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Research article

Quantitative proteomics reveals the therapeutic effects of RFAP against depression via pathway regulation of long-term depression and potentiation

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ABSTRACT

Ethnopharmacological relevance: RFAP is a compound extraction complex of four Traditional Chinese Medicine (TCM), including the dry bark of *Paeonia lactiflora* Pall. (Radix Paeoniae Alba), *Gardenia jasminoides* J. Ellis (Fructus Gardeniae), *Albizia julibrissin* Durazz. (Albizia julibrissin Durazz), and *Paeonia* × *suffruticosa* Andrews (Peony bark). Not only RFAP but also the individual ingredients have been commonly used for the treatment of depression in the clinic. However, the underlying mechanism of pharmacology is difficult to interpret since its holistic and multidrug nature.

Aim of the study: This study aimed to elucidate the potential antidepressant mechanism of RFAP in the treatment of chronic unpredictable mild stress (CUMS) rats' model via the quantitative proteomics approach.

Materials and methods: We established the CUMS rats' model and evaluated the efficacy of RFAP using multiple behavior assays, including the sugar preference test, open field test, and forced swimming test. Then label-free quantitative proteomics analyses were performed to evaluate the integrated changes of proteome profiling in control, CUMS, RFAP low dose, and RFAP high dose groups. Finally, we validated the critical changed proteins in the pathways of long-term depression and potentiation via RT-PCR and Western blotting assays.

Results: We successfully established the CUMS rats' model. The behavior assays indicated that the rats demonstrated a tendency to behavioral despair after four weeks. Label-free quantitative proteomics showed that 107 proteins were significantly upregulated and 163 proteins were downregulated in the CUMS group compared to the control group. These differentially expressed

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proteins were involved in long-term potentiation, long-term depression, nervous system development, neuronal synaptic structural constituent of ribosome, ATP metabolic process, learning or memory, and cellular lipid metabolic process. RFAP treatment partially restored the differentially expressed protein profile. The protective effect of RFAP on behavioral assessment were consistent with the results of proteomics.

Conclusions: The results indicated that RFAP exerted a synergistic effect on CUMS by regulating long-term inhibition and potentiation-related proteins.

1. Introduction

Depression is one of the most common psychiatric diseases which severely limits an individual's psychosocial functioning and quality of life [1]. More than 350 million people of all ages worldwide are affected by depression due to both internal and external factors [2,3]. Despite significant advances that have been made in the understanding of the pathophysiology and developing of multiple medical treatments, the pathogenesis and therapeutic mechanisms underlying this complex disorder are still unclear. Currently, clinical medications for the treatment of moderate and major depression include selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs), norepinephrine and specific serotonergic antidepressants (NaSSAs), which exert their antidepressant effects by blocking the reuptake of the neurotransmitters 5-hydroxytryptamine (5-HT) and norepinephrine (NE) uptake. Some of the first antidepressants developed, such as tricyclics and MAOIs, remain among the most effective drugs available, but are currently used in very small amounts. Most commercially available drugs have a variety of drawbacks, including slow onset of action, low response rates, nausea, weight gain, sleep problems, drug resistance and organ toxicity [1,4]. Moreover, there are few antidepressants particularly developed for mild to moderate depression. Therefore, more effective pharmacological strategies should be encouraged against depression and resolve unmet medical needs.

