Research Article

# Protein profiling identified mitochondrial dysfunction and synaptic abnormalities after dexamethasone intervention in rats with traumatic brain injury

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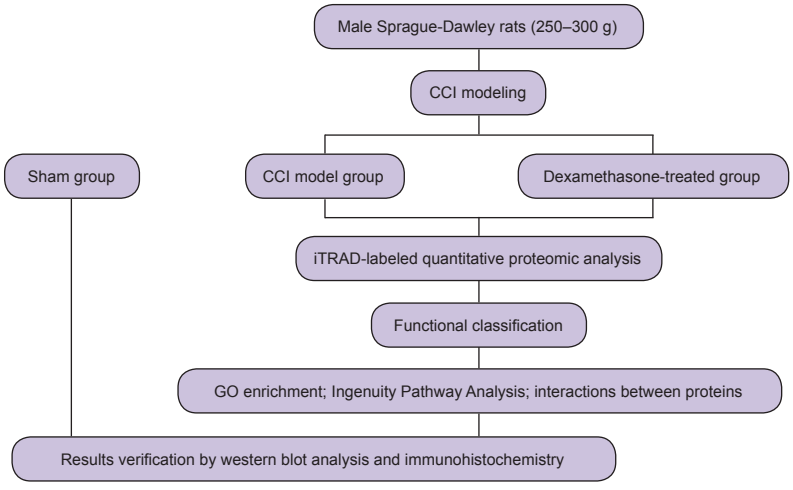
**Graphical Abstract** *iTRAQ-labeled proteomics to identify the protein expression change in the mitochondrial dysfunction pathway and synaptogenesis signaling pathway after TBI*




## Abstract

Dexamethasone has been widely used after various neurosurgical procedures due to its anti-inflammatory property and the abilities to restore vascular permeability, inhibit free radicals, and reduce cerebrospinal fluid production. According to the latest guidelines for the treatment of traumatic brain injury in the United States, high-dose glucocorticoids cause neurological damage. To investigate the reason why high-dose glucocorticoids after traumatic brain injury exhibit harmful effect, rat controlled cortical impact models of traumatic brain injury were established. At 1 hour and 2 days after surgery, rat models were intraperitoneally administered dexamethasone 10 mg/kg. The results revealed that 31 proteins were significantly upregulated and 12 proteins were significantly downregulated in rat models of traumatic brain injury after dexamethasone treatment. The Ingenuity Pathway Analysis results showed that differentially expressed proteins were enriched in the mitochondrial dysfunction pathway and synaptogenesis signaling pathway. Western blot analysis and immunohistochemistry results showed that Ndufv2, Maob and Gria3 expression and positive cell count in the dexamethasone-treated group were significantly greater than those in the model group. These findings suggest that dexamethasone may promote a compensatory increase in complex I subunits (Ndufs2 and Ndufv2), increase the expression of mitochondrial enzyme Maob, and upregulate synaptic-transmission-related protein Gria3. These changes may be caused by nerve injury after traumatic brain injury treatment by dexamethasone. The study was approved by Institutional Ethics Committee of Beijing Neurosurgical Institute (approval No. 201802001) on June 6, 2018.

**Key Words:** dexamethasone; Gria3; Maob; mass spectrometry; mitochondrial dysfunction; Ndufs2; Ndufv2; proteomics; synaptic abnormalities; traumatic brain injury

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## Introduction

Traumatic brain injury (TBI) is the second most common type of trauma following limb fractures and the leading cause of death and disability among young and middle-aged adults, causing a heavy burden on society and families (Maas et al., 2017). TBI can be divided into primary and secondary brain injuries. Secondary brain injury refers to neurological dysfunction and neuronal damage caused by a series of neurobiochemical processes triggered by primary brain injury that can last for several weeks, months or even a lifetime (Wilson et al., 2017). A previous study has found that the mechanism of secondary brain injury is extremely complex and mainly includes mitochondrial dysfunction, apoptosis, and limbic system dysfunction and cognitive impairment caused by hypothalamus-pituitary-adrenal axis injury (Eakin and Miller, 2012; Zhou et al., 2020). The occurrence and development of injury during this period seriously affect the recovery and prognosis of neurological function in patients with TBI. Therefore, treatment of secondary nerve injury after TBI is an important target for TBI patients (Loane and Faden, 2010).

Glucocorticoids are secreted under stress conditions and are necessary for the survival of organisms. The high expression of mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) in the hippocampus makes this region the main target of glucocorticoids in the brain (Reul and de Kloet, 1985). MR and GR interact with each other, and they may have complementary or opposite functions because of the difference of the cell type and conditions. It is very important for hippocampus' normal functions and cell survival to maintain a balance between the expression and activation of MR and GR. Based on the findings from experiment and clinic studies, a hypothesis of the balance between MR and GR, which argues that the reason for hypothalamus-pituitary-adrenal axis dysregulation may be related to the imbalance in MR:GR signaling pathways, thereby causing susceptibility to stress-related neurodegeneration and mental disorders (De Kloet et al., 1998; de Kloet et al., 2018). Supporting this hypothesis is evidence that changes in the spinous properties of cortical neurons that occur during the dexamethasone-induced sleep awakening cycle can be restored by supplementing MR with a corticosterone substitute (Ikeda et al., 2015); and the neuropsychological side effects and sleep disorders caused by dexamethasone can be reversed by the replacement dose of cortisol in young patients with acute lymphoblastic leukemia (Warris et al., 2016).

