iTRAQ-Based Protein Profiling in CUMS Rats Provides Insights into Hippocampal Ribosome Lesion and Ras Protein Changes Underlying Synaptic Plasticity in Depression

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Hippocampal atrophy is one of the key changes in the brain implicated in the biology of depression. However, the precise molecular mechanism remains poorly understood due to a lack of biomarkers. In this research, we used behavioral experiments to evaluate anxiety and anhedonia levels in depressed rats using chronic unpredictable mild stress (CUMS) modeling. We also used isobaric tag for relative and absolute quantitation (iTRAQ) to identify the differentially expressed hippocampal proteins between depressed and normal rats. Bioinformatics analyses were also performed for a better understanding. The results showed that CUMS rats had higher anxiety and anhedonia levels than control rats, along with hippocampal lesions. Through iTRAQ and bioinformatics analyses, we found that ribosome proteins were significantly downregulated and Ras proteins exhibited a mixed change in the hippocampus of depressed rats. These findings suggest that the expression of hippocampal ribosome lesions and Ras proteins is significantly different in depressed rats than in control rats, providing new insights into the neurobiology of depression.

1. Introduction

Approximately 50% of suicide victims worldwide suffer from depression or another mood disorder, which makes depression one of the leading causes of disease burden [1, 2]. Efforts have been made to understand the biology of depression. Several theories have been raised regarding the issue. One of the dominant theories is the monoamine hypothesis, which postulates that a deficit of certain neurotransmitters is responsible for depression [3]. The monoamine hypothesis is based on the observation that many antidepressants increase neurotransmitters at synaptic levels [4]. However, the limitation of the monoamine hypothesis is revealed by the 1-2-week therapeutic lag of these antidepressants, such as selective serotonin reuptake inhibitors (SSRIs) [5]. Additionally, researchers have found that serotonin knockout animals do not have typical depressive behaviors [6]. Therefore, the monoamine hypothesis might oversimplify the problem.

The inflammation theory has also been raised, stressing the role of inflammation in depression. Various reviews have found that depression patients have high levels of inflammatory cytokines, such as interleukin-1β, IL-6, and tumor necrosis factor-α (TNF-α) [7]. When exposed to stress, the translation of inflammatory cytokines is activated. Overexpressed inflammatory cytokines travel through the blood-brain barrier (BBB) or are released by microglia and influence brain function [8]. Studies have also demonstrated that anti-inflammatory therapies can alleviate the symptoms [9].

Among all these theories, stressor exposure has proven to be the most robust factor associated with the development of depression [10]. In response to stressors, the hypothalamic-pituitary-adrenal (HPA) axis is activated. Long-term exposure causes HPA axis dysfunction and high levels of...
glucocorticoids, which result in cell loss and compromise neurogenesis in the hippocampus [11]. Therefore, atrophy of the hippocampus is considered one of the main features of the depressed brain, which has been repeatedly observed in humans and rodents [12, 13].

Recent studies have also called attention to the role of disrupted synaptic plasticity (the ability of synapses to strengthen or weaken over time) in depression. Studies have demonstrated that synapse number is significantly reduced in certain crucial brain regions, such as the prefrontal cortex (PFC) and hippocampus [14]. In addition, depressed animals have impaired hippocampal long-term potentiation (LTP), which is a pattern of synaptic activity in which a long-lasting increase in synaptic strength is observed [15].

Proteomic technologies are the ideal techniques for the detection and investigation of biomarker candidates, owing to the high sensitivity and analytical performance that can be achieved and the ability to generate large datasets through the identification of large and ever-increasing numbers of proteins [16–18]. Isobaric tag for relative and absolute quantitation (iTRAQ) is a proteomic approach that can determine the amount of proteins from different sources in a single experiment [19]. This technology has been used to outline the proteomic profiles of cancers [20]. Currently, a number of biomarkers for bladder cancer have been detected in urine and tissue using this technique [21].

In this study, we identified differentially expressed proteins in the depressed and normal hippocampus using iTRAQ. Regarding the abundance of the proteins and their biological information, bioinformatics analyses were performed to identify possible proteins underlying the biology of depression.

2. Methods

2.1. Animals. A total of 25 Wistar rats (males; weight 180-200 g; Southern Medical University Experimental Animal Center) were acclimated to an SPF facility (temperature 24 ± 2°C, humidity 50%-60%) at Southern Medical University, China. Rats were housed individually with a constant 12 h light/dark cycle (lights on/off at 07:00/19:00) unless otherwise noted. Rats were bred normally for at least 6 days for adaption before the CUMS paradigm. Food and water were available ad libitum. Rats were randomly assigned into 2 groups: control (n = 10) and CUMS (n = 15).

2.2. CUMS Paradigm. CUMS rats underwent a 21-day chronic unpredictable mild stress procedure. On each of the 21 consecutive days, rats were exposed to a random stressor (Figure 1(a)). These stressors included water deprivation (24 h), food deprivation (24 h), wet bedding (24 h), light-dark reversal (24 h), stroboscopic lighting (12 h), immobilization (2 h), cold swim (4°C, 5 min), warm swim (45°C, 5 min), level shaking (5 min), and tail clamping (3 min).

