following TBI by inhibiting NCOA4-mediated ferritinophagy

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Abstract

Traumatic brain injury (TBI) often results in persistent neurological dysfunction, which is closely associated with white matter injury. While the mechanisms underlying white matter injury after TBI remain unclear, recent research has implicated ferroptosis, a form of programmed cell death, in cognitive impairment after TBI. Ferritinophagy, a selective autophagic process that degrades ferritin and releases free iron. Here, we established a rat model of TBI and examined the expression of NCOA4, which mediates ferritin degradation through autophagy in lysosomes, to investigate whether ferritinophagy contributes to white matter injury after TBI. Our results showed that NCOA4 was overexpressed in the rat model of TBI, and knockdown of NCOA4 using shNCOA4 lentivirus infection inhibited ferroptosis induced by ferritinophagy. Furthermore, we found that treatment with ISRIB, a small molecule that selectively inhibits the integrated stress response, attenuated NCOA4-mediated ferritinophagy and improved white matter injury. These findings suggest that NCOA4-mediated ferritinophagy is a critical mechanism underlying white matter injury after TBI, and that ISRIB may hold promise as a therapeutic agent for treating this injury.

1. Introduction

Traumatic brain injury (TBI) is a leading cause of disability and mortality worldwide(1), often resulting in long-lasting cognitive, behavioral, and emotional deficits, including depression and anxiety(2, 3). Increasing evidence suggests that the cognitive and behavioral deficits following TBI are associated with white matter injury(4). Despite this, the underlying mechanisms of white matter injury after TBI remain poorly understood.

ISRIB is a small molecule integrated stress response inhibitor that can reverse the effects of eIF2 phosphorylation in stressed cells, resulting in improved memory and spatial learning abilities in mice(5–7). However, ISRIB has no impact on translation or mRNA synthesis in non-stressed cells(8). Previous studies have demonstrated that ISRIB can improve long-term depression caused by Alzheimer's disease(9, 10) and severe depression(11), as well as promote motor function recovery after spinal cord injury(12). These findings suggest that ISRIB may have potential as a therapeutic strategy for neural injuries. However, its reparative effect on white matter injury after TBI has been rarely mentioned.

Several studies have shown that ferroptosis following TBI can lead to cognitive and motor impairment(13–15). The coordination of intracellular iron homeostasis is typically regulated by multiple molecular mechanisms, including uptake, export, and release(16). As the primary iron chelating and storage protein in cells, ferritin plays a crucial role in iron metabolism by reducing the concentration of free iron. NCOA4 has been identified as the selective cargo receptor for ferritinophagy(17), which facilitates the transport of ferritin to lysosomal degradation. However, it is uncertain whether ferritinophagy is involved in ferroptosis following TBI.
Therefore, our study aims to investigate the potential role of ferritinophagy in white matter injury following TBI and explore the effectiveness of ISRIB in mitigating such injury.

2. Results

2.1. NCOA4-mediated ferritinophagy occurs after TBI

To investigate the occurrence of NCOA4-mediated ferritinophagy following traumatic brain injury (TBI), we utilized western blot analysis to assess the expression of FTH1 and NCOA4 (Fig. 1A). Previous studies have indicated that NCOA4 binds to FTH1 and facilitates its transportation to lysosomes for autophagic degradation (18). Our findings indicated a gradual increase in NCOA4 levels from 6 hours to 7 days following TBI (p < 0.05, Fig. 1B), while FTH1 levels exhibited a decreasing trend at 6 and 12 hours, followed by an increase from 1 to 7 days (p < 0.05, Fig. 1C). Similar to an ischemic stroke model, where ferritinophagy and mRNA-mediated FTH1 synthesis occur simultaneously resulting in a decrease followed by an increase in FTH1 levels (19), we aimed to validate whether this situation also occurs in the TBI model by conducting qRT-PCR analysis. Results showed a continuous increase in FTH1 mRNA levels from 6h to 7d (p < 0.05, Fig. 1D). To further confirm the occurrence of ferritinophagy in TBI, we targeted ATG5 and NCOA4 in rats by intracerebroventricular injection of shATG5 and shNCOA4 lentiviruses, respectively. Western blot results indicated increased levels of ATG5 and LC3II/I ratio in the TBI + shCtrl group compared to the Sham group (p < 0.05, Fig. 1G-H). Successful knockout of ATG5 (p < 0.05, Fig. 1E) led to a significant decrease in LC3II/I ratio and NCOA4 expression, while FTH1 increased, compared to the TBI + shCtrl group (p < 0.05, Fig. 1H-J), indicating that suppression of autophagy resulted in an increase in FTH1. Similarly, NCOA4 knockout (p < 0.05, Fig. 1K) reversed the reduction in FTH1 levels (p < 0.05, Fig. 1L-N), further confirming the involvement of NCOA4-mediated ferritinophagy following TBI.