Accumulating studies have shown that the neuronal survival in target structures is closely related to the continuous activation of MR, while the inhibition of neurogenesis and the increase of neuronal apoptosis are related to the excessive or long-term activation of GR, which subsequently leads to cognitive, emotional and stress disorders (McCullers et al., 2002; Czekajlo and Milbrandt, 2005). According to the latest guidelines for the treatment of TBI in the United States (Brain Trauma Foundation et al., 2007), high-dose glucocorticoids cause neurological damage; therefore, the routine use of high-dose glucocorticoids after TBI is not recommended. Further clinical studies investigating the mechanisms of glucocorticoid-induced neurological damage and the dosage and forms of glucocorticoid administration should be performed before the implementation of high-dose glucocorticoids in clinical practice. Our previous study found that corticosterone can protect hippocampal neurons and reduce damage to spatial learning and memory by adjusting the MR/GR balance (Zhang et al., 2020a). Additionally, use of GR agonist dexamethasone increases cell loss in the hippocampus and paraventricular nucleus, aggravates spatial memory impairment, and increases the incidence of critical illness-related corticosteroid insufficiency and mortality (Zhang et al., 2019, 2020a, b). To further explore the causes of the harmful effects of high-dose glucocorticoids on trauma and provide a theoretical basis for the rational clinical application of glucocorticoids, the purpose of this study was to determine rat hippocampal protein expression during the acute phase following trauma using isobaric tabs for relative and absolute quantitation (iTRAQ)-based proteomics. To discover important proteins closely related to the decline in mitochondria and cognitive function, we analyzed differentially expressed proteins under different conditions and identified a group of proteins that may be closely related to the occurrence and development of brain injury and may serve as the basis for subsequent work.

## Materials and Methods

### Animals

Sex has a profound effects on the outcome of ischemic or traumatic brain injury, which could be due to the differences of sex steroid hormones between males and females (Roof and Hall, 2000; Spychala et al., 2017). Therefore, we only used male rats to remove the effects of sex hormone and ensure the data homogeneity. Male specific pathogen-free/viral antibody-free Sprague-Dawley rats (aged 6–7 weeks, weighing 250–300 g) were purchased from Beijing Vital River Laboratory (Beijing, China; licence No. SCXK (Jing) 2016-0006). The rats were kept in clean animal rooms, and the average room temperature and relative humidity were controlled at 22 ± 2°C and 55 ± 5%, respectively. During the modeling process, the animals were allowed to eat and drink water freely, and the room temperature was kept at 23 ± 1°C. The animal room was maintained under an alternating light (8:00–20:00)/dark (20:00–8:00) cycle. All experiments were performed in accordance with the standards established by the National Institutes of Health for the Care and Use of Laboratory Animals, and the experimental protocols were approved by the Institutional Ethics Committee of Beijing Neurosurgical Institute (approval No. 201802001) on June 6, 2018.

### Rat controlled cortical injury model and drug administration

The rats were anesthetized with isoflurane inhalation and placed on a stereotaxic frame (RWD Life Science Co., Shenzhen, China). The scalp was cut, the soft tissue was separated, and a bone window approximately 1.5 mm wide was opened between the right side of bregma and lambda using a high-speed drill (approximately 6 mm in diameter; RWD Life Science Co.) without damaging the underlying dura. Controlled cortical injury (CCI) model was established using a PCI 3000 PinPoint Precision Cortical Impactor (Hatteras Instruments, Cary, NC, USA). The model parameters are as follows: the diameter of the impactor was 5 mm, the impact velocity was 2.8 m/s, the impact depth was 2 mm, and the holding time was 85 ms (Taylor et al., 2010). After surgery, the skull cap was repositioned and sutured well with dental acrylic. The wound was sutured. The rats were randomly divided into the CCI model and dexamethasone-treated groups. Dexamethasone (Cat# D4902, Sigma-Aldrich Corp., St. Louis, MO, USA) was dissolved in sterile 0.9% NaCl solution containing dimethyl sulfoxide (at a final concentration < 1%). The injection volume was less than 2 mL per day. The dexamethasone-treated group was given dexamethasone (10 mg/kg) intraperitoneally within 1 hour after surgery, and the CCI model group was given the same volume of solvent through the abdominal cavity within 1 hour after injury. Dexamethasone (10 mg/kg) was given again between 9:00 and 11:00 on the second day after TBI. The sham group underwent the same procedure without percussion, and was administered intraperitoneally the same volume of solvent within 1 hour after injury.

### iTRAQ-labeled quantitative proteomic analysis

Forty-eight hours after CCI, the rats were sacrificed under isoflurane inhalation and hippocampal tissues were collected from the CCI model and dexamethasone-treated groups. Total protein was extracted using nondenatured protein lysate

(Applygen, Beijing, China), and protein concentration was detected by a Pierce Bicinchoninic acid assay protein assay kit (Thermos, Waltham, MA, USA). One hundred micrograms of protein from each group was denatured, reduced and alkylated according to the instructions of AB Sciex (Framingham, MA, USA). Protein digestion was performed with 20 μg trypsin at 37°C overnight. According to the instructions of the quantitative proteomics kit (AB Sciex, Framingham, MA, USA), the iTRAQ reagent was added to each tube with 150 μL isopropanol, and the tube was vortex-shocked and centrifuged; the iTRAQ reagent was added to the dissolution buffer-dissolved condensed peptide segment (the digestible protein products of the hippocampal tissues from five CCI model rats and five dexamethasone-treated rats were labeled 118 and 121, respectively), vortex-shocked and reacted at room temperature for 2 hours; then, 100 μL water was added to terminate the reaction. The labeled digested products were freeze-dried under a vacuum. The digested products were mixed and analyzed by liquid chromatography (Eksigent, Framingham, MA, USA) (strong cation exchange column 4.6 × 250 mm, 5 μm-C18, 100 Å). After separation with a chromatographic column, the digested products were divided into 48 components and then collected by vacuum freeze-drying. The 48 components were mixed and redivided into 10 components for the analysis by nano-liquid chromatography (LC) (Eksigent, MA, USA) (desalination column: 350 μm × 0.5 mm, 3 μm-C18 120 Å and analysis column: 75 μm × 150 mm, 3 μm-C18 120 Å) and protein mass spectrometry (MS). The ProteinPilot™ software (Applied Biosystems) package can identify and annotate protein MS data. The software package uses the Mascot 2.2 search engine and SwissProt database to search and compare protein peptides. The specific parameters are as follows: peptide mass error, ±20 ppm; fragment mass error, ±0.1 Da; and peptide false detection rate, ≤ 0.01.

