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ORIGINAL RESEARCH

Neuroprotective Mechanism of MOTS-c in TBI Mice: Insights from Integrated Transcriptomic and Metabolomic Analyses

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Background: Traumatic brain injury (TBI) is a condition characterized by structural and physiological disruptions in brain function caused by external forces. However, as the highly complex and heterogenous nature of TBI, effective treatments are currently lacking. Mitochondrial open reading frame of the 12S rRNA-c (MOTS-c) has shown notable antinociceptive and anti-inflammatory effects, yet its detailed neuroprotective effects and mode of action remain incompletely understood. This study investigated the neuroprotective effects and the underlying mechanisms of MOTS-c.

Methods: Adult male C57BL/6 mice were randomly divided into three groups: control (CON) group, MOTS-c group and TBI group. Enzyme-linked immunosorbent assay (ELISA) kit method was used to measure the expression levels of MOTS-c in different groups. Behavioral tests were conducted to assess the effects of MOTS-c. Then, transcriptomics and metabolomics were performed to search Differentially Expressed Genes (DEGs) and Differentially Expressed Metabolites (DEMs), respectively. Moreover, the integrated transcriptomics and metabolomics analysis were employed using R packages and online Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Results: ELISA kit method showed that TBI resulted in a decrease in the expression of MOTS-c. and peripheral administration of MOTS-c could enter the brain tissue after TBI. Behavioral tests revealed that MOTS-c improved memory, learning, and motor function impairments in TBI mice. Additionally, transcriptomic analysis screened 159 differentially expressed genes. Metabolomic analysis identified 491 metabolites with significant differences. Integrated analysis found 14 KEGG pathways, primarily related to metabolic pathways. Besides, several signaling pathways were enriched, including neuroactive ligand–receptor interaction and retrograde endocannabinoid signaling.

Conclusion: TBI reduced the expression of MOTS-c. MOTS-c reduced inflammatory responses, molecular damage, and cell death by down-regulating macrophage migration inhibitory factor (MIF) expression and activating the retrograde endocannabinoid signaling pathway. In addition, MOTS-c alleviated the response to hypoxic stress and enhanced lipid β-oxidation to provide energy for the body following TBI. Overall, our study offered new insights into the neuroprotective mechanisms of MOTS-c in TBI mice.

Keywords: MOTS-c, TBI, transcriptomics, metabolomics, neuroprotective

Introduction

Traumatic brain injury (TBI) involves a condition characterized by structural and physiological disruptions in brain function caused by external forces.[1] An estimated 50 million cases of TBI occur globally each year, making it a major public health concern.[2] TBI is accountable for almost one-third of all injury-related fatalities and is a leading cause of

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death among adults.[3] The neurological prognosis of TBI is poor, mainly related to diffuse brain injury and secondary injury.[4] The etiology of TBI is complex, involving both immediate and long-lasting neuronal damage resulting from both primary and secondary cerebral trauma. Primary injury refers to the initial physical damage to the brain tissue that occurs at the moment of impact. Primary injuries can be classified into several types: diffuse axonal injury (DAI), contusion and penetrating injury. Secondary injury is marked by extensive neuronal necrosis, cerebral tissue edema, disruption of the blood–brain barrier (BBB), increased oxidative stress, with an exaggerated inflammatory response. Various molecular components, mediators, chemical agents, and pro-inflammatory cytokines initiate this inflammatory cascade. Following traumatic brain injury, regeneration of neurons fails, inflammation occurs, and nerve atrophy leads to poor prognosis. Neuroinflammation can cause acute secondary injury after traumatic brain injury and is associated with chronic neurodegenerative diseases.[5,6] Currently, the main treatments include using diuretics and hypertonic salt solutions to alleviate brain edema, administering antibiotics to reduce the risk of intracranial infection, and applying neuroprotective drugs to protect the nerves following TBI.[7,8] Unfortunately, there are no effective therapies available to alleviate neuroinflammation. It is important to explore additional treatment options to address the neuroinflammation associated with TBI and therapies to promote reparative processes.[9]

Mitochondrial open reading frame of the 12S rRNA-c (MOTS-c) is a short open reading frame (sORF) within the mitochondrial 12S rRNA. It encodes a 16-amino-acid peptide and was found by Lee et al in 2015.[10] MOTS-c is widely discovered in various tissues and plasma. One study showed that MOTS-c appeared to regulate mitochondrial function by promoting mitochondrial biogenesis and improving mitochondrial quality control mechanisms, such as mitophagy. By enhancing mitochondrial function, MOTS-c might help protect neurons from oxidative stress and energy depletion, which were common features of neurodegenerative diseases and traumatic brain injury.[10] MOTS-c has been reported to play the part of significant antinociceptive and anti-inflammatory functions in mice, as well as an increase in anti-inflammatory factors.[11] MOTS-c has been shown to have anti-inflammatory properties by inhibiting the production of pro-inflammatory cytokines and promoting the activation of anti-inflammatory pathways. By reducing neuroinflammation, MOTS-c might mitigate neuronal damage and promote neuroprotection in various neurological disorders.[12] MOTS-c might exert neurotrophic effects by promoting the survival and growth of neurons. It has been suggested that MOTS-c enhances the expression of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), which play crucial roles in neuronal survival, synaptic plasticity, and neurogenesis. In in vitro cultures of HEK293 cells subjected to metabolic stress, extracellular MOTS-c translocated into the nucleus in an AMP-activated protein kinase (AMPK) dependent manner to regulate gene expression. It also activated various antioxidant kinases, playing a crucial role in enhancing cellular resistance, maintaining cellular metabolic homeostasis, and providing cellular protection.[13] MOTS-c increased the survival of mice during Methicillin-resistant Staphylococcus aureus (MRSA) infection by reducing pro-inflammatory cytokine levels like tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), and increasing anti-inflammatory cytokine interleukin-10 (IL-10).[14] A recent study showed that MOTS-c enhanced Memory through Inhibiting Neuroinflammation.[15] MOTS-c might protect neurons from oxidative stress by enhancing antioxidant defense mechanisms and reducing the production of reactive oxygen species (ROS). By maintaining redox balance, MOTS-c may prevent oxidative damage to neuronal cells and promote their survival.[10]

In recent years, omics technology has rapidly advanced and greatly contributed to the study of the interrelationships among various factors. Transcriptomics is the study of all mRNA transcripts produced within an organism. Comparing samples under different conditions, transcriptomics identifies differentially expressed genes and further explores the functions and regulatory mechanisms of these genes in biological processes.[16] Transcriptomic analysis elucidates changes in gene expression patterns following TBI, revealing crucial genes associated with injury severity, pathological stages, and prognosis. Metabolomics is the study of all small-molecule metabolites within an organism. Metabolomics reveal the structure, regulation, and associations with organismal function and disease by investigation of changes in metabolites within metabolic pathways.[17] Metabolomics has been used in the understanding of the pathophysiological mechanisms of TBI, and the differential metabolites might be used for the prediction of outcome, monitoring treatment response and prognosis of post-injury recovery.[18] Integrating transcriptomics and metabolomics is now widely used to analyze the pathophysiology, molecular mechanisms, and therapeutic approaches for diseases.[19–21] This integration is also widely applied in various other fields, such as zoology, botany, and environmental science.[22–24] Integrated

transcriptomic and metabolomic analyses play a pivotal role in studying TBI and its treatment by offering a holistic understanding of TBI pathophysiology, enhancing our knowledge of the disease mechanisms. Building on the background mentioned above, here we explored the neuroprotective effects of MOTS-c on TBI model mice. We employed the Morris water maze (MWM) test, open field test (OFT), and Balance beam test (BBT) to assess the behavioral characteristics and memory abilities of mice before and after MOTS-c treatment. Furthermore, we integrated metabolomics and transcriptomics to explore the correlations between metabolism and gene expression, providing a deeper understanding of the mechanisms by which MOTS-c protects the brains of TBI mice. This study aims to identify a new target and offer a fresh, systematic perspective on TBI therapy.