RFAP is a TCM formula extraction commercially available in the USA (EasyMind™ Chenland. Inc), which is composed of the dry bark of *Paeonia lactiflora* Pall. (Chinese name called Shaoyao, Radix Paeoniae Alba), *Gardenia jasminoides* J. Ellis (Chinese name called Zhizi, Fructus Gardeniae), *Albizia julibrissin* Durazz. (Chinese name called Hehuan, Albizia julibrissin Durazz), and *Paeonia* × *suffruticosa* Andrews (Chinese name called Mudanpi, Peony bark) [5]. These four herbal medicines have been reported their antidepressant effects from published studies, such as Gang et al. has validated that RFAP acts as an antidepressant in rats under prolonged restraint stress via the cAMP/PKA/CREB/BDNF signaling pathway [5]. Radix Paeoniae Alba is a well-known Chinese herb with a variety of bioactivities, which could be a Chinese herbal medicine remedy for the treatment of mental illnesses [6]. In the context of chronic treatment, paeoniflorin isolated from this compound also shown possible antidepressant-like effects. The active components of Fructus Gardeniae have been experimentally shown to have antidepressant properties, mainly primarily through the stimulation of AMPAR-mTOR signaling and regulation of synaptic plasticity enhancement [8]. Albizia julibrissin Durazz is one of the most commonly used herbs in the treatment of depression and anxiety disorders, and its active component exhibited therapeutic efficacy for mood disorders by acting on serotonin transporter (SERT) through a novel potential mechanism of action [9]. In addition, Peony bark extraction has been shown to have antidepressant effects, which are associated with modulation of hypothalamic-pituitary-adrenal axis function, inhibition of oxidative stress and upregulation of neurotrophic hormones [10–12]. Traditionally, depression has been treated with Traditional Chinese medicine (TCM). Its holistic, polypharmacological and multi-targeted nature is well in line with the therapeutic thinking of systems medicine in the treatment of depression, while its mechanisms are complex.

Based on previous studies, we investigated the RFAP (EasyMind™), which combined the extraction of these four herbal medicines. In the pathophysiology of depression, a method of combining the whole and multidrug formulation of TCM with multi-system targets is proposed. Therefore, the study of system-level mechanisms of action is essential to better understand the mechanisms of action of TCM in the treatment of depression. To investigate the synergistic effects and underlying mechanisms of TCM formulation, omics methods have been considered as the proper ways. Proteomics is one of the powerful tools to study the profiling changes in the protein level in order to understand what pathways have been changed for the treatments and targets.

In this study, we established a CUMS rat model to assess the pharmacological effects of RFAP. Then, the label-free quantitative proteomics analysis was performed to reveal the changes in proteomic profile and underlying molecular mechanisms in different experimental groups. The present study provided evidence that several critical signaling pathways were changed in vivo and were restored by the RFAP treatment.

2. Methods and materials

2.1. RFAP formula preparation and component analysis

The RFAP formula (EasyMind™, Chenland Nutritionals, Inc., Irvine, CA, USA) was prepared by mixing Radix Paeoniae Alba, Fructus Gardeniae, Albizia julibrissin Durazz, and Peony bark at a respective proportion in 52.1%, 27.0%, 18.7%, and 2.2%. The extract of each component was prepared following a previous study [5]. The component analysis of RFAP were performed on1220 Infinity II LC System (Agilent) with HPLC Column (4:6 × 250 mm, 5 μm, Agilent 5 TC) at 30 °C. The injection volume was 3 μL. Mobile phase A was 100% water with 0.1% formic acid, and mobile phase B was a 0.1% acetonitrle with 0.1% formic acid. The flowrate was 1 $mL{\cdot}min^{-1}$ with mobile phase B from 5% to 95% for 80 min.

2.2. Chronic unpredictable mild stress (CUMS) rat model and RFAP treatment

Sixty male Wister rats (SPF grade, 8 weeks, 200–220 g) were obtained from Pengyue Experimental Animal Center in Jinan, China (Certificate of Conformity: NO. SCXK(Lu) 2018-0003). The experimental protocol was authorized by the Animal Care and Ethics Committee of Beijing University of Chinese medicine (Permit Number 2019-014).