**Bioinformatics analysis**

A Gene Ontology (GO) analysis was performed via GENEONTOLOGY (http://geneontology.org), and rat (*Rattus norvegicus*) was selected as the analysis background and species. Ingenuity Pathway Analysis software (Qiagen, Dusseldorf, Germany) was used to analyze the enrichment of the differentially expressed proteins in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and identify proteins and pathways related to mitochondrial dysfunction and synaptogenesis after TBI and treatment with dexamethasone. The interaction between the proteins was analyzed by the CluePedia program in Cytoscape software (http://www.cytoscape.org/).

**Western blot analysis and immunohistochemical verification**

Western blot analysis and immunohistochemistry were performed to verify the expression of nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) Fe-S protein 2 (Ndufs2, nicotinamide adenine dinucleotide dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial (Ndufv2), monoamine oxidase (Maob) and glutamate receptor 3 (Gria3) and verify the reliability of the iTRAQ technology.

***Western blot analysis***

Rats were sacrificed under isoflurane inhalation, the hippocampal tissue (100 mg) was collected, and tissue lysate was added ($n$ = 5/group). The tissue was ground by an electric homogenizer (IKA, Staufen, Germany) and centrifuged for 20 minutes at 12,000 × $g$, and the supernatant was collected.

Protein was quantified by a bicinchoninic acid assay kit and denatured in a metal salt bath. According to the molecular mass of the target protein, a 10% separation gel and a 4% concentrated gel were prepared. The voltage was adjusted from 80 V to 120 V when bromophenol blue dye entered the separation gel for approximately 1 hour, and electrophoresis was completed when the dye reached the bottom of the gel. Then, polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) was blocked with 5% skimmed milk in Tris-buffered saline containing 1% Tween 20 at room temperature for 1 hour. The target proteins were detected by corresponding primary antibodies at 4°C overnight. Then, the membrane was washed with Tris-buffered saline containing 1% Tween-20 three times for 10 minutes per wash. The membrane was incubated with a goat anti-rabbit (Millipore, Cat# AP132P, 1:2000) or anti-mouse (Millipore, Cat# AP124P, 1:2000) IgG (H + L) horseradish peroxidase-conjugated secondary antibodies at room temperature for 1.5 hours. After washing with Tris-buffered saline containing 1% Tween 20, electrochemiluminescence images were obtained. The following primary antibodies were used in these experiments: Ndufs2 (1:1000; Cat# ab192022, Abcam, Cambridge, UK), Ndufv2 (1:1000; ab183715, Abcam), Maob (1:500; Cat# HPA002328, Atlas Antibodies AB, Bromma, Sweden), Gria3 (1:400; Cat# AGC-010, Alomone Labs, Jerusalem, Israel), and β-actin (1:2000; Sigma-Aldrich Corp.). The blots were visualized by chemiluminescence (Millipore, MA, USA). The images were quantified using the Bio-Rad Quantity One software. β-Actin was used as an internal control for the hippocampal fractions.

***Immunohistochemistry***

Rats were anesthetized with isoflurane inhalation 48 hours after surgery ($n$ = 5/group). Paraformaldehyde (4%) was perfused into the heart, and the brain was removed for histochemical analysis. Paraffin-embedded sections (4 μm) were dewaxed in water and then placed in 3% hydrogen peroxide for 15 minutes at room temperature, followed by high-pressure boiling with ethylenediaminetetraacetic acid repair solution for 3 minutes. Then, the sections were allowed to cool naturally. The sections were washed with phosphate-buffered saline (PBS) three times for 5 minutes per wash and then blocked in normal goat serum (Zhongshan Goldenbridge Biotechnology, Beijing, China) for 20 minutes at 37°C. The sections were incubated with the primary antibodies (Ndufs2, 1:250, Cat# ab192022, Abcam; Ndufv2, 1:300, Cat# ab183715, Abcam; Maob, 1:600, Cat# HPA002328, Atlas Antibodies AB, Bromma, Sweden; and Gria3, 1:700, Cat# AGC-010, Alomone Labs, Jerusalem, Israel) overnight at 4°C. The sections were washed with PBS three times for 5 minutes per wash and then incubated with polymer adjuvant (ready to use; Zhongshan Goldenbridge Biotechnology, Beijing, China) for 20 minutes at 37°C. The sections were washed again with PBS three times for 5 minutes per wash and then incubated with horseradish-peroxidase-conjugated anti-rabbit IgG polymer (Cat# PV-9001, Zhongshan Goldenbridge Biotechnology, Beijing, China) for 20 minutes at 37°C. The sections were washed with PBS three times for 5 minutes per wash, developed in 3,3′-diaminobenzidine for nuclear staining and microscopic observation, dehydrated, and sealed. Under a light microscope, the whole section was scanned by a digital slice scanning system (AT2, Leica Biosystems, Buffalo Grove, IL, USA). For quantification of immunoreactive cells, five fields of view were randomly selected under a high-power field (100× for the quantification and 200× to obtain representative images of the animals in all groups), and the number of immunoreactive cells in each image was counted and quantified using IPP 6.0 software (produced by Media Cybernetics Incorporation, Rockville, MD, USA). The data are presented as the average density of the immunoreactive cells within the ipsilateral traumatic hemisphere ($mm^2$).