Materials and Methods

Animals and Treatment

The C57BL/6 male mice in the experiments were procured from Pengyue Experimental Animal Breeding Co. Ltd. in Jinan, Shandong, China. They were aged 6–7 weeks old and weighed 20–25g. They were then housed in a standard condition at 25°C with a 12-h light–dark cycle and had access to food and water ad libitum. After a week of acclimating to a standard diet, 7 mice served as a control (CON) group with sham-operated (only the scalp was incised and sutured after anesthesia), 14 mice underwent a TBI modeling procedure based on established methodologies in prior literature.[25–27] 24 hours after TBI induction, the 14 mice were randomly divided into two groups: the TBI group (n=7) and the MOTS-c group (n=7). In the MOTS-c group, mice received intraperitoneal injections of MOTS-c (Macklin Reagent, Shanghai, China) at a dose of 5 mg/kg/day for two weeks, while the TBI group and the CON group received equivalent doses of normal saline. The dosage and administration of MOTS-c were based on established literature.[10,28–31] All experimental procedures strictly followed the guidelines for laboratory animals' management established by the State Committee of Science and Technology of the People's Republic of China. Additionally, they were approved by the University Ethics Committee under reference number JNRM-2023-Dw-105.

Establishment of TBI Mouse Model

The TBI model was established following the cortical impact method, as described in previous literature.[32–34] Experimental C57BL/6 mice were intraperitoneally anesthetized with pentobarbital sodium to induce a painless and unconscious state. Following partial skin preparation and disinfection, a 10-mm-long midline scalp incision was made to expose the skull. They were then securely immobilized on a stereotaxic apparatus. Subsequently, we used a high-speed surgical hand drill to create a 3-mm-diameter circular craniotomy (2.0 mm rostral and lateral from bregma) over the left frontal cortex while preserving the integrity of the dura mater. Using a 2.5-mm-diameter rounded metal tip, we employed a controlled cortical impact (CCI) device (CCI Model 6.3; Custom Design, USA) to induce a moderate-severe TBI on the exposed brain, with a velocity of 4 m/s, a depth of 1.5 mm, and a contact time of 100 ms. Afterward, the bone flap was promptly replaced and sealed, and the scalp was sutured.

Behavioral Tests

Balance Beam Test (BBT)

BBT offers a valuable way to evaluate the physical coordination and balance of mice with impact-induced cortical injuries. Mice that have experienced cortical impact lesions often exhibit contralateral slipping on the beam. This experimental procedure typically used a narrow balance beam, measuring about 100 centimeters in length and 12 millimeters in width. The mice were placed at one end of the balance beam, and their behavior was closely observed or recorded. By analyzing the time taken by the mice to cross the beam and the score of mice (The more times the paw slipped on the beam, the lower the score), it was performed to assess the likelihood of impairment or the extent of impact. After two consecutive days of training, three times daily (6am, 10am and 2pm), we carefully recorded the time taken by the mice crossing the beam and the scores in the third day.

Morris Water Maze Test (MWM)

MWM is a commonly employed animal behavioral experiment designed to assess the spatial learning and memory abilities of mice, considering their inherent navigation instincts. The methodology in this study closely adheres to a documented protocol.[35] The MWM apparatus comprised a circular tank, 120 cm in diameter and 50 cm in height, filled with water at a controlled temperature of 23 ± 2°C and a depth of 15.5 cm. To limit visibility in the tank, non-toxic, tasteless white ink was added to the water, making it opaque. An escape platform, transparent and measuring 6 cm in diameter and 14.5 cm in height, was strategically located at the midpoint of one quadrant, hidden just 1 cm below the water's surface. During each trial, the mice had a maximum of 60 seconds to find the submerged escape platform. If they did not succeed within this time, they were gently placed on the platform and given a 10-second break to observe and learn spatial information. After four consecutive days of training, three times daily (8am, 12am and 4pm), we carefully recorded the mice's paths and the time they spent in the target quadrant (where the platform had been previously) in the fifth day. This data was collected using Visu Track software (Shanghai NewSoft, Shanghai, China).

Open Field Test (OFT)

OFT is used to assess the mice's spontaneous behavior, exploratory inclinations, and anxiety levels in a novel environment. The test occurred in a dimly lit room and employed a rectangular container, with 50cm ×50cm ×40cm in length, width and height. Mice were allowed to freely explore their surroundings for 5 minutes after they were placed in the central area of the container. A video camera was situated directly above the center of the floor, and the Visu Track software was used to automatically track their movements. This software created trace maps and recorded essential behavioral parameters, such as distance traveled, movement speed, and total motion duration. After each trial, the experimental equipment was meticulously cleaned and dried with a 75% ethanol solution to prepare for the assessment of the next mouse. The total distance traveled from the three days (once a day) was used for further analysis.

Sample Preparation

C57BL/6 mice were humanely euthanized by cervical dislocation 24 hours after behavioral tests. Subsequently, the cerebral cortex tissues were carefully and promptly extracted under ice-cold conditions and rinsed with a PBS solution. These harvested tissues were placed in cryotubes and securely stored at −80°C. The cerebral cortex tissues from three mice in the TBI and MOTS-c group were randomly selected for transcriptomic analysis.

Enzyme-Linked Immunosorbent Assay (Elisa) Kit Method

The cerebral cortex tissues and phosphate buffer saline (PBS) were fully ground in the homogenizer at the ratio of 1g: 9mL on the ice. Then, it was centrifuged 5000×g for 10 minutes. The supernatants were stored at −80 degrees until the trial day.

The supernatant was dissolved on ice, and then the expression levels of MOTS-c in the different groups were measured using a competitive Elisa kit (Abbexa LTD, Cambridge, UK). The specific procedural requirement was performed according to the kit's instructions. A Biotek Elx800 Microplate reader (BioTek Instruments Inc., Winooski, VA, USA) was used to read the absorbance values at 450nm. A Gen5 data analysis program was used to calculate the concentrations of MOTS-c. The resulting values were expressed as ng/mL.

Transcriptomics Analysis

Transcript analysis was performed by RNA sequencing as previously described.[36] Total RNA was extracted from the samples using the Trizol reagent (Invitrogen, CA, USA) following the manufacturer's instructions. Then, reverse transcriptase was used to synthesize single-stranded complementary deoxyribonucleic acid (cDNA), with messenger Ribonucleic Acid (mRNA) as the template. Subsequently, a 2×150bp paired-end sequencing (PE150) was conducted using an Illumina Novaseq™ 6000. StringTie was used to determine mRNA expression levels by calculating fragments per kilobase per million (FPKM). Differentially expressed mRNAs and genes were identified with a log2 (fold change) >1 or log2 (fold change) <-1 and statistical significance ($q < 0.05$, corrected p-value) using R package (version 3.5.1). Finally, Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and (KEGG) pathway enrichment analyses

were conducted for the Differentially Expressed Genes (DEGs) to identify significantly enriched terms based on the hypergeometric distribution, and figures were generated.

Metabolomics Analysis

25mg samples were placed in a 500μL extraction solution of methanol, acetonitrile, and water in a 2:2:1 ratio. Then, the samples were ground at 35 Hz for 4 minutes and sonicated in an ice-water bath for 5 minutes. After repeating this process three times, the samples were frozen at −40°C for 60 minutes and then centrifuged at 12,000 rpm for 15 minutes. Finally, the supernatant was collected for further analysis. LC-MS/MS (Liquid Chromatography–Tandem Mass Spectrometry) analysis was conducted using a Ultra High Pressure Liquid Chromatography (UHPLC) system with a Ultra Performance Liquid Chromatography Ethylene Bridged Hybrid (UPLC BEH) amide column (2.1 mm×100 mm, 1.7 μm) connected to a Q-Exactive plus quadrupole-orbitrap mass spectrometer equipped with heated electrospray ionization (ESI) source (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic conditions were set as follows: auto-sampler temperature at 4°C; 2 μL injection volume; mobile phase A containing 25 mmol/L ammonium acetate and 25 ammonia hydroxides in water; and mobile phase B composed of acetonitrile. The ESI source operated in both positive and negative ion modes with these parameters: sheath gas flow rate of 30 Arb, Aux gas flow rate of 25 Arb, capillary temperature set to 350°C, full MS resolution at 120,000, MS/MS resolution at 7500, and spray voltage at 3.6 kV (positive) or −3.2 kV (negative), respectively. Then, the raw data underwent processing steps, including peak detection, extraction, alignment, and integration, and was subsequently matched with the mass spectrometry database for analysis. The data were normalized, processed, and imported into SIMCA (V16.0.2). Model validity was assessed using orthogonal partial least-squares discriminant analysis (OPLS-DA) in SIMCA. Metabolites with variable importance in projection (VIP) values >1.0 in the OPLS-DA model and a p-value <0.05 in a Student's *t*-test were considered Differentially Expressed Metabolites (DEMs). Finally, the KEGG pathway library was used to analyze the pathways of DEMs.