2.3. Behavioral Experiments. The sucrose preference test (SPT) was used to assess anhedonia. After a 2-day habituation phase, rats were housed singly for 24 h without any food or water. Then, the rats were presented with two identical bottles containing either sucrose solution (1%) or pure water for 1 h. The sucrose preference rate was calculated as the amount of sucrose solution consumed relative to the total fluid consumed.

One day after the SPT, an open-field test (OFT) was performed to evaluate anxiety. The open-field arena (100 × 100 × 40 cm) was equally divided into 25 square areas. The 9 grids in the center were defined as the central region. Rats were individually placed into the arena for 5 min. Distance travelled and time spent in the central zone were analyzed using video cameras with associated software (Smart 2.0).

The behavioral experiments and weighing of the animals were performed before and after the CUMS paradigm (Figure 1(a)).

2.4. Hippocampus Tissue Acquisition. After the behavioral experiments, rats were exposed to 25% pentobarbital sodium (50 mg/kg, intraperitoneal injection) and subsequently decapitated. Brains were instantly dissected, and all attached tissues were removed. Hippocampus tissues were separated, rinsed with phosphate-buffered saline (PBS), immediately frozen in liquid nitrogen, and stored at -80°C until analyses (3 rats/group). For hematoxylin and eosin (H&E) staining, cornu ammonis (CA) 1 was separated, fixed in 4% paraformaldehyde, embedded in paraffin, sliced, deparaffinized, and stained for routine H&E staining and histological examination (6 rats/group).

2.5. Protein Preparation, iTRAQ Isobaric Labeling, and SCX Separation. Hippocampus tissues were ground into powder in liquid nitrogen using lysis buffer (Roche). Then, the samples were ultrasonically disrupted on ice. Supernatants were collected after centrifugation (10,000g, 30 min, 4°C), and protein concentrations were determined using an enhanced bicinchoninic acid (BCA) Protein Assay Kit (P0010; Beyotime Biotechnology Ltd., Beijing, China), according to the manufacturer’s instructions. The protein samples (200 µg) were mixed with dithiothreitol, alkylated with iodoacetic acid, and then treated with trypsin (protein-trypsin ratio = 50:1, 12 h).

Protein peptides (100 µg) from each group were labeled using an iTRAQ Reagent-8plex Multiplex Kit (AB SCIEX, Framingham, MA, USA). The samples were labeled as 113 (control 1), 114 (control 2), 115 (CUMS 1), 116 (CUMS 2), and 117 (CUMS 3). The labeled samples were pooled and further fractionated offline using the ÄKTApurifier 100 (GE Healthcare Life Sciences) with a strong cation exchange column (PolySULFOETHYL A™; PolyLC Inc., Columbia, MD, USA). The retained peptides were eluted with buffer A (10 mM KH2PO4 in 25% ACN (acetonitrile), pH 3.0) and buffer B (10 mM KH2PO4 and 500 mM KCl in 25% ACN, pH 3.0) with a flow rate of 0.7 ml/min.

2.6. LC-MS/MS Analysis. Eluted fractions were lyophilized using a centrifugal speed vacuum concentrator (CentriviPap® Complete Vacuum Concentrator; Labconco, Kansas City, MO, USA) and dissolved in formic acid (5 µl, 0.5%). Equivalent amounts of peptides from each fraction were mixed and
then subjected to reversed-phase nanoflow LC-MS/MS analysis using a high-performance liquid chromatography (HPLC) system (EASY-nLC™, Thermo FisherScientific) connected to a hybrid quadrupole/time-of-flight mass spectrometer equipped with a nanoelectrospray ion source. The peptides were separated on a C18 analytical reversed-phase column with mixtures of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in ACN). A full MS scan was conducted using a Q Exactive™ mass spectrometer (Thermo FisherScientific) with a flow rate of 600 nl/min. Mass spectrometry was then performed using a mass spectrometer (Q Exactive HF, Thermo FisherScientific).

2.7 Protein Identification and Quantification. Raw MS/MS data were searched against the UniProt database (last modified on April 22, 2017) using Mascot 2.2 and ProteomeDiscoverer™ 1.4 software (Thermo FisherScientific). A peptide false discovery rate (FDR) ≤ 0.01 was used as the identification standard. Protein quantification was based on the total intensity of the assigned peptides. The average of labeled sample mixes was used as a reference and was based on the weighted average of the intensity of the reported ions in each peptide identified. The final protein ratios were normalized to the median average protein content of the 8-plex samples. A 1.2-fold cutoff was set to identify upregulated and downregulated proteins.

2.8 Bioinformatics Analysis. The functional enrichment analysis of significantly changed proteins was performed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis with the online software DAVID (https://david.ncifcrf.gov/). Corrected P values < 0.1 were considered significantly enriched. Protein-protein interaction (PPI) networks were retrieved from STRING (https://string-db.org/) using Cytoscape software (Version 3.6.1, https://cytoscape.org/). The Markov cluster algorithm (MCL) was then performed to determine topological clusters of the network using Cytoscape software.