**Statistical analysis**

All statistical analyses were performed using SPSS statistics version 19.0 (IBM Corp., Armonk, NY, USA), and the results are expressed as the mean ± standard deviation (SD). Then, a least significant difference *post hoc* analysis through one-way analysis of variance was used to compare the differences among the three groups. Results with a $P$-value < 0.05 were considered statistically significant.

## Results

### Proteomics analysis of hippocampal tissues in CCI model and dexamethasone-treated rats

To investigate the proteins differentially expressed between the CCI model and dexamethasone-treated groups, we employed iTRAQ-based proteomics. We chose a high dose of dexamethasone (10 mg/kg) because this dose aggravated neuronal apoptosis in the hippocampus after TBI, which is directly associated with increased mortality and morbidity (Chen et al., 2013). The iTRAQ-labeled protein MS data identified 3134 proteins in this study. Among these proteins, the expression of 48 proteins significantly differed between the dexamethasone-treated and CCI model groups ($P < 0.05$).

Compared to the CCI model group, Volcano plots illustrated that 31 differentially expressed proteins were upregulated with a fold change greater than 1.2, and 12 differentially expressed proteins were downregulated with a fold change lower than 0.9 in the dexamethasone-treated group (**Figure 1A**). The fold changes and *P*-values of the differentially expressed proteins between the groups are shown in **Table 1**.

**Table 1** | **Differentially expressed proteins with fold changes between the Dex-treated and the CCI model groups**

| Accession | Protein name | Gene symbol | CCI + Dex/CCI (fold change) | *P*-value |
|---|---|---|---|---|
| P16086 | Spectrin alpha chain, non-erythrocytic 1 | *Sptan1* | 2.884032 | 9.71E-12 |
| Q9QWN8 | Spectrin beta chain, non-erythrocytic 2 | *Sptbn2* | 3.221069 | 6.22E-07 |
| P06238 | Alpha-2-macroglobulin | *A2m* | 1.958845 | 2.49E-06 |
| P46101 | Dipeptidyl aminopeptidase-like protein 6 | *Dpp6* | 0.253513 | 0.000162 |
| P02770 | Serum albumin | *Alb* | 0.972747 | 0.000573 |
| Q9ERH3 | WD repeat-containing protein 7 | *Wdr7* | 2.070141 | 0.001842 |
| P12346 | Serotransferrin | *Tf* | 0.758578 | 0.00208 |
| P47819 | Glial fibrillary acidic protein | *Gfap* | 2.228435 | 0.00264 |
| P01026 | Complement C3 | *C3* | 0.717794 | 0.002932 |
| Q641Y2 | Nicotinamide adenine dinucleotide dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial | *Ndufs2* | 2.051162 | 0.00308 |
| Q62910 | Synaptojanin-1 | *Synj1* | 0.704693 | 0.003748 |
| P30427 | Plectin | *Plec* | 1.342765 | 0.004041 |
| Q03555 | Gephyrin | *Gphn* | 2.290868 | 0.007823 |
| P08413 | Calcium/calmodulin-dependent protein kinase type II subunit beta | *Camk2b* | 2.070141 | 0.008551 |
| Q62950 | Dihydropyrimidinase-related protein 1 | *Crmp1* | 1.258925 | 0.009871 |
| Q63041 | Alpha-1-macroglobulin | *A1m* | 0.765597 | 0.010889 |
| O55166 | Vacuolar protein sorting-associated protein 52 homolog | *Vps52* | 1.614359 | 0.011282 |
| P25809 | Creatine kinase U-type, mitochondrial | *Ckmt1* | 0.580764 | 0.014806 |
| P02764 | Alpha-1-acid glycoprotein | *Orm1* | 2.511886 | 0.01558 |
| Q62638 | Golgi apparatus protein 1 | *Glg1* | 2.051162 | 0.016479 |
| P06686 | Sodium/potassium-transporting ATPase subunit alpha-2 | *Atp1a2* | 2.884032 | 0.017413 |
| Q7TT49 | Serine/threonine-protein kinase MRCK beta | *Cdc42bpb* | 2.070141 | 0.018356 |
| P31596 | Excitatory amino acid transporter 2 | *Slc1a2* | 1.584893 | 0.018601 |
| Q6UPE1 | Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial | *Etfdh* | 0.809096 | 0.018601 |
| P19492 | Glutamate receptor 3 | *Gria3* | 3.404082 | 0.020192 |
| P48004 | Proteasome subunit alpha type-7 | *Psma7* | 1.380384 | 0.02093 |
| Q2IBD4 | Cortactin-binding protein 2 | *Cttnbp2* | 1.853532 | 0.023572 |
| P25286 | V-type proton ATPase 116 kDa subunit a isoform 1 | *Atp6v0a1* | 1.599558 | 0.023622 |
| P34058 | Heat shock protein HSP 90-beta | *Hsp90ab1* | 0.47863 | 0.026206 |
| P11442 | Clathrin heavy chain 1 | *Cltc* | 0.824138 | 0.029249 |
| Q8VHK2 | Caskin-1 | *Caskin1* | 1.527566 | 0.034518 |
| Q5EB62 | Solute carrier family 25 member 46 | *Slc25a46* | 0.242103 | 0.035607 |
| P86252 | Transcriptional activator protein Pur-alpha (Fragments) | *Pura* | 0.586138 | 0.036156 |
| O08722 | Netrin receptor UNC5B | *Unc5b* | 1.180321 | 0.036556 |
| P30904 | Macrophage migration inhibitory factor | *Mif* | 2.208005 | 0.037476 |
| P11960 | 2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial (Fragment) | *Bckdha* | 1.584893 | 0.037983 |
| Q5BJR4 | Protein prune homolog 2 | *Prune2* | 10.18591 | 0.038273 |
| P54758 | Ephrin type-A receptor 6 | *Epha6* | 1.037528 | 0.038546 |
| Q9ER34 | Aconitate hydratase, mitochondrial | *Aco2* | 1.037528 | 0.038651 |
| P07936 | Neuromodulin | *Gap43* | 0.559758 | 0.039749 |
| P34926 | Microtubule-associated protein 1A | *Map1a* | 1.770109 | 0.039777 |
| Q07266 | Drebrin | *Dbn1* | 1.584893 | 0.040201 |
| P29457 | Serpin H1 | *Serpinh1* | 1.706082 | 0.043466 |
| Q9R1K8 | RAS guanyl-releasing protein 1 | *Rasgrp1* | 0.937562 | 0.043764 |
| O35814 | Stress-induced-phosphoprotein 1 | *Stip1* | 1.940886 | 0.04378 |
| Q3KR86 | MICOS complex subunit Mic60 (Fragment) | *Immt* | 2.032357 | 0.044228 |
| P19643 | Amine oxidase [flavin-containing] B | *Maob* | 1.555966 | 0.044403 |
| P19234 | Nicotinamide adenine dinucleotide dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial | *Ndufv2* | 1.393157 | 0.047255 |