Integrative Analysis of Metabolomics and Transcriptomics

Integrative analysis of metabolomics and transcriptomics was employed for a comprehensive assessment of the neuroprotective effect of MOTS-c on TBI mode mice. The Spearman algorithm was applied to analyze correlations between DEMs and DEGs, and heatmaps were generated with cluster mapping. Afterwards, all DEGs and DEMs were cross-referenced and mapped to pathways using the online KEGG database (http://www.kegg.jp/). Moreover, enrichment analysis was conducted. R packages (version 3.5.1) were utilized to integrate the KEGG annotations and enrichment results of the two omics.

Statistical Analysis

Data analysis was performed using SPSS 22.0 and R packages (version 3.5.1). GraphPad Primer 8.0 (GraphPad Prism, GraphPad Software, La Jolla, CA) and R packages (version 3.5.1) were employed to generate plots. All data were presented as means ± standard deviation (means ± SD). The independent sample *t*-test was employed to compare group differences; One-way analysis of variance (ANOVA) was used to compare differences among multiple groups; The Student's *t*-test was used to identify differential metabolites. P <0.05 was considered statistically significant.

Results

TBI Reduced the Expression of MOTS-c, and Peripheral Administration of MOTS-c Could Enter the Brain Tissue After TBI

We conducted the ELISA assay to explore the effect of TBI on MOTS-c in the cortex of mice. The results showed that the expression level of MOTS-c in CON group was significantly more compared to the TBI group ($p<0.05$); After intraperitoneal injection of MOTS-c, the expression level of MOTS-c in the cortex of TBI mice was significantly higher than that of TBI group ($p<0.05$) (Figure 1A). This told us that TBI reduced the expression levels of MOTS-c in the cerebral cortex of mice, and when the BBB was disrupted by TBI, MOTS-c could enter the cerebral cortex of mice exerting its effects (Specific information is presented in Supplementary Table 1).

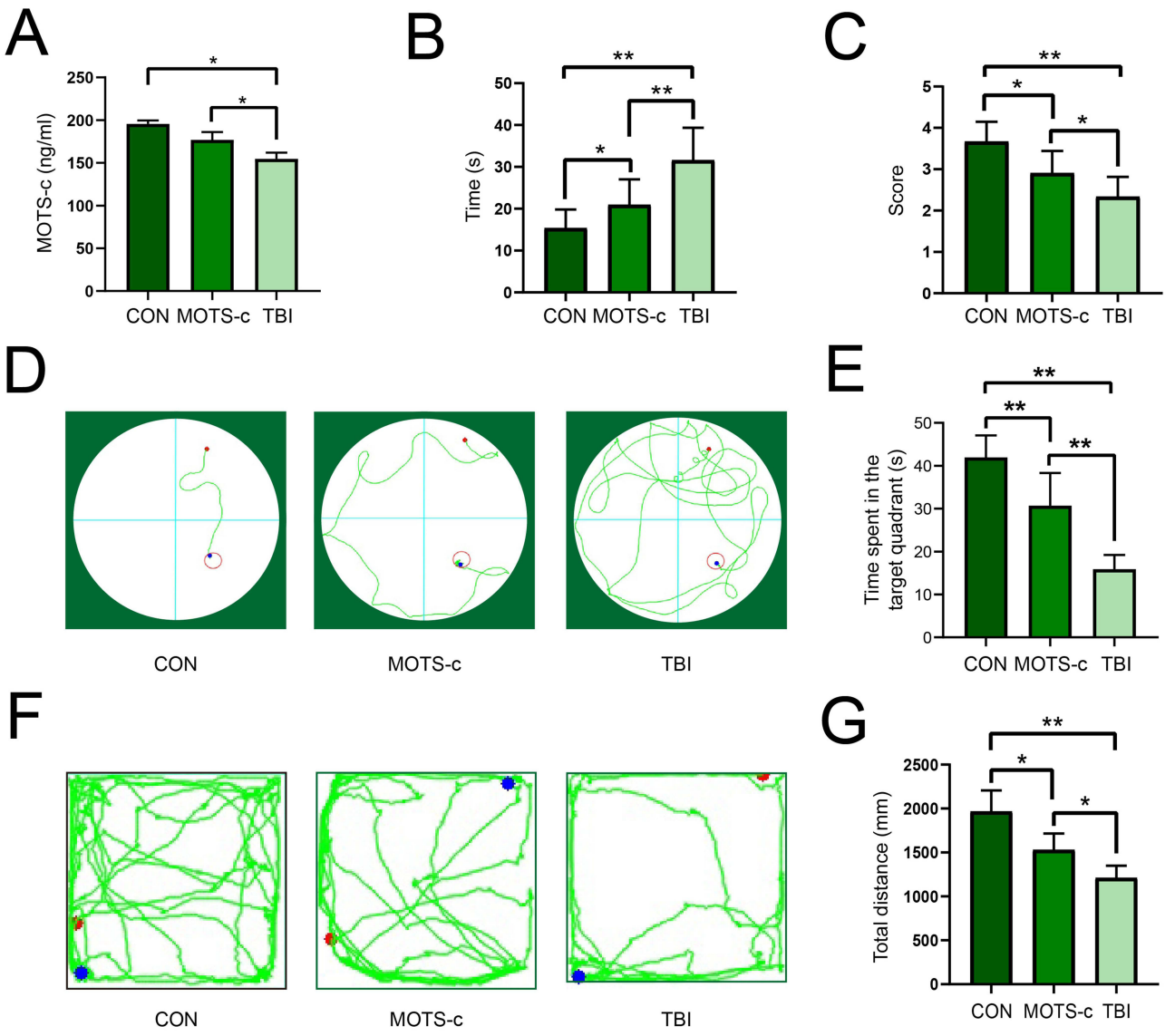

Figure 1 The levels of MOTS-c and experimental behavioral results of mice in different groups (n=7).
Notes: (**A**) Determination of levels of MOTS-c by ELISA in different groups. (**B**–**G**) Experimental behavioral results in different groups. (**B**) Time spent by mice on the beam in the balance beam test (BBT). (**C**) scores of mice in the BBT test. (**D**) Representative trajectory plot of the mice in the Morris water maze (MWM). (**E**) Time spent by mice in the target quadrant in the MWM test. (**F**) Representative trajectory plot of the mice in the open field test (OFT). (**G**) Total distances traveled by mice in the OFT test (All data were expressed as mean ± SD. *p<0.05; **p<0.01).
Abbreviations: CON, control; MOTS-c, mitochondrial open reading frame of the 12S rRNA-c; TBI, traumatic brain injury.