2.9 Statistical Analysis. All data are expressed as the mean ± SEM. Differences in behavioral results and differentially expressed proteins were evaluated using Student’s t-test. All statistical analyses were carried out using SPSS software (version 20.0, SPSS Inc., USA). P < 0.05 was considered statistically significant.
3. Results

3.1. Stressors Cause Behavioral and Hippocampal Abnormalities. We used a 21-day chronic unpredictable mild stress (CUMS) paradigm to model human depression in rats. Animals were exposed to chronic unpredictable mild stressors for 21 days (Figure 1(a)). To measure appetite, anxiety, and anhedonia levels, body weight, open-field test (OFT), and sucrose preference test (SPT) were used, respectively.

The CUMS paradigm resulted in a lower weight in CUMS rats than in control rats (Figure 1(b)). CUMS rats also had decreased time spent in the central zone and distance travelled in the OFT, indicating increased anxiety (Figures 1(c) and 1(d)). In the SPT, CUMS rats had a lower sucrose preference rate than control rats, indicating anhedonia (Figure 1(e)).

The hippocampus is one of the important brain regions involved in the biology of depression. Hippocampus tissue was obtained for histology examination. H&E staining showed that control rats had a thick hippocampal pyramidal cell layer as well as densely, closely, and regularly arranged cells in CA1. In contrast, CUMS rats had a thin pyramidal cell layer, widened intercellular spaces, and irregularly and loosely arranged cells. Therefore, tissue damage and cell apoptosis occurred in the hippocampus of CUMS rats (Figures 1(f) and 1(g)).

3.2. Changes in the CUMS Hippocampal Proteomic Profile. To further understand the mechanism of depression, we used iTRAQ to identify differentially expressed hippocampal proteins between groups. Based on the iTRAQ-LC-MS/MS analysis results, a total of 3511 proteins and 18,381 peptides were identified (peptide false discovery rate (FDR) ≤ 0.01). Most of the identified proteins (75.39%) had molecular weights in the range of 10-80 kDa (Figure 2(a)). Approximately 60.84% of the identified proteins had more than 2 peptides (Figure 2(b)). Fifty-two quantified proteins with \( P < 0.05 \) and an expression change of higher than 1.50-fold or lower than 0.67-fold between the CUMS and control groups were manually selected (Table 1). Thirty differentially expressed proteins were upregulated, and 22 were downregulated after the CUSM paradigm.

3.3. Functional Annotation Enrichment of the Differentially Expressed Proteins. To understand the biological meaning behind the large list of proteins differentially expressed between groups and the underlying mechanism of depression, differently expressed proteins were subjected to enrichment analysis using the DAVID website. The identified enriched biological themes included biological process, molecular function, cellular component, and KEGG pathway. Enrichment analyses were performed on all differentially expressed proteins and then on upregulated and downregulated proteins separately for better understanding.

For all the differentially expressed proteins, the enrichment analysis results showed that, in terms of biological process, most of the differentially expressed proteins were involved in response to peptide hormone (7.69%, \( P = 0.01 \)), regulation of cell morphogenesis (5.77%, \( P < 0.01 \)), negative regulation of NF-kappaB transcription factor activity (5.77%, \( P = 0.02 \)), response to prostaglandin F (3.85%, \( P = 0.03 \)), isoprenoid biosynthetic process (3.85%, \( P = 0.06 \)), positive regulation of protein autophosphorylation (3.85%, \( P = 0.06 \)), multicellular organism aging (3.85%, \( P = 0.09 \)), response to metal ion (3.85%, \( P = 0.09 \)), and Rap protein signal transduction (3.85%, \( P = 0.09 \)). Regarding molecular function, most of the differentially expressed proteins were annotated as being associated with calcium ion binding (11.54%, \( P = 0.07 \)). In terms of cellular components, most
### Table 1: Differentially expressed proteins between the CUMS and control groups.