CCI: Controlled cortical injury; Dex: dexamethasone.

### Bioinformatics analysis of hippocampal tissues in CCI model and dexamethasone-treated rats

#### *GO annotation and enrichment analysis*

To further assess the effect of dexamethasone on the proteins with differential accumulation patterns, a GO annotation analysis of all proteins was performed to generally describe the location of the proteins in cells and the types and proportions of proteins participating in biological processes and molecular functions. The specific results are shown in **Figure 1**. The GO enrichment analysis of the proteins that were differentially expressed between the CCI model and dexamethasone-treated groups showed that the following terms were enriched in the cellular component category: cell, cell part, membrane, organelle, membrane part, organelle part, protein-containing complex and synapse part. The terms cellular process, metabolic process, cellular component organization or biogenesis, biological regulation and developmental process were enriched in the biological process category. The terms binding, catalytic activity, molecular transducer activity, transporter activity, molecular function regulator and structural molecule activity were enriched in the molecular function pathway.

#### *KEGG pathway annotation and enrichment analysis*

To characterize the signaling pathways enriched by the altered proteins, a KEGG pathway enrichment analysis was adopted. The results of the enrichment analysis of the core pathways using Ingenuity Pathway Analysis software showed that 10 classic pathways (**Figure 2**), including acute phase response signaling, clathrin-mediated endocytosis, LXR/RXR activation, FXR/RXR activation, mitochondrial dysfunction, atherosclerosis signaling, iron homeostasis signaling, the synaptogenesis signaling pathway, the glutamate receptor signaling pathway, and the Sertoli cell-Sertoli cell junction signaling pathway, exhibited the most significant enrichment. Among these proteins, Aco2, Maob, Ndufs2 and Ndufv2 were enriched in the mitochondrial dysfunction pathway; Camk2b, Epha6, Gria3, and Rasgrp1 were enriched in the synaptogenesis signaling pathway; and Gria3 and Slc1a2 were enriched in glutamate receptor signaling. The above proteins were all upregulated in the dexamethasone treatment group compared with CCI model group.

#### *Protein-protein interaction analysis*

Subsequently, we determined the relationship between the proteins analyzed by Cytoscape software (**Figure 3**), and the results showed that Ndufs2 interacted with Ndufv2, which also interacted with other proteins, such as Aco2, Etfdh, and Immt. Maob could interact with Sptan1, and Gria3 could interact with Camk2b and Slc1a2, which are enriched in the synaptogenesis signaling pathway and glutamate receptor signaling, respectively.

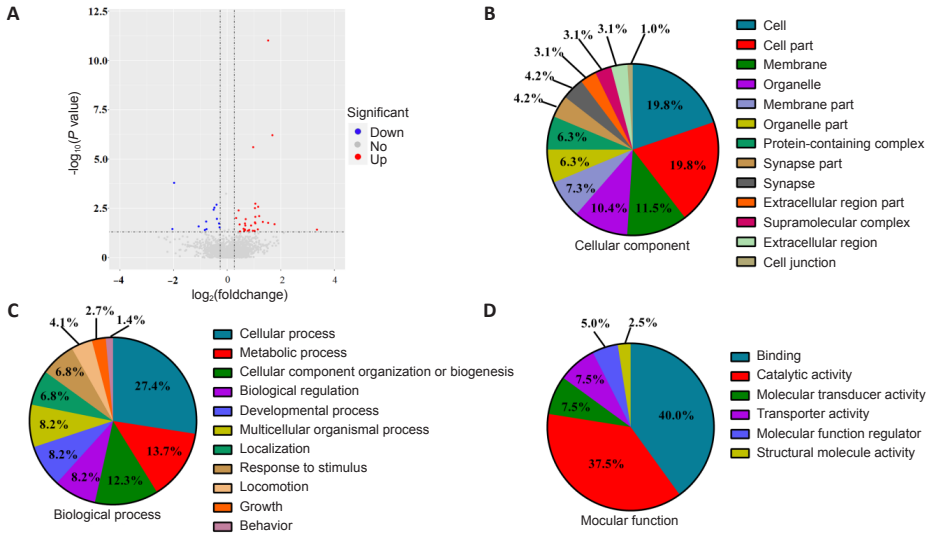


**Figure 1 | Determination of differentially regulated proteins by isobaric tabs for relative and absolute quantitation-labeled quantitative proteomic analysis in dexamethasone-treated and CCI model groups.**