MOTS-c Ameliorates the Memory, Learning, and Motor Behavior Impairment of TBI Mice

Balance Beam Test (BBT) results showed that mice in the CON group spent less time on the balance beam compared to the TBI group, achieving higher composite scores (Figure 1B and C). Mice in the CON group displayed better coordination and stability, with fewer foot slips compared to the TBI group. After injections of MOTS-c, the time spent less and scores achieved higher in the TBI mice (Specific information is presented in Supplementary Table 2). MWM results indicated that mice in the TBI group spent significantly less time in the target quadrant compared to the CON group, yet the time was increased after injections of MOTS-c in the TBI mice (Figure 1D and E), suggesting improved memory and learning abilities by MOTS-c (specific information is presented in Supplementary Table 3). OFT results demonstrated that the mice in the TBI group walked significantly decreased distances compared to the CON

group, and they spent less time in the central zone. After injections of MOTS-c in the TBI mice, they walked longer distances and spent more time in the central zone (Figure 1F and G). It indicated MOTS-c increased activity and reduced anxiety of the mice (Specific information is presented in Supplementary Table 4). In summary, the behavioral experiments revealed that MOTS-c has the potential to improve memory, learning, and motor behavior functions of TBI mice.

Transcriptomics Analysis of the TBI and MOTS-c Groups

Transcriptomics analysis was employed to analyze DEGs in the cerebral cortex between TBI and MOTS-c groups. Principal Component Analysis (PCA) revealed a significant difference in gene expression profiles between the TBI and MOTS-c groups (Figure 2A). $|\log2FC|\geq1$ and $q<0.05$ were used as strict criteria for identifying DEGs. The results showed that 159 genes were significantly changed in the MOTS-c group compared to the TBI group, with 88 genes up-regulated and 71 genes down-regulated. Detailed information regarding the DEGs is provided in Supplementary Table 5. The expression of genes in both groups was visualized through hierarchical clustering analysis heatmaps (Figure 2B) and the volcano plot (Figure 2C). Subsequently, we performed GO and KEGG pathway analyses of the DEGs. It was shown that the Top 25 (biological process), the Top 15 (cellular component), and Top 10 (molecular function) terms according to the number of DEGs in the histogram of GO enrichment classification (Figure 3A). Our results revealed that the most prominent GO terms were related to the cytoplasm, nucleus, membrane, and protein binding. To further investigate the mechanism underlying the neuroprotective effects of MOTS-c on TBI model mice, we conducted KEGG pathway analysis to validate the enriched pathways of these DEGs. According to q value of the DEGs, Top 20 enrichment pathways are shown in Figure 3B. As we can see, the most significantly enriched pathway was the nucleotide-binding oligomerization domain (NOD)-like receptor signaling pathway. Specific information on the pathways is presented in Supplementary Table 6. In conclusion, these findings suggested significant changes in gene expression within the cerebral cortex following MOTS-c treatment for TBI.

Metabolomics Analysis of the TBI and MOTS-c Groups

Metabolomic analysis of the TBI and MOTS-c groups was performed to assess the altered metabolic profile of the cerebral cortex tissues. OPLS-DA was made for multivariate group analysis and it showed that the samples within the groups were clustered together. In contrast, a clear separation trend was observed between the two groups, showing significant differences between the two groups (Figure 4A). We screened significantly DEMs by univariate statistical analysis, and a total of 187 up-regulated metabolites and 304 down-regulated metabolites were obtained (VIP>1 and $P<0.05$). Specific information on the metabolites is shown in Supplementary Table 7. The changes in metabolites of two groups were shown by a volcano map (Figure 4B). As we can see in the volcano plots, each point represented a peak; The X-axis and the Y-axis represented the fold change and the p-value, respectively. The unsupervised hierarchical clustering analysis of 491 DEMs further indicated the different expression patterns between the two groups (Figure 4C). We then analyzed the relevant metabolic pathways involved in the metabolites by using the KEGG pathway library (Figure 5). The results showed that the pathway of differential metabolites enrichment mainly involved metabolism-related pathways, including the Glycerophospholipid metabolism pathways, D-Amino acid metabolism pathways and Arginine and proline metabolism. Specific information on metabolic pathways is shown in Supplementary Table 8.

Integrated Analysis of Metabolomics and Transcriptomics

To understand the changes occurring in cerebral cortex, the potential relationship between DEMs and DEGs was further analyzed. We first conducted a Spearman algorithm to further analyze the interaction between DEMs and DEGs (Figure 6B). S-1-Propenyl-L-cysteine, Glycine, Uracil, and Methylmalonic acid exhibited positive correlations with the macrophage migration inhibitory factor (MIF) and the reduced nicotinamide adenine dinucleotide ubiquinone oxidoreductase core subunit protein 7 (NDUFS7) genes. Additionally, glycerophosphocholine and Glycerylphosphorylethanolamine were positively correlated with the choline acetyltransferase (CHAT) gene. Based on the above analysis, we identified 14 KEGG pathways involving both the transcriptome and metabolome (Figure 6A). Our findings revealed that these DEGs and DAMs were primarily associated with pathways related to metabolism, glycerophospholipid metabolism, and neuroactive

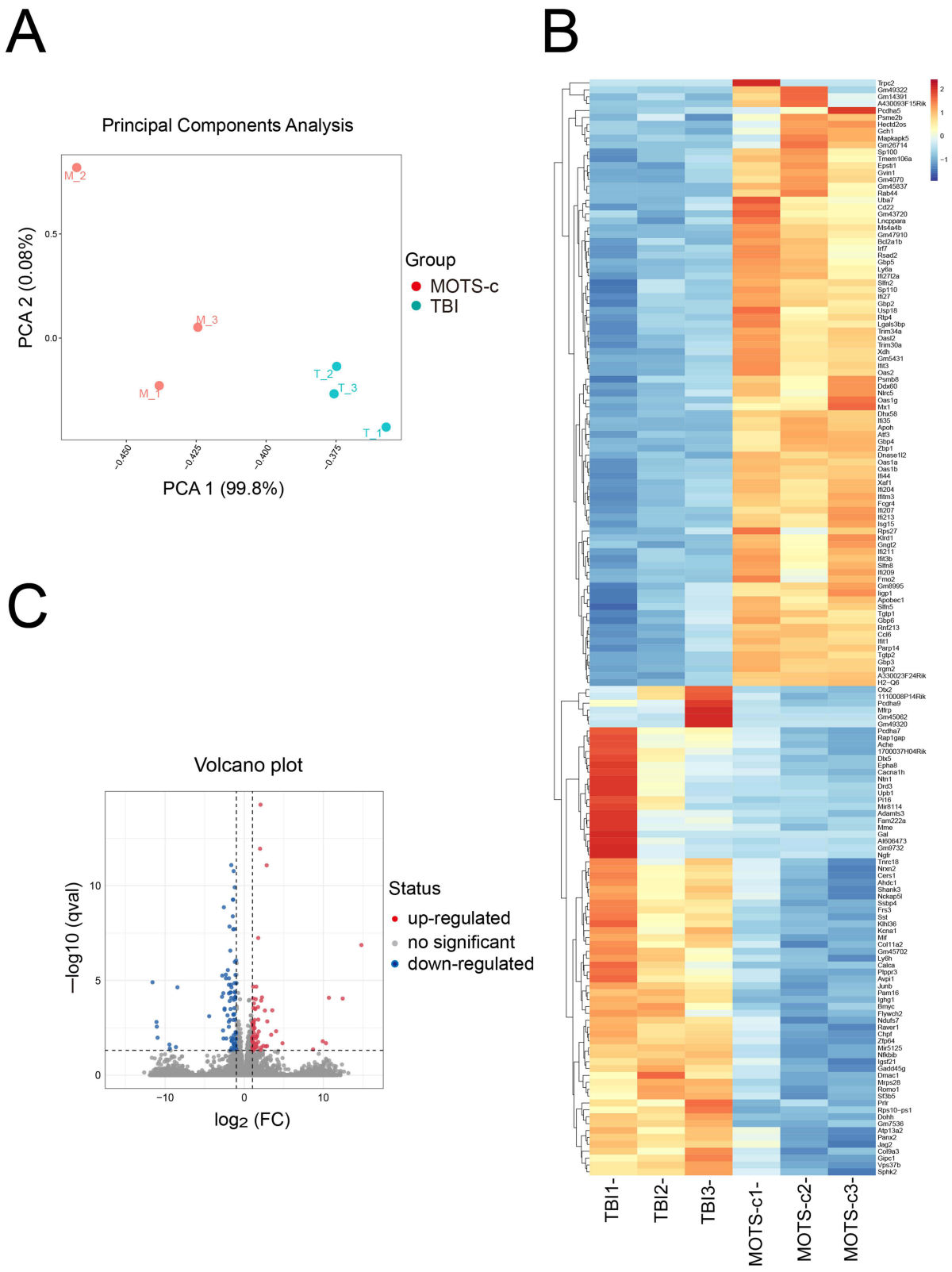

Figure 2 Transcriptomics analysis of MOTS-c VS TBI groups (n=3).