| Uniprot accession | Protein name | Gene | Fold change (CUMS/control) | P  |
|-------------------|--------------|------|-----------------------------|----|
| F1LZP5            | Protein Tgm7 | Tgm7l1 | Undetected in control group / |   |
| G3V7Q3            | Coiled-coil domain-containing protein 127 | Ccdc127 | Undetected in control group / |   |
| Q5Y886            | Itch E3 ubiquitin ligase | Itch | Undetected in control group / |   |
| B4F797            | RGD1311345 protein (fragment) | RGD1311345 | Undetected in control group / |   |
| G3V6E7            | Fibromodulin | Fmod | Undetected in control group / |   |
| Q8VH9             | Nesprin-1 | Syn1 | Undetected in control group / |   |
| P04177            | Tyrosine 3-monoxygenase | Th | Undetected in control group / |   |
| P10688            | 1-Phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta-1 | Plcd1 | Undetected in control group / |   |
| D3ZJB8            | Ariadne homolog 2 (Drosophila) (predicted), isoform CRA_a | Arie2 | 2.15 | 0.04 |
| Q9R1B1            | Mitochondrial import inner membrane translocase subunit Tim10 B | Timm10b | 2.05 | 0.01 |
| D3ZEK6            | Protein Gipc3 | Gipc3 | 1.97 | 0.04 |
| D3ZEB8            | Protein Upf3b | Upf3b | 1.96 | 0.01 |
| Q2MCP5            | Protein Wdr45b | Wdr45b | 1.88 | 0.01 |
| P61023            | Calcineurin B homologous protein 1 | Chp1 | 1.86 | 0.001 |
| D3ZJ2             | Protein Esy2 | Esy2 | 1.61 | 0.02 |
| Q08581            | Syntaxin-5 | Stx5 | 1.56 | 0.01 |
| D4A634            | Protein Rabp6 | Rabp6 | 1.37 | 0.03 |
| A0A0G2K132        | Protein Fmnl2 | Fmnl2 | 1.37 | 0.01 |
| D3ZZU4            | Protein Tmem160 | Tmem160 | 1.35 | 0.05 |
| Q568Z4            | Signal peptidase complex subunit 3 | Spcs3 | 1.34 | 0.02 |
| Q99PJ4            | Diphosphomevalonate decarboxylase (fragment) | Mvd | 1.33 | 0.02 |
| Q6A4YQ4           | Transmembrane protein 109 | Tmem109 | 1.30 | 0.03 |
| P0C5H9            | Mesencephalic astrocyte-derived neurotrophic factor | Manf | 1.29 | 0.02 |
| P61227            | Ras-related protein Rap-2b | Rap2b | 1.25 | 0.03 |
| A0A0G2JTW1        | Protein Rap2a | Rap2a | 1.25 | 0.04 |
| Q66H80            | Coatamer subunit delta | Arcn1 | 1.25 | 0.04 |
| P63170            | Dynine light chain 1, cytoplasmic | Dynll1 | 1.24 | 0.02 |
| B2GV08            | Adaptor-related protein complex 1, sigma 2 subunit (predicted), isoform CRA_a | Ap1s2 | 1.23 | 0.02 |
| F1LNV5            | Calcium uptake protein 1, mitochondrial | Micu1 | 1.22 | 0.02 |
| Q3KRD0            | Aspartate-TRNA ligase, mitochondrial | Dars2 | 1.20 | 0.03 |
| Q642E3            | CDK5 regulatory subunit associated protein 3, isoform CRA_b | Cdk5rap3 | Undetected in CUMS group / |   |
| D4A1R8            | Copine-1 | Cpe1 | 0.83 | 0.01 |
| D3ZY2             | Mitochondrial ribosomal protein S5 (predicted) | Mrps5 | 0.82 | 0.01 |
| D4AEG7            | Protein Tbc1d13 | Tbc1d13 | 0.81 | 0.02 |
| A0A0G2K189        | Protein Scm3 | Scm3 | 0.81 | 0.01 |
| Q5K00             | 39S ribosomal protein L46, mitochondrial | Mrpl46 | 0.81 | 0.01 |
| P16975            | SPARC | Sparc | 0.81 | 0.02 |
| D4A6W6            | Protein RGD1561333 | RGD1561333 | 0.80 | 0.03 |
| F1LSW7            | 60S ribosomal protein L14 | Rpl14 | 0.80 | 0.01 |
| F1LP34            | Acidic leucine-rich nuclear phosphoprotein 32 family member B | Anp32b | 0.80 | 0.03 |
| G3V8U9            | Proteasome subunit beta type | Psmb4 | 0.79 | 0.05 |
| P22791            | Hydroxymethylglutaryl-CoA synthase, mitochondrial | Hmgcs2 | 0.79 | 0.02 |
| B0BN94            | Protein FAM136A | Fam136a | 0.76 | 0.03 |
| D4A4Z9            | Protein Ktn1 | Ktn1 | 0.75 | 0.02 |
| D4A2Z6            | Protein Sec63 | Sec63 | 0.74 | 0.03 |
of the differentially expressed proteins were predicted to be in the endomembrane system (5.77%, \( P = 0.06 \)). For KEGG pathways, most of the differentially expressed proteins were involved in terpenoid backbone biosynthesis (3.85%, \( P = 0.08 \)) and protein export (3.85%, \( P = 0.08 \)) (Figure 3(a)).

For upregulated proteins, only biological process enrichment was found, and most of the upregulated proteins were involved in the positive regulation of protein autophosphorylation (6.67%, \( P = 0.03 \)), Rap protein signal transduction (6.67%, \( P = 0.05 \)), protein K48-linked ubiquitination (6.67%, \( P = 0.07 \)), protein K63-linked ubiquitination (6.67%, \( P = 0.07 \)), and microvillus assembly (6.67%, \( P = 0.09 \)) (Figure 3(b)).

For downregulated proteins, enrichment analyses results showed that, in terms of biological process, most of the downregulated proteins were involved in the regulation of cell morphogenesis (9.09%, \( P = 0.04 \)), inner ear development (9.09%, \( P = 0.07 \)), lung development (9.09%, \( P = 0.10 \)), and response to cAMP (9.09%, \( P = 0.10 \)). Regarding molecular function, most of the downregulated proteins were annotated as being associated with NF-kappaB binding (9.09%, \( P = 0.07 \)), the structural constituents of ribosomes (18.18%, \( P = 0.02 \)), and poly(A) RNA binding (27.27%, \( P = 0.10 \)). In terms of the cellular component, most of the downregulated proteins were predicted to be in the endomembrane system (13.64%, \( P = 0.01 \)) and membrane (40.91%, \( P = 0.08 \)) (Figure 3(c)). KEGG pathway enrichment was not found in downregulated proteins.