(A) Volcano plot analysis of differentially expressed proteins in CCI rats treated with dexamethasone or not. Blue, gray or red color indicates the downregulated, unchanged or upregulated proteins in the dexamethasone-treated and CCI model groups, respectively. (B–D) Cellular component analysis (B), biological process analysis (C) and molecular function (D) of these proteins in CCI rats treated with dexamethasone or not. CCI: Controlled cortical injury.

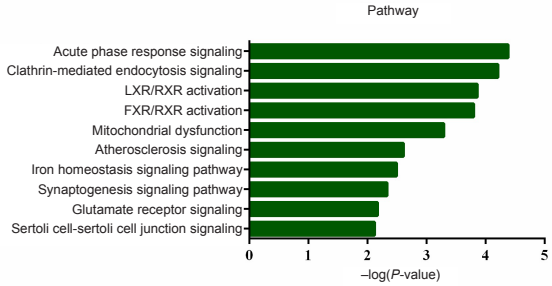


**Figure 2 | Diagram of ingenuity pathway analysis of signaling pathway proteins that are differentially expressed in the dexamethasone-treated and CCI model groups.**

Acute phase response signaling, clathrin-mediated endocytosis, LXR/RXR activation, FXR/RXR activation, mitochondrial dysfunction, atherosclerosis signaling, iron homeostasis signaling, the synaptogenesis signaling pathway, the glutamate receptor signaling pathway, and the Sertoli cell-Sertoli cell junction signaling pathway were the most significant enrichment pathways. CCI: Controlled cortical injury.

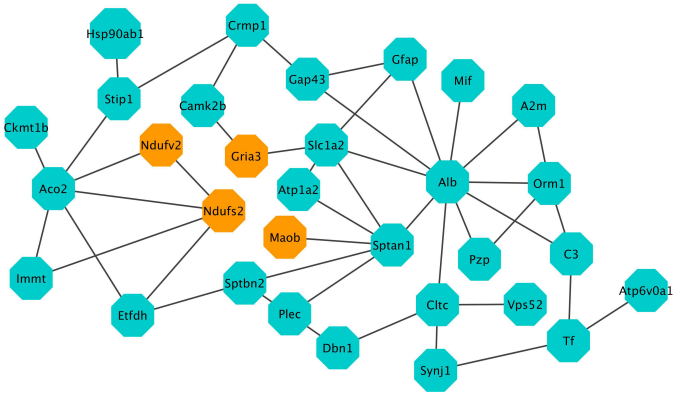


**Figure 3 | Interactions between proteins in controlled cortical injury rats treated with dexamethasone or not were analyzed by Cytoscape software.**

Ndufs2 interacted with Ndufv2, which also interacted with Aco2, Etfdh, and Immt. Maob could interact with Sptan1, and Gria3 could interact with Camk2b and Slc1a2, which are enriched in the synaptogenesis signaling pathway and glutamate receptor signaling, respectively. Gria3: Glutamate receptor 3; Maob: monoamine oxidase; Ndufs2: nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) Fe-S protein 2; Ndufv2: nicotinamide adenine dinucleotide dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial.

### Result verification by western blot analysis and immunohistochemistry

Based on the results of iTRAQ-labeled proteomics, we first selected Maob, Ndufs2 and Ndufv2 proteins, which are enriched in the mitochondrial dysfunction pathway, to confirm mitochondrial dysfunction in TBI caused by dexamethasone. Second, we detected the expression of Gria3 in the synaptogenesis signaling pathway and glutamate receptor signaling and confirmed that dexamethasone interfered with the abnormal expression of Gria3, resulting in abnormal synaptic plasticity in the model of CCI.

To verify the changes in protein accumulation measured in the iTRAQ analysis, we performed western blot analysis and immunohistochemistry to analyze selected proteins. As shown in **Figure 4**, the results showed that protein levels of Gria3, Maob and Ndufs2 were significantly decreased in the hippocampus of CCI rats ($P < 0.05$ or $P < 0.01$, *vs*. sham group), treatment with dexamethasone significantly increased the levels of Gria3, Maob, Ndufs2 and Ndufv2 in the hippocampus after TBI ($P < 0.05$ or $P < 0.001$, *vs*. CCI model group), which is essentially consistent with the proteomics results (**Figure 4**).

Consistent with the western blot analysis results, rats in the sham group exhibited clearly visible Gria3-, Maob-, Ndufs2- and Ndufv2-immunoreactive cells. The number of Gria3-, Maob-, Ndufs2- and Ndufv2-immunoreactive cells in the dexamethasone-treated group was significantly greater than that in the CCI model group. The number of Gria3-, Maob-, Ndufs2- and Ndufv2-immunoreactive cells in the CCI model group was similar to that in the sham group (**Figure 5**). Then we investigated the effects of dexamethasone on Gria3, Maob, Ndufs2 and Ndufv2 expression in CCI rats. The positive rate of Gria3, Maob, Ndufs2 and Ndufv2 expression in the dexamethasone-treated group was significantly higher than that in the CCI model group ($P < 0.01$).