Notes: (**A**) PCA of transcriptome profiles separated the MOTS-c and TBI groups. (**B**) Hierarchical clustering based on the DEGs. Red and green represent up-regulation and down-regulation, respectively. (**C**) Volcano plot of the DEGs. The red and blue points represent the up-regulated and down-regulated genes, respectively (DEGs: | log2FC|≥1 and q<0.05).

Abbreviations: MOTS-c, mitochondrial open reading frame of the 12S rRNA-c; TBI, traumatic brain injury.

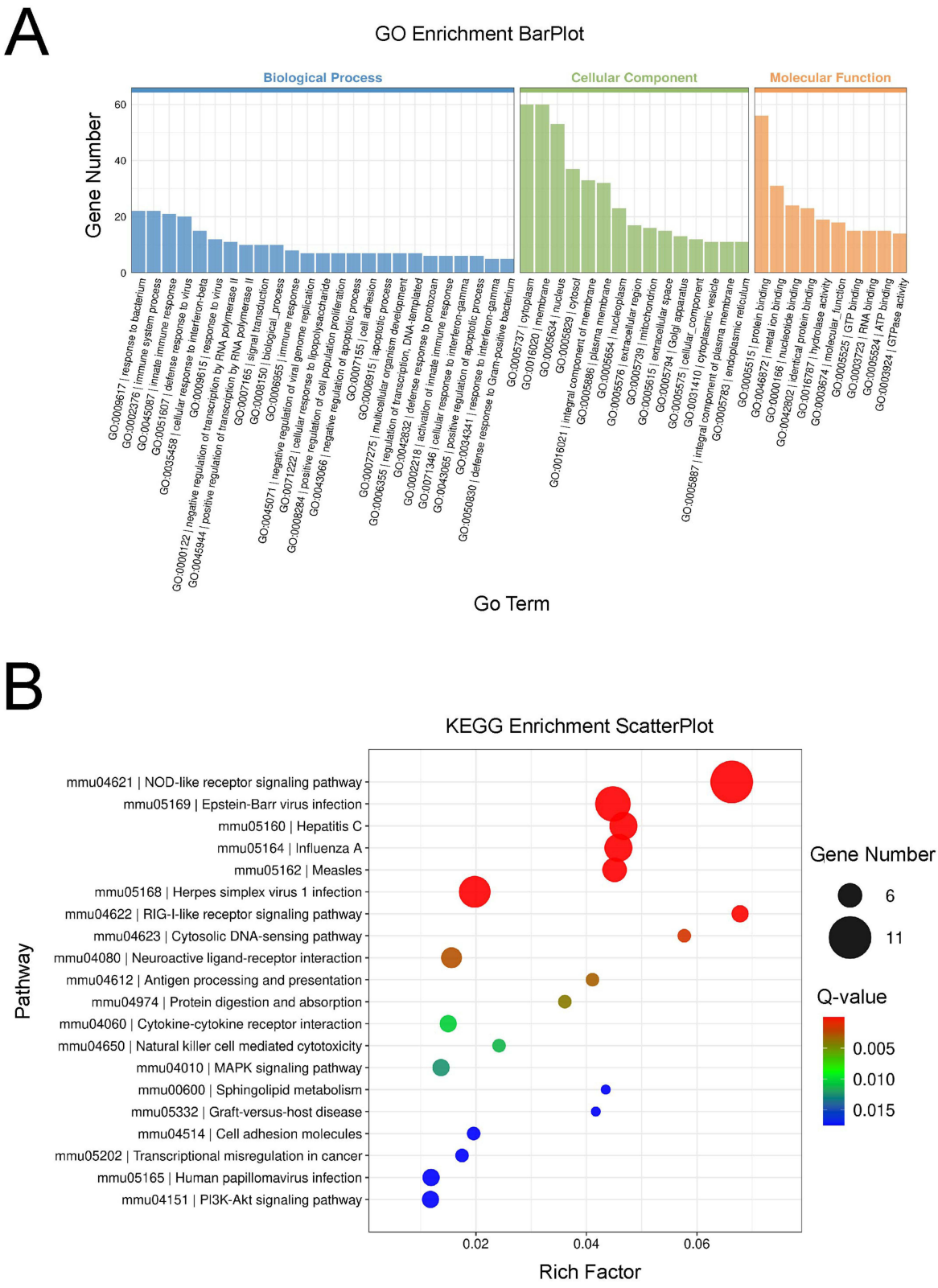

Figure 3 GO terms and KEGG pathway analyses of the DEGs (n=3).
Notes: (**A**) The enriched GO terms of the DEGs in cellular component, biological process and molecular function. (**B**) KEGG pathway enrichment analysis of the DEGs. Color represents q value and bubble size represents the number of genes enriched.