### 3.4. Protein-Protein Interaction Network of the Differentially Expressed Proteins

First, we retrieved the interaction network of all 52 differentially expressed proteins. MCL was performed to explore the strong connections between groups of nodes. Examining the main connected component of the network of upregulated proteins, we found 2 clusters of proteins, one of which consisted of Rap2b, Rap2a, Chp, Ap1s2, Fmod, and Syn1 (cluster 1). Both Rap2b and Rap2a belong to the family of Ras-related proteins, also known as Rap GTP-binding protein, one of the subfamilies of the Ras superfamily (Table 2). Members of this superfamily appear to regulate a diverse array of cellular events, including cell growth control, cytoskeletal reorganization, and protein kinase activation.

The other cluster consisted of Th, Stx5, Dynll1, and Arcn1 (cluster 2) (Figure 4(b)). These proteins are mainly involved in vesicle structure and trafficking. For instance, Stx5 is a member of the syntaxin or t-SNARE (target-SNAP receptor) family, which plays a crucial role in synaptic vesicle docking. Notably, Th is a rate-limiting enzyme in the synthesis of catecholamines, which is the process necessary for the formation of the dopamine (DA) precursor levodopa (L-DOPA). Hence, Th plays a key role in the biosynthesis of dopamine (Table 2).

As for the network of downregulated proteins, one cluster of downregulated proteins contained Mrps5, Msmb4, Mrpl44, RGD1561333, Anp32b, Fam136a, and Rpl14 (cluster 3). These proteins are mostly ribosome translation related. For example, Mrps5, Mrpl44, and Rpl14 are all ribosomal subunit proteins, and RGD1561333 and Anp32b are both involved in translation (Table 3).

There was also a cluster of proteins, Rac3, Tbc1d13, Neo1, Arhgap12, and Dnajc27, that was predominantly downregulated (Figure 4(c)), and which was mainly relevant to the Ras superfamily of small GTP-binding proteins and subsequent signaling pathways. For example, Rac3 and Dnajc27 belong to the Rho and Rab protein families, respectively, which are subfamilies belonging to the Ras superfamily. Tbc1d13 binds to Rab GTPase (Table 3).

### 4. Discussion

After exposure to stressors for 21 days, the CUMS rats exhibited less time spent in the central zone, less distance travelled, and lower sucrose preference in the OFT and SPT, as well as decreased weight, indicating elevated anhedonia and anxiety levels. Hippocampus lesions were also observed. These results suggest that the depression model was successfully established. To understand the hippocampal

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Table 1: Continued.

| Uniprot accession | Protein name                                      | Gene     | Fold change (CUMS/control) | \( P \) |
|-------------------|--------------------------------------------------|----------|---------------------------|--------|
| G3V7J2            | Interferon-inducible double-stranded RNA-dependent protein kinase activator A | Prkra    | 0.72                      | 0.02   |
| Q5PQZ8            | Selenoprotein T                                  | Selt     | 0.70                      | 0.02   |
| A0A0G2K7G2        | Protein Agfg2                                    | Agfg2    | 0.66                      | 0.03   |
| M0RST4            | Protein Rac3                                     | Rac3     | 0.64                      | 0.02   |
| A0A0G2K1Z2        | Protein Arhgap12                                 | Arhgap12 | 0.61                      | 0.05   |
| F1M0Z6            | Neogenin                                         | Neo1     | 0.57                      | 0.04   |
| Q6IML7            | DnaJ homolog subfamily C member 27               | Dnajc27  | 0.35                      | 0.01   |
proteomic changes underlying the mechanism of depression, we used LC-MS/MS analysis and bioinformatics analysis to identify the significantly changed proteins between the CUMS and control groups. We found GO enrichment in the GO term "Rap protein (a subfamily of Ras superfamily) signal transduction" among all differently expressed proteins and in the "structural constituent of ribosome" among downregulated proteins (Figure 3). Similarly, in the MCL clustering analyses, some identified clusters are involved in ribosomal translation and are relevant to the Ras superfamily (Figure 4). Together, these findings suggest that hippocampal ribosome lesions and Ras protein changes underlie the mechanism of depression.