In addition, the results of the immunofluorescence analysis revealed that the TBI group exhibited a higher expression of LC3, an autophagic marker, and a lower expression of FTH1 when compared to the sham group. Besides, the colocalization of LC3 and FTH1 was enhanced in the TBI group. Conversely, NCOA4 knockout led to a reversal of these results (p < 0.05, Fig. 1P-R). Collectively, these findings suggest that ferritinophagy is induced after TBI.

2.2. ISRIB attenuates white matter injury after TBI by inhibiting ferritinophagy

2.2.1. ISRIB mitigates white matter injury after TBI

An increasing number of studies have found that white matter injury after TBI is involved in long-term neurological impairment after TBI. To explore whether ISRIB can alleviate white matter injury after TBI, diffusion tensor imaging (DTI) scans were performed (Fig. 2A). According to a previous report (20), DTI is an MRI technique specifically designed to visualize the structure of white matter in the brain. Fractional anisotropy (FA) is a measure of DTI analysis. In general, higher FA values correspond to greater structural
integrity due to increased alignment of axonal fibers in the white matter. Our findings revealed significantly decreased FA values in the TBI + Vehicle group compared to the Sham group. However, treatment with ISRIB led to significant restoration of FA values in rats (p < 0.05, Fig. 2B).

To assess the integrity of the myelin sheath, transmission electron microscopy (TEM) was used. TEM results at 3 days after TBI revealed myelin sheath dissolution and de-myelination in the TBI + Vehicle group, with a decreased number of myelinated axons. In contrast, the sham group showed normal myelin sheath thickness in the white matter, with a large number of evenly distributed myelinated axons. However, the TBI + ISRIB group showed less severe demyelination (Fig. 2C). Furthermore, compared to the sham group, the TBI + Vehicle group had a higher g-ratio, indicating a thinner myelin sheath. Conversely, treatment with ISRIB rescued the increase in g-ratio (p < 0.05, Fig. 2D).

To assess the potential of ISRIB in promoting neurological function recovery after TBI, we evaluated rat performance in the NSS and Morris water maze tests across groups. The findings revealed a clear neurological damage in both tests following TBI. However, treatment with ISRIB significantly improved neurological function (p < 0.05, Fig. 2E-G). These results indicate that ISRIB may alleviate white matter injury.

2.2.2. ISRIB weakens ferroptosis after TBI by inhibiting ferritinophagy

To investigate whether ISRIB alleviate white matter injury by inhibiting ferritinophagy, first we used western blot (Fig. 3A). The results demonstrated that ISRIB treatment significantly increased FTH1 expression and decreased NCOA4 expression compared to the vehicle group (p < 0.05, Fig. 3B-C). Immunofluorescence analysis revealed that ISRIB treatment reduced the co-localization of LC3 with FTH1 (p < 0.05, Fig. 1R).

Moreover, the levels of free iron and MDA were reduced, and the level of GSH was increased in brain tissue following ISRIB treatment compared to the vehicle group (p < 0.05, Fig. 3D-F). Collectively, these findings suggest that ISRIB weakens ferroptosis after TBI by inhibiting ferritinophagy.