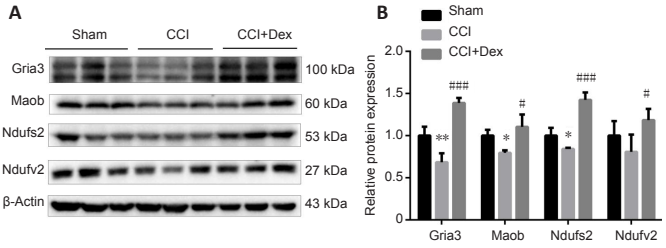


**Figure 4 | Effect of Dex on the expression of Gria3, Maob, Ndufs2 and Ndufv2 proteins in the hippocampal tissue of CCI rats detected by western blot analysis.**

(A) Bands of target proteins. (B) Quantitative results of protein expression, which was normalized by β-actin. The data are presented as the mean ± SD ($n = 5$). *$P < 0.05$, **$P < 0.01$, *vs*. sham group; #$P < 0.05$, ###$P < 0.001$, *vs*. CCI model group (one-way analysis of variance followed by the least significant difference analysis). CCI: Controlled cortical injury; Dex: dexamethasone; Gria3: glutamate receptor 3; Maob: monoamine oxidase; Ndufs2: nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) Fe-S protein 2; Ndufv2: nicotinamide adenine dinucleotide dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial.

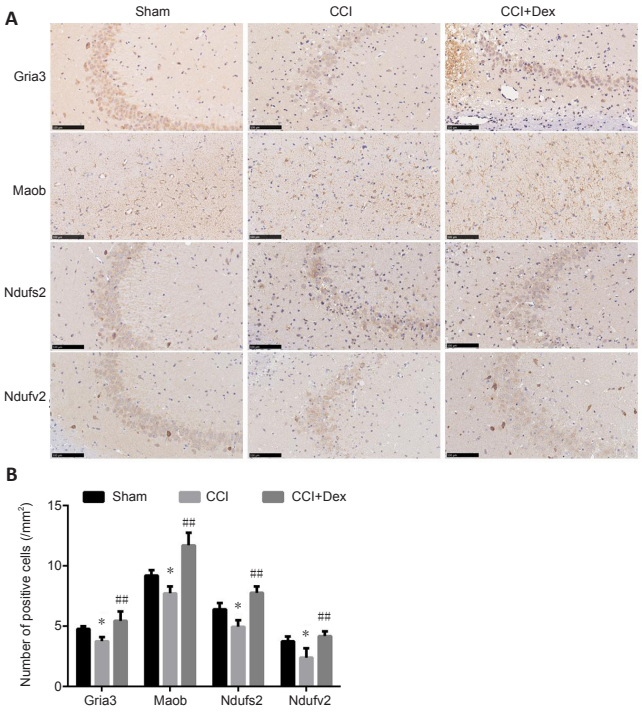


**Figure 5 | Effect of Dex on the Gria3, Maob, Ndufs2 and Ndufv2 positive cells in ipsilateral traumatic hemisphere of CCI rats detected by immunohistochemistry.**

(A) Dexamethasone treatment reversed decrease of Gria3, Maob, Ndufs2 and Ndufv2 positive cells (brown) in ipsilateral traumatic hemisphere. Dexamethasone significantly increased the levels of Gria3, Maob, Ndufs2 and Ndufv2 in the hippocampus after traumatic brain injury. Scale bars: 100 μm. (B) Quantitative results of number of positive cells. The data are presented as the mean ± SD ($n = 5$). *$P < 0.05$, *vs*. sham group; ##$P < 0.01$, *vs*. CCI model group (one-way analysis of variance followed by least significant difference test). CCI: Controlled cortical injury; DEX: dexamethasone; Gria3: glutamate receptor 3; Maob: monoamine oxidase; Ndufs2: nicotinamide adenine dinucleotide dehydrogenase (ubiquinone) Fe-S protein 2; Ndufv2: nicotinamide adenine dinucleotide dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial.

## Discussion

Quantitative proteomics is used to analyze whole protein in a complex system with accurate quantitative methods (Gygi et al., 1999; Qin et al., 2020). Compared with two-dimensional difference gel electrophoresis and traditional two-dimensional gel electrophoresis (Issaq and Veenstra, 2008), quantitative proteomics analyses based on isotope labeling of biological MS have higher sensitivity and accuracy. Among these methods, iTRAQ combined with nano-LC-MS/MS technology can obtain more qualitative and quantitative information regarding proteins. Peptide labeling with an iTRAQ kit has become a main method of high-throughput quantitative proteomics. In this study, iTRAQ quantitative proteomics technology was used to explore proteins related to abnormal mitochondrial function and synaptic plasticity in TBI. Eight proteins that were differentially expressed between the CCI model and dexamethasone-treated groups were enriched in the mitochondrial dysfunction pathway, synaptogenesis signaling pathway and glutamate receptor signaling, and these proteins were significantly upregulated.

A recent study has found that GRs have good nuclear translocation characteristics in many cell lines and that GRs translocate to mitochondria (Koufali et al., 2003). Mitochondria produce the energy required by the body through the Krebs tricarboxylic acid cycle and oxidative phosphorylation. Mitochondria play an additional important role in the regulation of intracellular calcium levels, cytoprotection, and neuroplasticity. Studies have shown that mitochondrial morphology is usually abnormal after TBI and that mitochondrial dysfunction is a key factor in neuronal apoptosis and necrosis after TBI (Mazzeo et al., 2009; Cheng et al., 2012; Balan et al., 2013). Low-dose glucocorticoids can inhibit the activity of the transcription factor nuclear factor-κB, block cyclooxygenase-2, reduce interleukin-1 level, and enhance various mitochondrial functions (Tang et al., 2013; Kasahara and Inoue, 2015). High-dose glucocorticoids can exacerbate neuronal apoptosis in the hypothalamus and hippocampus after trauma (Zhu et al., 2013; Zhang et al., 2020a). This study first confirmed the potential damaging effect of dexamethasone on mitochondrial and synaptic function after TBI. Our results further indicated that dexamethasone activates multiple signaling pathways in TBI, upregulates the levels of Ndufs2, Ndufv2, and Maob in the

mitochondrial dysfunction pathway, and upregulates the expression of Gria3 in the synaptogenesis signaling pathway and glutamate receptor signaling.