4.1. Ribosome and Depression. Ribosomes serve as the workplace of RNA translation, which makes them vital organelles for protein synthesis [22]. In the neural system, ribosomes are known to contribute to neuron development. Moreover, rapid, local activation of protein synthesis in ribosomes is required for synaptic plasticity [23]. Ribosomes not only exist in the soma of neurons but also play an important role in axons and synapses. RNA is transferred to its postsynaptic destination and subsequently translated in the postsynaptic ribosome [24]. A recent study also demonstrated that presynaptic protein synthesis in the ribosome is essential for the long-term plasticity of neurotransmitter gamma-aminobutyric acid (GABA) release [25].
Figure 4: String network with MCL cluster shown. Protein-protein interaction networks with MCL clusters of all differentially expressed proteins (a), upregulated proteins (b), and downregulated proteins (c). Network nodes: proteins (upregulations are represented by red nodes, downregulations are represented by blue nodes, and higher expression changes are represented by larger nodes); edges: associations (stronger associations are represented by darker lines).
| Cluster | Gene/displayed name | Biological process | Molecular function | Cellular component |
|---------|---------------------|--------------------|-------------------|-------------------|
| 1       | Syne1               | Golgi organization, brain development, response to light stimulus, establishment of nucleus localization, muscle cell differentiation, positive regulation of receptor-mediated endocytosis, regulation of dendrite morphogenesis, cytoskeletal anchoring at nuclear membrane, nuclear matrix anchoring at nuclear membrane | Actin binding, receptor binding, structural molecule activity, protein binding, lamin binding, enzyme binding, identical protein binding, protein homodimerization activity, poly(A) RNA binding, actin filament binding | Nucleus, nuclear envelope, nucleoplasm, cytoplasm, Golgi apparatus, spindle, integral component of membrane, sarcomere, midbody, nuclear membrane, LINC complex, dendritic spine, postsynaptic membrane, perinuclear region of cytoplasm |
|         |                     | Microvillus assembly, platelet activation, negative regulation of cell migration, positive regulation of protein autophosphorylation, Rap protein signal transduction, regulation of protein tyrosine kinase activity, platelet aggregation, establishment of endothelial intestinal barrier | GTP binding, GDP binding, protein domain-specific binding | Cytosol, plasma membrane, bicellular tight junction, membrane, cell-cell contact zone, membrane raft, recycling endosome, recycling endosome membrane, extracellular exosome |
|         | Rap2b               | Positive regulation of protein phosphorylation, small GTPase-mediated signal transduction, microvillus assembly, negative regulation of cell migration, actin cytoskeleton reorganization, positive regulation of protein autophosphorylation, Rap protein signal transduction, cellular protein localization, cellular response to drug, establishment of protein localization, establishment of epithelial cell apical/basal polarity, regulation of JNK cascade, regulation of dendrite morphogenesis, protein localization to plasma membrane | GTPase activity, GTP binding | Intracellular, cytosol, plasma membrane, membrane, recycling endosome membrane, extracellular exosome |
|         | Rap2a               | Microtubule bundle formation, negative regulation of protein phosphorylation, negative regulation of protein kinase activity, protein export from nucleus, negative regulation of phosphatase activity, calcium ion-regulated exocytosis, calcium-mediated signaling, membrane docking, cytoplasmic microtubule organization, negative regulation of protein ubiquitination, negative regulation of protein autophosphorylation, negative regulation of NF-kappaB transcription factor activity, positive regulation of sodium : proton antipporter activity, negative regulation of protein import into nucleus, transcytosis, protein stabilization, positive regulation of | Protein kinase inhibitor activity, transporter activity, calcium ion binding, protein binding, microtubule binding, kinase binding, calcium-dependent protein binding | Golgi membrane, nucleus, cytoplasm, endoplasmic reticulum, endoplasmic reticulum-Golgi intermediate compartment, cytosol, plasma membrane, focal adhesion, microtubule cytoskeleton, transport vesicle, extracellular exosome |
|         | Chp                 | | | |
| Cluster | Gene/displayed name | Biological process                                                                                                                                                                                                                                                                                                                                 | Molecular function | Cellular component                                                                 |
|---------|---------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|-----------------------------------------------------------------------------------|
| Ap1s2   |                     | protein transport, protein oligomerization, regulation of intracellular pH, positive regulation of protein glycosylation, membrane organization, membrane fusion, negative regulation of calcineurin-NFAT signaling cascade, cellular response to acidic pH, positive regulation of protein targeting to membrane, regulation of neuron death | Protein transporter activity | Golgi apparatus, membrane coat, intracellular membrane-bounded organelle           |
|         |                     | Intracellular protein transport, visual learning, vesicle-mediated transport, synaptic vesicle recycling, fat cell differentiation, neuromuscular process controlling balance, adipose tissue development |                     |                                                                                   |
|         |                     | Transcription, DNA-templated, regulation of transcription, DNA-templated, nitric oxide biosynthetic process, transport, apoptotic process, microtubule-based process, substantia nigra development, intracellular retrograde transport, neurotransmitter metabolic process, negative regulation of phosphorylation, negative regulation of catalytic activity, motile cilium assembly, cilium morphogenesis, positive regulation of nonmotile primary cilium assembly |                     |                                                                                   |
|         | Dynll1              | Retrograde vesicle-mediated transport, Golgi to ER, adult locomotor behavior, protein transport, cerebellar Purkinje cell layer maturation, pigmentation, Golgi vesicle transport Motor activity, enzyme inhibitor activity, protein binding, protein C-terminus binding, enzyme binding, protein domain-specific binding, nitric oxide synthase regulator activity, protein homodimerization activity, dynein intermediate chain binding |                     | Kinetochore, nucleus, cytoplasm, mitochondrion, centrosome, cytosol, cytoskeleton, cytoplasmic dynein complex, microtubule, cilium, COP9 signalosome, membrane, extracellular exosome, mitotic spindle |
|         | Arcn1               | Response to hypoxia, synaptic transmission, dopaminergic, response to amphetamine, dopamine biosynthetic process from tyrosine, fatty acid metabolic process, sphingolipid metabolic process, heart development, visual perception, sensory perception of sound, learning, memory, mating behavior, locomotor behavior, regulation of heart contraction, response to water deprivation, response to light stimulus, response to herbicide, response to salt stress, organ morphogenesis, response to metal ion, response to zinc ion, multicellular organism aging, response to organic cyclic compound, response to activity, aminergic neurotransmitter loading | Poly(A) RNA binding | Golgi membrane, cytoplasm, endoplasmic reticulum, Golgi apparatus, membrane, COPI vesicle coat, COPI-coated vesicle, intracellular membrane-bounded organelle |
|         | Th                  | Monooxygenase activity, tyrosine 3-monooxygenase activity, protein binding, ferrous iron binding, ferric iron binding, amino acid binding, oxygen binding, enzyme binding, protein domain-specific binding, tetrahydrobiopterin binding, dopamine binding |                     | Nucleus, cytoplasm, mitochondrion, smooth endoplasmic reticulum, cytosol, synaptic vesicle, cytoplasmic side of plasma membrane, axon, dendrite, cytoplasmic vesicle membrane, cytoplasmic vesicle, melanosome membrane, neuron projection, neuronal cell body, terminal bouton, perikaryon |
In our study, the expression of ribosome proteins was significantly decreased in the hippocampus of depressed (CUMS) rats, especially the expression of ribosomal subunit proteins Mrps5, Mrpl46, and Rpl14 (Figure 4(a)). Similar studies have also revealed ribosome lesions in depression patients and animal models [26–28]. Interestingly, both Mrps5 and Mrpl46 belong to the family of mitochondrial ribosomal proteins (MRPs).