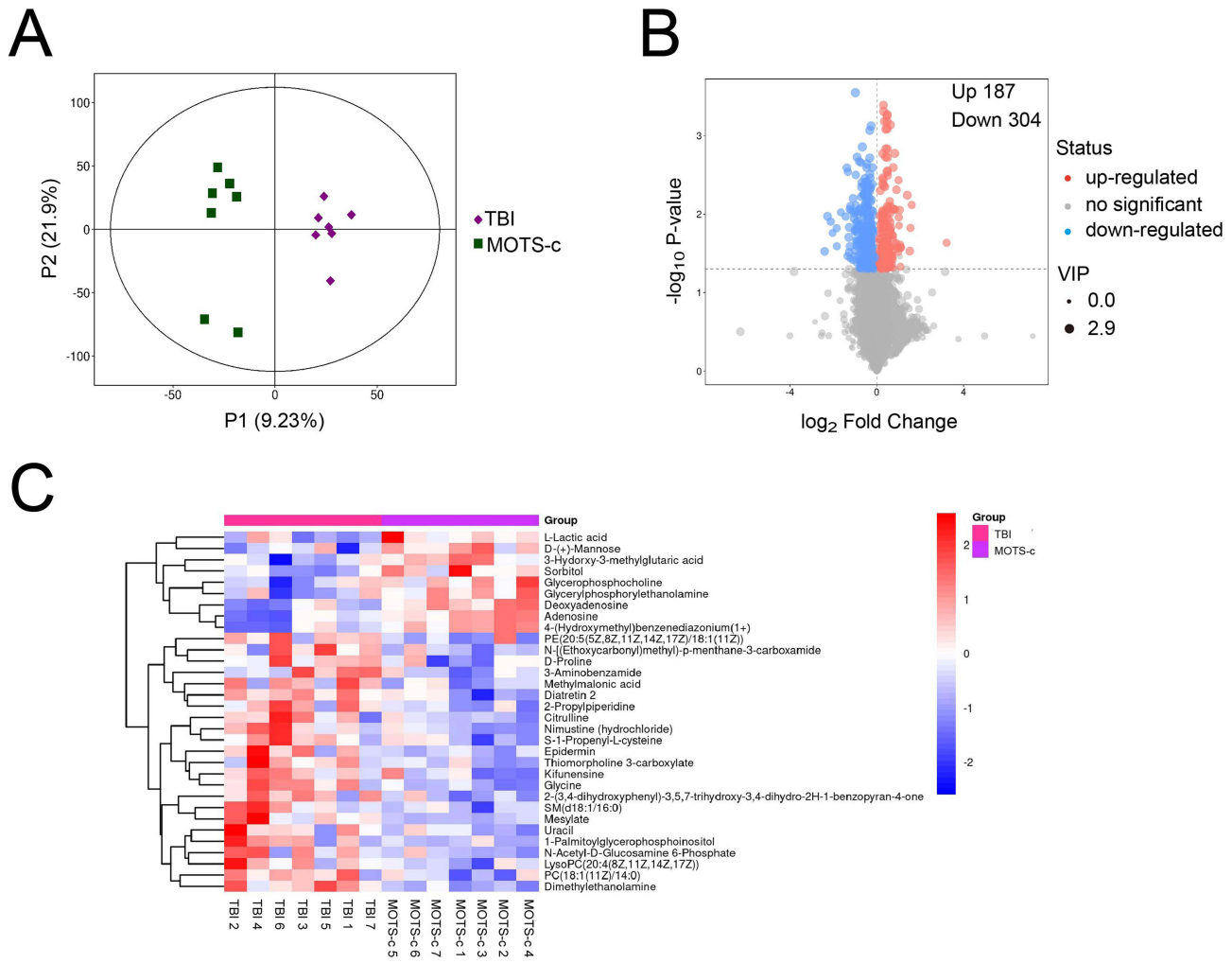

Figure 4 Metabolomics analysis of MOTS-c VS TBI groups (n=7).
Notes: (**A**) Score scatter plot of the OPLS-DA model. (**B**) Volcano plot. The red and blue points represent the up-regulated and down-regulated metabolites, respectively. The bubble size represents the VIP value. (**C**) Heatmap of hierarchical clustering analysis for the MOTS-c and TBI groups. Red and blue represent up-regulation and down-regulation, respectively (DEMs: VIP>1.0 and p<0.05).
Abbreviations: MOTS-c, mitochondrial open reading frame of the 12S rRNA-c; TBI, traumatic brain injury.

ligand–receptor interactions (Figure 6C). Specific information on pathways is shown in Supplementary Table 9. These results suggested that the changes that occur in the cerebral cortex were closely associated with DEMs and DEGs.

Discussion

The complex nature of TBI pathogenesis, which included brain tissue injury, intracranial bleeding, brain edema, a subsequent cascade of inflammatory reactions, cellular damage, and metabolic disturbances, had posed challenges for the development of effective and targeted treatments.[37] Minimizing the inflammatory response and maintaining stable brain metabolism were crucial strategies to mitigate secondary damage in severe TBI.[38] MOTS-c was a mitochondrial-related biological molecule that played a crucial role in regulating metabolism and anti-inflammation. However, due to the protective effect of the blood–brain barrier, many proteins and peptides could not enter the central nervous system to exert their effects.[39,40] Some researchers used cell-penetrating peptides to transport proteins or peptides into cells for functional purposes.[41] MOTS-c by intraperitoneal injection did not cross the BBB and entered the central nervous system. When the blood–brain barrier was breached after TBI, its protective function was lost, allowing many macromolecules to freely enter and exit the central nervous system. In this study, we initially ascertained that TBI reduced the expression levels of MOTS-c and peripheral administration of MOTS-c can enter the brain tissue after TBI. Subsequent

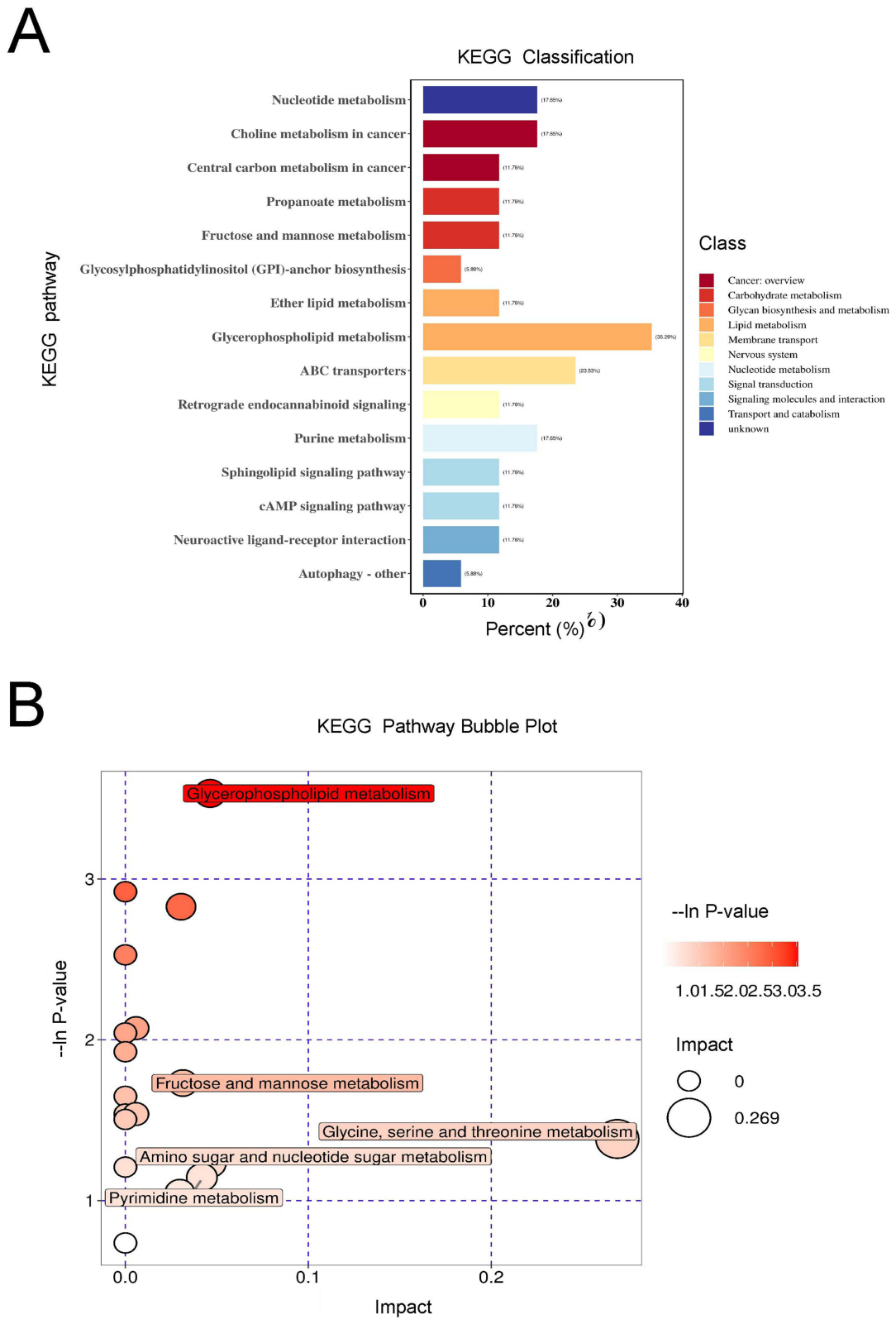

Figure 5 Pathway analysis of the DEMs (n=7).
Notes: (**A**) KEGG Classification of the TBI and MOTS-c groups. (**B**) Bubble plot of the MOTS-c and TBI groups. Color represents p value and bubble size represents the pathway impact.