Ndufs2 is a subunit of mitochondrial complex I that has oxidoreductase activity, participates in the energy metabolism pathway and can reflect the body's oxidative phosphorylation level. Complex I dysfunction is the most common cause of mitochondrial disorders and can lead to many clinical symptoms, such as neurological diseases, cardiomyopathy, liver damage and myopathy (Loeffen et al., 2001; Dunham-Snary et al., 2019). Furthermore, Ndufv2, which is involved in encoding complex I, is related to mitochondrial oxidative phosphorylation. Ndufs2 and Ndufv2 show a high degree of protein interaction in networks with differentially expressed genes in pathways related to neurological diseases. Complex I is also a main source of reactive oxygen species. Oxidative stress caused by reactive oxygen species is a major cause of neurological diseases (Odashima et al., 2006; Shavali et al., 2008; Murphy, 2009; Nishioka et al., 2010). In this study, we found that high-dose dexamethasone can increase the expression of Ndufs2 and Ndufv2 in CCI rats. It is speculated that high-dose dexamethasone may damage mitochondrial function through Ndufs2 and Ndufv2, cause the release of reactive oxygen species, and then aggravate brain damage.

Mao is a flavin-dependent protein considered to be a mitochondrial enzyme. This enzyme is located on the outer membrane of mitochondria. Mao isoenzymes are divided into type a (Maoa) and type b (Maob) (Johnston, 1968). Among these enzymes, Maob, which widely exists in the brain, heart and adrenal gland in humans and animals, is an important candidate gene in the study of behavioral molecular genetics. Maob is an important candidate gene in behavioral molecular genetic research, and its main role is oxidizing and degrading monoamines, such as serotonin, dopamine, norepinephrine and other neurotransmitters, in the brain and peripheral tissues. Maob affects motor, emotional, and cognitive behavior by regulating the expression levels of these neurotransmitters (Nagatsu, 2004). An abnormal increase in human Maob activity is related to various neuropsychiatric diseases, such as Parkinson's disease, Alzheimer's disease, depression, and schizophrenia (Jo et al., 2014; Ziegler and Domschke, 2018; Zhou et al., 2019). The inhibition of Maob with propargyl amphetamine can significantly reduce lipid peroxidation in the prefrontal cortex, striatum and hippocampus and significantly increase the activity of glutathione peroxidase in the prefrontal cortex and hippocampus (Kiray et al., 2006), suggesting that Maob-mediated metabolism is a source of oxidative stress. In this study, we observed that high-dose dexamethasone upregulated the level of Maob in TBI rats, suggesting that high-dose dexamethasone may be a mechanism underlying the learning and memory loss in TBI rats because it upregulates Maob and promotes oxidative stress.

Glutamate is the most important excitatory amino acid in the central nervous system in mammals. Glutamate receptors (O'Rourke and Boeckx, 2020) can be divided into the following two categories: metabotropic receptors, which are coupled to G proteins to regulate the activity of ion channels and enzymes on the cell membrane, and ionotropic receptors, including N-methyl-D-aspartate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors and kainic acid receptors, which mainly mediate the transmission of fast signals and are directly related to the generation and transmission of neuronal action potentials. Gria3 (also called glutamate receptor 3) is an important α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subtype (Levite, 2014) that is traditionally believed to exist mainly in neurons and astrocytes. After activation, Gria3 causes a large amount of Na+ and Ca2+ influx and a K+ efflux and then mainly mediates rapid excitatory synaptic transmission in the central nervous system. Relevant studies have shown that abnormal Gria3 gene function can cause neuronal damage, which manifests as mental retardation and epilepsy, and participate in migraine (Miyamoto et al., 2012). Recently, increasing attention has been paid to the expression and function of glutamate and its receptors in other tissues or cells; for example, the activation of Gria3 in human T cells can induce T cell adhesion and chemotactic migration (Ganor et al., 2003), suggesting that Gria3 participates in a wider range of physiological and pathological processes. The results of this study show that Gria3 could be expressed in neurons or glial cells in the hippocampus, and the Gria3 levels were upregulated after TBI, suggesting that Gria3 may cause excitatory synapse transmission, neuronal damage and learning and memory performance decline or loss.

Some results obtained in this study provide clues suggesting that the administration of only high-dose dexamethasone could promote TBI neurogenesis, and targeted protein analyses could be the focus of the next step. However, in this study, proteomics research was performed only at the 48-hour time point during the acute phase of brain trauma. Furthermore, based on a previous study (Zhang et al., 2019), only high-dose dexamethasone was selected for the analysis. Therefore, proteomics research and the neurological effects caused by different doses of dexamethasone should be studied in the future.

In summary, this study used iTRAQ-labeled proteomics to identify the upregulation of Maob, Ndufs2, Ndufv2 and Gria3 in mitochondrial function and the synaptic plasticity pathway after TBI. These results indicate that these proteins may represent mechanisms of nerve injury induced by high-dose dexamethasone after trauma and provide a theoretical basis for posttraumatic brain protection, the combined use of brain protective agents for brain injury and the clinical standardized application of glucocorticoids.

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