Research has found that MRPs are evolutionarily conserved proteins that serve as metabolic and longevity regulators. MRPs play a crucial role in activating the mitochondrial unfolded protein response (UPR<sub>mt</sub>) and therefore maintaining the balance of mitochondrial-nuclear proteins and extending lifespan [29]. Lifespan enhancers such as rapamycin and resveratrol also share this mechanism [30]. UPR<sub>mt</sub> activation has been observed in a mouse model of depression.

| Cluster | Gene/displayed name | Biological process | Molecular function | Cellular component |
|---------|---------------------|--------------------|--------------------|--------------------|
| Stx5    | Stx5                | IntraGolgi protein transport, ER to Golgi vesicle-mediated transport, early endosome to Golgi transport, retrograde transport, endosome to Golgi, positive regulation of protein catalytic process, vesicle docking, vesicle fusion with Golgi apparatus, Golgi disassembly, cell-cell adhesion, regulation of Golgi organization | SNARE binding, SNAP receptor activity, protein binding, protein N-terminus binding, cadherin binding involved in cell-cell adhesion | Golgi membrane, nucleoplasm, endoplasmic reticulum, Golgi apparatus, cytosol, cell-cell adherens junction, integral component of membrane, SNARE complex, vesicle, endoplasmic reticulum-Golgi intermediate compartment membrane |
| Cluster | Gene/displayed name | Biological process                                                                 | Molecular function                                                                 | Cellular component                                                                 |
|---------|---------------------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| 3       | Mrps5               | Translation                                                                         | Structural constituent of ribosome, poly(A) RNA binding                            | Mitochondrion, mitochondrial small ribosomal subunit, cytosolic small ribosomal subunit |
|         | Psmb4               | Negative regulation of inflammatory response to antigenic stimulus, proteolysis involved in cellular protein catabolic process | Lipopolysaccharide binding, threonine-type endopeptidase activity                   | Proteasome complex, nucleus, cytoplasm, proteasome core complex, extracellular exosome |
| 3       | Mrp146              | /                                                                                  | Structural constituent of ribosome, hydrolase activity                              | Nucleoplasm, mitochondrion, mitochondrial large ribosomal subunit, cell junction     |
|         | RGD1561333          | Cytoplasmic translation                                                             | Structural constituent of ribosome, poly(A) RNA binding                            | Nucleolus, focal adhesion, membrane, cytosolic large ribosomal subunit               |
| 3       | Anp32b              | /                                                                                  | Protein binding, histone binding, RNA polymerase binding                           | Nucleus, nucleolus, cytoplasm, extracellular exosome                                  |
|         | Fam136a             | /                                                                                  | rRNA processing, translation, ribosomal large subunit biogenesis                   | Cytoplasm, mitochondrion                                                             |
| 3       | Rpl14               | /                                                                                  | Structural constituent of ribosome, poly(A) RNA binding                            | Cytoplasm, ribosome, membrane, cytosolic large ribosomal subunit, extracellular exosome |
| 3       | Tbc1d13             | Intracellular protein transport, regulation of vesicle fusion, activation of GTPase activity, regulation of cilium assembly | GTPase activator activity, Rab GTPase binding                                      | Intracellular, endomembrane system                                                  |
|         |                     | Neuron migration, regulation of transcription, DNA templated, cell adhesion, axon guidance, myoblast fusion, positive regulation of BMP signaling pathway, regulation of axon regeneration, negative regulation of axon regeneration, negative regulation of protein secretion, iron ion homeostasis, negative regulation of neuron death, regulation of neuron migration | Receptor activity, signaling receptor activity, coreceptor binding, cadherin binding, BMP receptor binding | Nucleoplasm, Golgi apparatus, plasma membrane, integral component of plasma membrane, cell surface, membrane, neuronal cell body, intracellular vesicle, plasma membrane protein complex |
| 4       | Neo1                | /                                                                                  | /                                                                                   | /                                                                                  |
| 4       | Arhgap12            | Morphogenesis of an epithelial sheet, signal transduction                           | /                                                                                   | /                                                                                  |
| 4       | Dnajc27             | MAPK cascade, positive regulation of ERK1 and ERK2 cascades, regulation of MAPK export from nucleus | GTPase activity, GTP binding                                                       | Nucleus, mitochondrion                                                             |
caused by chronic restraint [31]. These studies and ours provide a new strategy in depression intervention to use rapamycin and resveratrol as supplements to alleviate depression by changing mitochondrial translation.