3. Discussion

Traumatic Brain Injury (TBI) is a leading cause of morbidity and mortality worldwide, with long-term consequences that can severely impact patients' quality of life. The pathophysiology of TBI involves two types of injury: primary and secondary. Primary injury refers to brain damage caused directly by the external force, including increased intracranial pressure, bleeding, edema, and axonal injury due to nerve and vascular tissue stretching(21). On the other hand, secondary injury refers to cellular and molecular events that occur within hours to days after the initial trauma, including neuroinflammation, oxidative stress, synaptic injury, and protein aggregation(22). White matter injury is a common result of secondary injury, leading to a range of cognitive and motor impairments as well as psychological issues. The morphological changes of white matter injury include widespread axonal damage and demyelination(23,
Currently, no specific treatment exists for white matter injury, and management focuses on supportive care and rehabilitation. Further research is required to develop effective neuroprotective and regenerative therapies for this debilitating condition.

ISRIB was first discovered in 2013 by a team of scientists at the University of California, San Francisco. They identified the drug using a high-throughput screening approach and found that it could reverse the effects of ISR activation in cells(5). Since then, several studies have demonstrated that ISRIB can improve cognitive function and promote neuronal regeneration in animal models of neurological disorders(9–11). Previous studies have shown that(25) treatment with ISRIB in TBI mice restored long-term memory and cognitive deficits observed in TBI mice. In our study, we found that ISRIB can alleviate white matter injury following TBI.

Iron is an essential element for nearly all living organisms, participating in a wide range of biological reactions such as oxygen transport, oxidative phosphorylation, and enzyme-catalyzed reactions required for cell proliferation(26, 27). While iron is critical for these reactions, its availability must be strictly controlled because high levels of free iron can generate harmful reactive oxygen species through the Haber-Weiss/Fenton reaction(28). In times of iron excess, iron is stored in ferritin and released when needed. The main pathway for releasing iron from ferritin is through NCOA4-mediated selective autophagy, where NCOA4 binds to ferritin and transports it to the lysosome, where ferritin is degraded, and iron is released for cellular use, known as ferritinophagy(17). Ferritinophagy plays a critical role in maintaining cellular iron homeostasis. Dysregulation of ferritinophagy has been implicated in various diseases, including neurodegenerative disorders(29–31), cancer(32, 33), and iron overload disorders such as hemochromatosis(34). Studies have shown that the induction of ferritinophagy can alleviate the symptoms of these diseases by reducing the iron burden within the cells. Our research suggests that the increase in intracellular free iron after TBI is due to NCOA4-mediated ferritinophagy and the ferritinophagy is also involved in white matter injury following TBI.

In conclusion, our results demonstrate that ISRIB can suppress ferroptosis caused by ferritinophagy following TBI, thereby improving white matter injury. Although the preclinical data on ISRIB looks promising, several challenges need to be addressed before the drug can be tested in humans. One major concern is the potential for off-target effects, as the ISR is a crucial mechanism for maintaining cellular homeostasis. Additionally, the optimal dosage and treatment duration are still unknown. ISRIB represents a promising new avenue for the treatment of neurological disorders. Further research is needed to fully understand its mechanisms of action and potential side effects, but the preclinical data suggests that it may be a valuable addition to the current arsenal of neurological drugs.

4. Materials and Methods

4.1. Animal
Adult male Sprague-Dawley (SD) rats (8–10 weeks old, weighing 220 ± 20g) were purchased from the Experimental Animal Center of Chongqing Medical University (Experimental Animal Use License: SYXX (Yu) 2018–0003). The rats were housed in SPF-class animal rooms with ad libitum access to food and water and subjected to a reverse 12:12h light-dark cycle. The ambient temperature was maintained between 20–25 °C.

4.2. Traumatic brain injury model

SD rats were randomly assigned to either the CCI or sham groups for all experiments. A standardized rat-controlled cortical impact (CCI) model was produced using the craniocerebral injury instrument (TBI.0310, PSI, USA) to simulate reproducible craniocerebral trauma. The rats were weighed and anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (50mg/Kg). Once anesthesia was successful, a 5-mm diameter craniectomy was performed at the following coordinates: 4 mm posterior to bregma and 2.5 mm lateral to the right of the midline. The impact parameters were set to 5m/s, a depth of 5mm, and a dwell time of 250ms. Following the impact, the rats were rewarmed on an electric blanket. The sham group only underwent craniectomy surgeries without impingement.