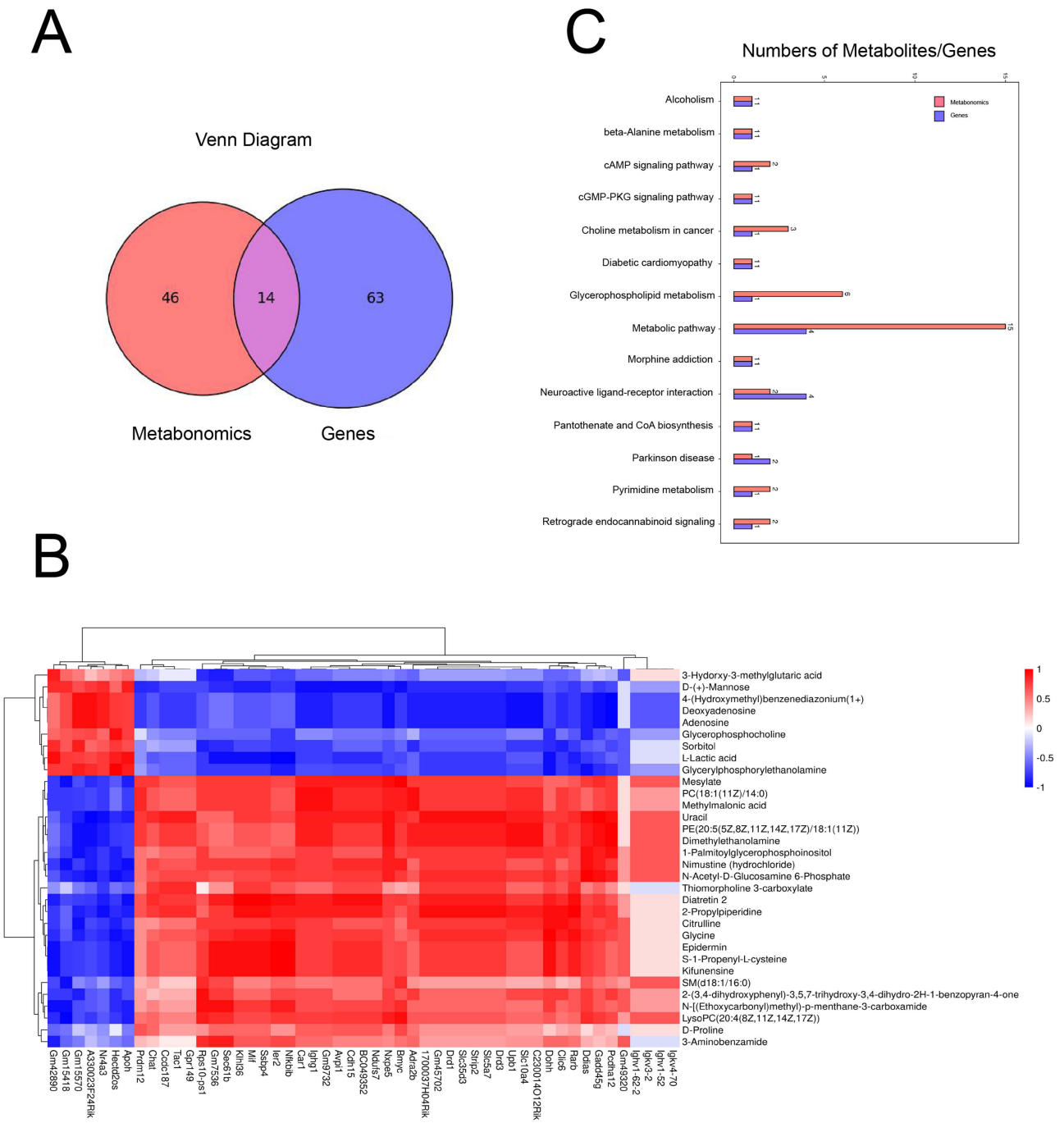

Figure 6 Integrated analysis of metabolomics and transcriptomics data.

Notes: (**A**) Venn plot of metabolomics and transcriptomics. The red and blue circles represent the enriched pathways of metabolomics and transcriptomics, respectively. The purple represents the enriched pathways involving both the transcriptome and metabolome. (**B**) The correlations of DEGs and DEMs by Spearman algorithm. Red and green represent the positive and negative correlation, respectively. The color labels represent Spearman correlation coefficient values. (**C**) KEGG pathway enrichment of differential genes and metabolites. The red and blue bars represent the metabolites and genes, respectively. Numbers represent the number of DEMs and DEGs involved in this pathway, respectively.

animal behavior test showed that MOTS-c had a neuroprotective effect on TBI model mice. Behavioral experiments demonstrated that MOTS-c improved the neural function of TBI mice. This was primarily reflected in an increase in the total walking distance of mice during the OFT test, longer time spent in the target quadrant during the MWM test, reduced time spent on the balance beam, and a higher composite score in the BBT test. Then, we combined transcriptomics and metabolomics to explore the mechanisms that MOTS-c's neuroprotective effect in TBI mice. A total of 159

DEGs and 491 DEMs were identified between the MOTS-C group and the TBI group. KEGG pathway analysis revealed a total of 14 pathways associated with DEGs and DEMs. These pathways are primarily related to metabolic pathways, including Glycerophospholipid metabolism, Pantothenate and coenzyme A (CoA) biosynthesis, β-Alanine metabolism, and Nucleotide metabolism. Additionally, several signaling pathways were also enriched, such as neuroactive ligand–receptor interaction, retrograde endocannabinoid signaling, cyclic Adenosine monophosphate (cAMP) signaling pathway, and cyclic guanosine monophosphate-protein kinase G (cGMP-PKG) signaling pathway.

MOTS-c Mitigated the Excessive Inflammatory Response and Cell Death by Down-Regulating MIF Expression

MIF, initially identified and named by Bloom and Bennett in 1966,[42] is a multifunctional cytokine and a crucial immunomodulatory factor. MIF is implicated in various biological processes, such as cell proliferation, angiogenesis, and cell chemotaxis.[43] MIF has been associated with autoimmune diseases, cardiovascular diseases, Alzheimer's disease, and more.[44–46] Functioning as an inflammatory and stress regulator, MIF can directly or indirectly stimulate the release of inflammatory factors like tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), under stress conditions, including hypoxia and injury.[47] Furthermore, MIF induces apoptosis and necroptosis in endothelial cells by activating a receptor-interacting protein kinase 1 (RIPK1)-dependent pathway, leading to neural dysfunction and cognitive impairment in brain injury.[48–52] In our experiment, we observed a significant down-regulation of MIF expression in the MOTS-c group compared to the TBI group. Therefore, we summarized that damage to brain tissue and hypoxia after TBI activated MIF, releasing inflammatory factors like TNF-α, IL-6, and IL-1β either directly or indirectly. Additionally, MIF activated the RIPK1 receptor pathway, mediating neuronal apoptosis and functional disruption. Peripheral MOTS-c could inhibit the activation of MIF, thereby blocking the release of inflammatory factors and preventing the activation of the RIPK1 receptor pathway, safeguarding the integrity and function of nerve cells (Figure 7).