4.2. Ras Superfamily in Depression. The Ras superfamily is an evolutionarily conserved protein superfamily of small GTPases, including several subfamilies, such as Ras, Rho, Ran, Rab, and Arf GTPases, among which the Ras family itself is further divided into Ras, Ral, Rap, Rheb, Rad, and the recently included Rit and Miro [32]. Generally, these proteins are responsible for cell proliferation and survival [33]. Until now, the mechanism of Ras family proteins has primarily been discussed in terms of their role in tumorigenesis. However, recent studies have shown that the Ras superfamily has been involved in psychiatric disorders. Ras gene mutations are found in patients suffering from psychiatric and neurodevelopmental disorders [34].

Ras proteins activate and stimulate multiple downstream effector pathways by direct interactions, such as the Raf/Mitogen-activated protein kinase (MEK)/extracellular regulated protein kinase (ERK) cascade and the phosphoinositide 3-kinase (PI3K) signaling cascades [35]. These pathways mediate the control of various physiological processes. Taking PI3K signaling cascades as an example, the pathway has been found to be a necessary component in LTP [36]. Studies have also shown that these signaling cascades serve as key biochemical cascades in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) trafficking during synaptic plasticity in neurons and altered behavior [37].

Altered hippocampal synaptic plasticity is considered one of the underlying mechanisms of depression. In our research, the expression of proteins in the Ras superfamily changed significantly. The MCL clustering results showed a mixed change in these proteins, meaning some of the Ras proteins were upregulated and some downregulated. Upregulated proteins such as Rap2b and Rap2a belong to the Rap family (Figure 4(a)).

Notably, Ras and Rap proteins of the Ras subfamily function antagonistically [38]. In neurons, Ras plays a crucial role in synapse enforcement and LTP by promoting postsynaptic insertion of AMPAR. Rap weakens synapses and induces long-term depression (LTD) by increasing AMPAR internalization [39]. Our results show that typical Rap proteins, Rap2b and Rap2a, were upregulated in the CUMS hippocampus (Table 2), which indicates synaptic weakening and synaptic plasticity disturbances in depression.

On the other hand, of the downregulated proteins identified, Rac3 belongs to the Rho family and Dnaic27 belongs to the Rab family (Table 3). Specifically, Rho proteins are responsible for the morphogenesis of dendritic spines [40] and Rab for that of synaptic vesicles [41], which are both vital biological processes underlying synaptic plasticity. Therefore, we consider that Ras proteins are involved in hippocampal pathology changes by affecting hippocampal synaptic plasticity.

However, we did not conduct experiments examining the UPRmit or synaptic plasticity of hippocampal neurons in this study. Whether these proteins are responsible for the UPRmit and disrupted synaptic plasticity in the hippocampus is still unknown. Further research may be needed to draw a conclusion. Another possible limitation of the study is that we did not focus on a specific subfield of the hippocampus, such as the dentate gyrus (DG), CA1, CA2, CA3, or CA4. Because most of the identified proteins in this research are synapse-related, we would like to focus on the CA1 and DG in future studies. Indeed, synaptic plasticity in the CA1 is rather vulnerable in diseases, and adult neurogenesis still exists in the DG, which makes DG a subfield of high synaptic plasticity [42, 43].

Data Availability
The data used to support the findings of this study are available from the corresponding authors upon request.

Ethical Approval
This experiment was approved by the Southern Medical University Experimental Animal Ethics Committee (resolution no. L2015056).

Disclosure
Jialing Zhang and Zhinan Zhang are co-first authors.

Conflicts of Interest
The authors declare no conflict of interest in the present study.

Authors’ Contributions
Jialing Zhang, Zheng Zhong, Shanshan Qu, and Yong Huang conceived and designed the experiments. Jiping Zhang and Zengyu Yao performed the experiments. Zhinan Zhang analyzed the data.

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