4.3. Drug administration

ISRIB (Cat: A14302; AdooQ BioScience, USA) was initially dissolved in dimethyl sulfoxide (DMSO) to 4 mg/ml, then further diluted to a final concentration of 0.3125 mg/ml using PBS (35). ISRIB was delivered at a dosage of 2.5 mg/kg via i.p. injection as per a previous report (25). The vehicle group received an equivalent of vehicle (8%DMSO in PBS).

4.4. In vivo RNAi of ATG5 and NCOA4

The sequences used for RNAi targeting ATG5 and NCOA4 were GGCATTATCCAATTGGCCTAC and CTCCTTGTCAGTGGCTTAT, respectively. Lentivirus-based transfer system was performed by GenePharma Pharmaceutical Technology (Suzhou, China). One week prior to TBI modeling, rats received shATG5, shNCOA4, or shCtrl lentiviruses through lateral ventricular injection. After anesthesia with isoflurane, the rats were fixed under a stereotaxic device (RWD Life Science, China). Using a 10-µl Hamilton syringe (Hamilton, USA), 8µl of virus was injected at a rate of 0.5µl/min at the following coordinates: 1.5 mm posterior to bregma, 1.1 mm lateral from the midline, and 4.5 mm below the skull surface. The needle was left in place for 10 min to prevent reflux and then withdrawn at a slow rate. Finally, the scalp was sutured, and the rats were placed on an electric blanket to recover from anesthesia.

4.5. Western blot

We extracted the lysate from White matter tissue around the trauma using RIPA buffer (Cat: P0013B; Beyotime, China). Protein samples from each group (30ug/lane) and pre-stained molecular weight markers (Cat: 7E411H0, Vazyme, China) were separated by SDS PAGE (12, 15% gel). The isolated proteins were electrophoretically transferred to a PVDF membrane (Cat: ISEQ00010, Millipore, USA). The blots were then incubated in 10% non-fat milk in tris-buffer saline (TBS) at room temperature for 2.5 hours. Then the following antibodies were used for incubation at 4°C overnight: mouse anti-ATG5(1:2000,Cat:
66744-IG, Proteintech, China), rabbit anti LC3 (1:1000, Cat: #3868, Cell Signaling Technology, USA), rabbit anti FTH1 (1:1000, Cat: #4393, Cell Signaling Technology, USA), rabbit anti NCOA4 (1:1000, Cat: #66849, Cell Signaling Technology, USA), rabbit anti GAPDH (1:10000, Cat: D110016, Sangon Biotech, China). After washing with TBST, the blots were incubated with corresponding secondary antibody for 1 h at room temperature. Finally, the blots were performed with an ECL kit (Cat: WBKLS0100, Millipore, USA). Image J (National Institutes of Health, USA) software was used to determine the intensity of each band.

4.6. Immunofluorescence staining

The brain tissues collected from various groups were processed for paraffin embedding and sectioning. The paraffin sections were then dewaxed using a sequential treatment of xylene I and xylene II. Subsequently, they were hydrated in graded ethanol solutions of 100%, 95%, 85%, and 75% for 5 minutes each. After antigen retrieval using citric acid repair buffer, the sections were treated with 3% hydrogen peroxide for 15 minutes at room temperature while protecting them from light. Next, the sections were blocked with 3% bovine serum albumin (BSA)-PBS for 30 minutes at room temperature. The primary antibodies, FTH1 (1:200, Cat: #4393, Cell Signaling Technology, USA), LC3 (1:500, Cat: #3868, Cell Signaling Technology, USA), CC1 (1:500, Cat: OP80, Sigma-Aldrich, USA), and NCOA4 (1:100, Cat: A5695, ABclonal, China) were applied to the brain sections and incubated overnight at 4°C. On the following day, after washing the sections with PBS (pH 7.4), fluorescein-conjugated secondary antibody (Cat: AFIHC024, Aifang Biological, China) was applied for 30–50 minutes. The nuclei were counterstained with 6-diamidino-2-phenylindole (DAPI) for 10 minutes. Finally, the images were captured using a Laser Scanning Microscope (Pannoramic DESK, P250, 3D HISTECH, Hungary). The analysis was performed in a blinded manner using ImageJ software (NIH) with the region-of-interest (ROI) tool.