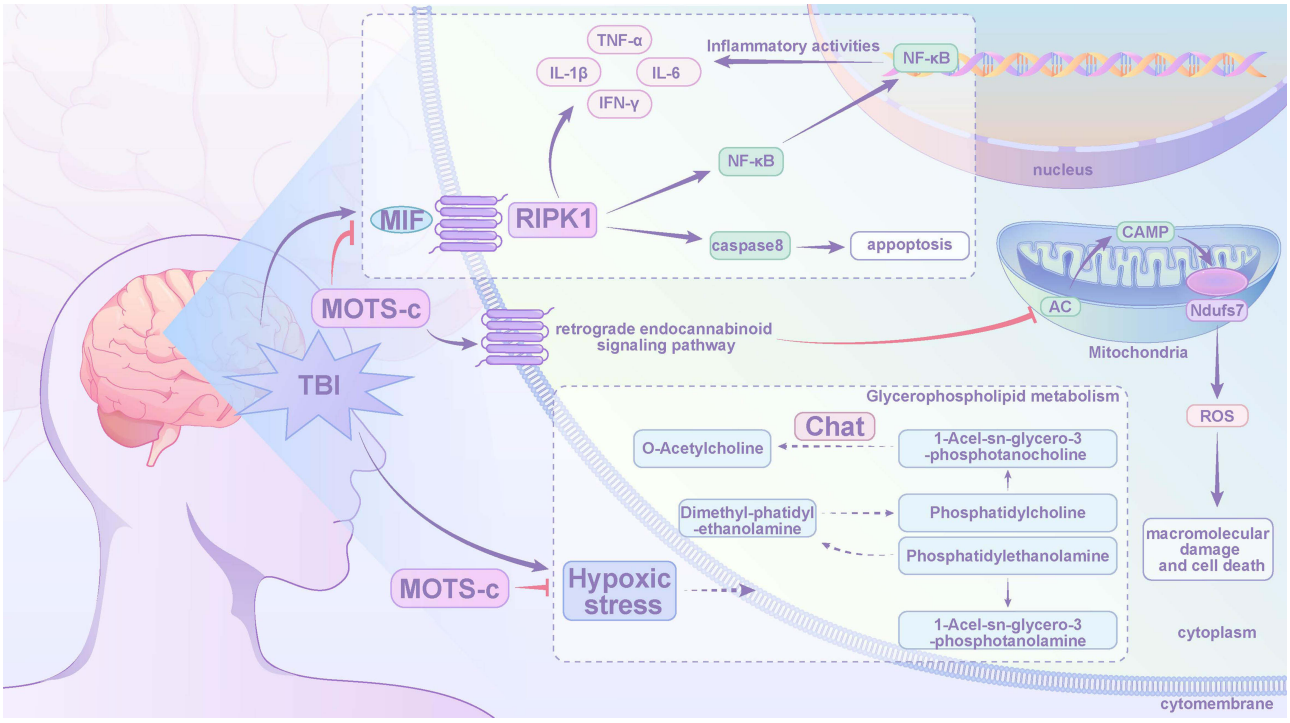

Figure 7 The possible neuroprotective mechanism by MOTS-c in the TBI mice.
Notes: down-regulation: MIF, RIPK1, TNF-α, IL-1, IL-6, IFN-γ, Ndufs7, ROS, Chat, Glycerophospholipid metabolism; up-regulation: retrograde endocannabinoid signaling pathway.

MOTS-c Inhibited Oxidative Phosphorylation to Produce Reactive Oxygen Species (ROS), Reducing Molecular Destruction and Cell Death by Activating the Retrograde Endocannabinoid Signaling Pathway

NDUFS7 encoded enzyme as one of the ubiquinone-binding sites of Complex I, involving in the electron transport system of the respiratory chain. ROS are radicals, ions, or molecules with a single unpaired electron in their outermost electron shell. Endogenous ROS can be generated through mitochondrial electron transport-chain reactions and nicotinamide adenine dinucleotide phosphate oxidase.[53,54] Various cellular stimuli, including pathogen invasion, inflammation, traumatic brain injury, growth factors, and calcium signaling, contribute to the overproduction of ROS.[55] Excessive ROS could result in plasma membrane damage and increased mitochondrial membrane permeability, followed by macromolecular damage and cell death.[56] The retrograde endocannabinoid signaling pathway, when activated by endocannabinoids, inhibits the adenylate cyclase-mediated CAMP-PKA pathway, subsequently suppressing oxidative phosphorylation and ROS production.[57,58] We generalized that TBI induced excessive production of ROS in brain tissue, which in turn caused damage to cell membranes, increased mitochondrial membrane permeability, and subsequent macromolecular damage and cell death. MOTS-c activated the endocannabinoid signaling pathway, further stimulating the cannabinoid receptor 1 (CB1), and through Gi/Go protein coupling, inhibiting the cAMP-PKA pathway mediated by adenyl cyclase (AC), reducing the activity of NDUFS7, further suppressing oxidative phosphorylation and ROS production. This was supported by the observed downregulation of the NDUFS7 gene and the enrichment of the retrograde endocannabinoid signaling pathway in our experiment (Figure 7).

MOTS-c Alleviated the Response to Hypoxic Stress and Enhanced Lipid β-Oxidation to Provide Energy for the Body Following TBI

A main cause of secondary brain injury after severe TBI was cerebral hypoxia.[59] This might be related to intracranial hypertension and low cerebral perfusion pressure. Cerebral hypoxia induced a series of problems, such as metabolic derangements, disruption of the microcirculation, and mitochondrial dysfunction, which aggravated the poor prognosis of TBI patients.[60] In a previous study, it was found that many metabolites of the glycerophospholipid pathway, such as phosphatidylcholine, phosphatidylethanolamine, phosphoinositide, phosphatidyl-N-dimethylethanolamine, and phosphatidic acids, were significantly upregulated in response to hypoxic stress, contributing to the maintenance of cell membrane stability and reduction in cell injury.[61] In our experiment, phosphatidylcholine, phosphatidylethanolamine, 1-Acyl-glycero-3-phosphocholine, Phosphatidyl-N-dimethylethanolamine, and choline acetyltransferase (encoded by the chat gene) were significantly upregulated in response to hypoxic stress by TBI in the TBI group. This was consistent with previous studies. However, after the application of MOTS-c, the results were reversed. This demonstrated that MOTS-c improved the hypoxic environment in brain tissue after TBI, alleviating the response to hypoxic stress, reducing the cellular demand for glycerophospholipid metabolites, and enhancing lipid β-oxidation to provide energy for the organism (Figure 7).

In omics experiments, due to various differences between individuals, such as genetic factors, phenotype differences, and experimental technique differences, ultimately led to differences in results among individuals within the same group. It was very difficult to completely eliminate these differences. Understanding these differences and minimizing them as far as possible was crucial for the correct interpretation of experimental results and the derivation of accurate conclusions. Based on previous literature on omics, results were considered reliable when there were at least 3 individual samples within the same group. Therefore, our individual samples in each group were at least 3, and the metabolomic experiments were even 7 individual samples due to the instability of metabolites. Additionally, although our experimental results showed a great deal of variability among some individuals within the group, the overall trend was the same, and the differences between groups were also significant. Therefore, we believed that our experimental results were reliable and accurate. Of course, considering that differences between individuals with 3 samples are still significant, we will use at least 7 samples to minimize the interference of individual differences on the experimental results as much as possible in future research.

Conclusion

In summary, we integrated the transcriptomic and metabolomic to explore the neuroprotective mechanism of MOTS-c on the TBI mice. We observed that MOTS-c mitigated the inflammatory response, decreased molecular damage, and reduced cell death by downregulating MIF expression and activating the retrograde endocannabinoid signaling pathway. In addition, MOTS-c alleviated the response to hypoxic stress and promoted the β-oxidation of lipids to provide energy for the body after TBI. Compared to previous research methods, multi-omics combination method was more innovative and comprehensive, making it easier to uncover the mechanisms of the neuroprotective effects of MOTS-c in mice after TBI. On the other hand, compared to other therapeutic strategies, MOTS-c possessed unique advantages in the treatment of TBI. It not only protected neurons from damage through various pathways but also regulated the brain tissue's hypoxic environment, reduced cellular membrane damage, and promoted energy metabolism. This offered new perspectives and approaches for the treatment of TBI. The findings of this study contributed to a better understanding of the neuroprotective mechanism of MOTS-c.

However, there were limitations in the present study. Due to peripheral administration of MOTS-c did not cross the BBB and entered the central nervous system, we could not demonstrate whether MOTS-C had the same neuroprotective effects on normal mice.

Furthermore, the mechanism needed to be further verified by experiments, including the expression of various molecules, genes and metabolites in the pathway. This will be our following work.

Additionally, considering the differences between mouse models and human pathophysiology, our conclusions are still a long way from being translated into clinical trials. We need do much work to facilitate this transformation as much as possible. Although our experimental TBI mice model is already very close to human pathophysiology after TBI, we can conduct experiments using TBI model in other animals to validate the reliability and consistency of the results. We also further validate the results obtained in animal models at the cellular and molecular levels. In summary, despite the differences between mice models and human pathophysiology, animal experiment results can still be effectively translated to the prevention, diagnosis, and treatment of human diseases through the design of experiments, rigorous data analysis, and the use of translational medicine research methods.

Ethics Approval

The animal study was reviewed and approved by the ethics committee of Jining First People's Hospital (protocol no. JNRM-2023-Dw-105).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; agreed to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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