4.7. qRT-PCR

Total RNA was extracted using RNAeasyTM animal RNA isolation kit with spin column (Cat: R0026, Beyotime, China) following the manufacturer’s protocol. For cDNA synthesis, 500ng RNA, 2ul 5x PrimeScript RT Master Mix (Perfect Real Time) (Cat: RR360A, Takara, Japan), and RNase Free dH2O were used. The RT-qPCR was performed as per the manufacturer’s instructions using TB Green Premix Ex TaqTM II (Tli RNases plus)(2X) (Cat: RR820A, Takara, Japan), Synthesized cDNA, and 0.4uM PCR primer on a Bio-Rad MJ Mini Option Real-Time PCR System. The primers (Tsingke, Chian) used in the experiments were as follows

Ferritin heavy chain 1 (FTH1): Forward − 5’CTGAGCCCTTTGCAACTTTCG-3’,
Reverse-5’GATCTGGCGTTGATGGCAG-3’;

ATG5: Forward − 5’ GGATTCCAACGTGCTTTACTCTC-3’,
Reverse-5’GTCAGTTACCAGCGTCAAATAGC-3’;

NCOA4: Forward − 5’ TCAGATTGTTACGGCCTCCC-3’,

Reverse-5’ATGGTGCTTTGGACCCCTTCT-3’.
Reverse-5'GGTCACTCAGCTCACGATGT-3';

GAPDH: Forward − 5’ GAAGGTGCTGTGAACGGAT-3’,
Reverse-5’ CCCATTTGATGTTAGCGGGAT-3’.

For gene expression analysis, the data were normalized to the reference gene GAPDH. The expression levels of the target genes were calculated using the 2- ΔΔCT method.

4.8. Transmission electron microscopy

After cardiac perfusion and isolation, brain tissue was pre-fixed using 3% glutaraldehyde, refixed with 1% osmium tetroxide. And then, dehydration was performed in series acetone. Next tissue was embedded in Ep812. After that, semi-thin sections were stained with toluidine blue for optical localization. Ultra-thin sections of 60–90 nm were prepared using a diamond knife, and the sections were stained with uranyl acetate and lead citrate. Lastly, graphs were observed by transmission electron microscopy (JEM-1400FLASH, JEOL). G-ratio was used to evaluate the extent of myelin damage, which is defined as the ratio of the axon diameter to the sum of the axon diameter and the myelin sheath thickness.

4.9. Diffusion Tensor Imaging (DTI)

As previous described(36), the degree of white matter damage was evaluated using diffusion tensor imaging (DTI). In our studies, DTI was performed using Bruker Biospec 70/20 USR (Brucker, Biospin, USA) system. DTI images were acquired using a single-shot echo-planar imaging (EPI) sequence in the coronal plane, which included 5 b values of 0 s/mm2 and 30 non-co-linear diffusion-weighted images with a b value of 1000 s/mm2. The acquisition parameters were as follows: repetition time (TR)/echo time (TE) = 3000/30ms, 128×128 matrix, 2.5×2.5 cm field-of-view, 15 slices, 0.5 mm slice thickness.

4.10. Free Iron Content

The free iron content was measured using the Iron Assay Kit (Cat: ab83366, Abcam, England) according to the manufacturer's instructions. Brain tissue was removed and homogenized, and the absorbance was measured at 593 nm.

4.11. MDA

The MDA content was measured using the MDA assay kit (Cat: A003-1-2, Nanjing Jian Cheng, China) according to the manufacturer's instructions. Brain tissue was removed and homogenized, and the absorbance was measured at 532 nm.

4.12. GSH

The GSH content was measured using the GSSH/GSH Quantification kit (Cat: G263, Dojindo, Japan) following the manufacturer's instructions. Brain tissue was removed and homogenized, and the absorbance was measured at 405 nm.

4.13. Measurements of Neurological Functions
The neurological functions of rats were evaluated using the neurological severity scores (NSS), as described in previous studies. The NSS was used to assess the neurological outcome of the rats at different time points, including before injury, and on the 1st, 3rd, 7th, 14th, and 21st days after TBI. The evaluation comprised ten different tasks to assess various aspects of rat behavior, such as general behavior, balance, alertness, and motor ability. Points were assigned for each failed task, with a higher score indicating a more severe neurological deficit and a worse outcome. Additionally, the cued learning ability of the rats was assessed using the Morris water maze test, as previously described (37), and their memory was tested by removing the hiding platform on the 20th day after TBI.

4.14. Statistical analysis

All data were presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, United States). The normality of the data was checked using the Shapiro-Wilk normality test in advance and then analyzed for statistical significance between groups was determined using one-way ANOVA or two-way ANOVA. P value less than 0.05 were considered statistically significant.

Declarations

Author Contributions: Conceptualization, Data curation, Formal analysis, Writing—original draft, and Writing—review and editing, W-ZZ and Y-DL, Methodology, Investigation, and Visualization, W-HD, Data curation, Resources, and Writing—review and editing, X-YL, Methodology, Investigation, and Data curation, W-QF and S-ST, Conceptualization, Resources, Supervision, and Writing—review and editing, XJ and Y-BD. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Laboratory Animal Center of Chongqing Medicine University in China (protocol code IACUC-CQMU-2021-0015) on 15 February 2021.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest

References


Figures
**Figure 1**

**TBI activates ferritinophagy** (A-C) The expression levels of NCOA4(B) and FTH1(C) at different time points after TBI were shown by WB. (D) The mRNA level of FTH1 after TBI were shown by qRT-PCR. (E) The mRNA level of ATG5 after ATG5 knockout were shown by qRT-PCR. (F-J) The expression levels of ATG5(G), LC3II/I ratio(H), NCOA4(I), and FTH1(J) after ATG5 knockout were shown by WB. (K) The mRNA level of NCOA4 after NCOA4 knockout was shown by qRT-PCR. (L-N) The expression levels of NCOA4(M)
and FTH1(N) after NCOA4 knockout were shown by WB. (O) Immunofluorescence analysis for FTH1 (green) colocalization with LC3 (red). Nuclei were counterstained with DAPI (blue). Scale bar= 50 µm. (P-R) Quantification of fluorescence intensity of FTH1(P), LC3(Q), and FTH1 colocalization with LC3(R). Error bars represent the mean ± SEM (n = 3 for each group). *p < 0.05, **p < 0.01, ##p>0.05 by one-way ANOVA with the respective controls.

Figure 2
ISRIB mitigates white matter injury after TBI (A) WMI was measured by DTI 7d after TBI. The colors of the FA maps indicate the principal axis of water diffusion (red, mediolateral, green, dorsoventral, blue, anteroposterior). (B) Quantification of average FA values. (C) Integrity of the myelin sheath was measured by TEM. Scale bar = 2 µm. (D) Quantification of G Ratio. *p < 0.05, **p < 0.01 by one-way ANOVA, n = 4. (E) Neurological severity scores. **p < 0.01 by two-way ANOVA, n = 4. (F) Learning latency (E) and probe trail in Morris water maze after TBI. **p < 0.01 by two-way ANOVA, n = 6. Error bars represent the mean ± SEM.
**Figure 3**

**ISRIB inhibits ferroptosis by suppressing ferritinophagy** (A-C) The expression levels of NCOA4(B) and FTH1(C) were shown by WB. (D) The free iron content. (E) The MDA content. (F) The GSH content. (G) Immunofluorescence analysis for CC1 (green) colocalization with NCOA4 (red). Nuclei were counterstained by DAPI (blue). Scale bar= 50 µm. (H) Quantification fluorescence intensity of CC1 colocalization with NCOA4. Error bars represent the mean ± SEM (n = 3 for each group). *p < 0.05, **p < 0.01 by one-way ANOVA with the respective controls.

We also used immunofluorescence analysis to evaluate the co-localization of CC1 (a marker of oligodendrocytes) and NCOA4 to investigate whether oligodendrocytes undergo ferritinophagy, as oligodendrocytes are myelin-forming cells (Figure 3G). The results demonstrated that the fluorescence co-localization intensity of CC1 and NCOA4 was higher in the vehicle group than in the sham group, while ISRIB treatment reversed this phenomenon (Figure 3B-C, p<0.01). These results suggest that oligodendrocytes undergo ferritinophagy after TBI, and that ISRIB improves white matter injury after TBI by inhibiting ferritinophagy in oligodendrocytes.