Chronic unpredictable mild stress had been considered as a reliable animal model for modeling depression disorder and screening antidepressants [13,14]. Initially, the drug was solvated and diluted in water. Rats were dosed daily via oral gavage from the day of modeling. In the CUMS model group, the same volume of saline was administered every day for 5 weeks. A total of 60 rats were randomly divided into five groups (n = 12 per group): the control group (saline, 10 mL kg^{-1}); CUMS model group (saline, 10 mL kg^{-1}); CUMS + Hypericum perforatum group (HP group, 186.67 mg/kg); CUMS + Radix Paeoniae Alba + Fructus Gardeniae + Albizia julibrissin Durazz + Peony bark low dose (RFAPL group, 70.35 mg/kg); and CUMS + Radix Paeoniae Alba + Fructus Gardeniae + Albizia julibrissin Durazz + Peony bark high dose (RFAPH group, 281.4 mg/kg). According to "Pharmacological Experiment Methodology Third Edition", the RFAP dose for rats is converted based on the equivalent dose factor of the human clinical dosage [5]. Rats were housed singly and subjected to 5 weeks of unpredictable mild stress. These stresses were administered in a randomized order with a gradual increase in intensity. 10 different stressors were introduced into the CUMS procedure, including day and night reversal (24 h), 45° cage tilt (12 h), restraint (30 min), moist bedding (12 h), food deprivation (12 h), 4 °C cold water swimming (10 min), strobe lighting (12 h), white noise (15 min), cage shaking (15 min), tail pinch (5 min) [15,16]. The three stressors were randomly arranged every day for the CUMS group(s), but without repetitive stressors on two consecutive days. Control rats were grouped and housed in a neutral environment, and all rats lived in comparable environmental conditions. In the CUMS procedure, behavioral tests were performed weekly on individual rats in the order of SPT and OFT, with at least a 2-h rest period between the two different tests. FST was measured before tissue collection.

2.3. Behavioral assays

Body weights of all rats were recorded weekly. The sucrose preference test (SPT) was used to assess symptoms of disorientation with depression-like behavior. As described in previous studies with minor modifications [17], all rats were housed individually and were free to choose between two bottles of water for 24 h, one with 1% sucrose solution and the other with tap water. To prevent possible effects of side-effects in terms of drinking water, change the position of the bottle and balance it within 12 h. Rats were not deprived of food or water before and during the test. The consumption of water and sucrose solutions for the control and experimental groups was estimated simultaneously by weighing the bottles. Sucrose intake was calculated as the amount of sucrose consumed per gram of body weight, in milligrams. The preference for sucrose was calculated as the percentage of sucrose solution consumed to the total amount of liquid drunk. The rats were randomly divided into five groups (n = 12 for each group). Each rat was tested a minimum of three times and calculated the average value. And then, intragroup mean and standard deviations (SD) were calculated for each group.

Forced swimming test (FST) [18] was used to assess the efficacy of antidepressant treatment. Rats were individually placed in transparent cylinders (40 cm high and 20 cm in diameter) containing 30 cm of water (23–25 °C) and left in the water for 5 min. The duration of immobility was recorded during the test. Rats were judged to be immobile when they floated passively on the water surface without struggling, making only slow movements to keep their heads above the water.

Open Field Test (OFT) [19] was used to assess spontaneous exploratory activity and evaluate anxiety levels in rats. Rats were placed in the center of the open field analysis system (Shanghai Xinruan Technology Company Limited, China) to record and analyze the activities of rats in the experiment box. Locomotor activity was detected through beam-interruptions of the rats within 6 min and the computer analysis system analyzed the movement trajectory of the rats. The behavioral indicators (total distance, center area distance, time in center area and rearing time) were recorded. Throughout the test, the room was kept quiet and the equipment was cleaned with 75% ethanol after each test.

Euthanasia was performed by CO2 asphyxiation in the 5th week, and blood and hippocampus tissue were harvested.

2.4. Protein extraction, digestion, and enrichment

Frozen rat hippocampal samples were quickly ground and then dissolved in 0.20 mL of 0.1 M Tris–HCl (pH 7.6) and rapidly homogenized with a Tissuelyser® (Jingxin Industrial Development Co., Ltd., Shanghai, China) at 60 Hz, −20 °C for 120 s. Total protein was extracted and digested according to the protocol of our previous paper [20].

2.5. Label-free quantification proteomics analysis and bioinformatic analysis

The peptide analysis and the label-free proteomic quantification were procedure in accordance with the previously reported method [21]. Data was collected by Xcalibur™ software (Thermo Fisher Scientific, USA).

All raw files were analyzed by the MaxQuant software (version 1.6.3.4) and compared with the UniProt rat protein database (version 20180903, 8033 sequences). A target-decoy based strategy was used to achieve a peptide and protein false discovery rate ≤1%. Search parameters were set as described previously [22]. The following criteria were used to identify differentially expressed proteins: unique peptide ≥2, *p*-value ≤0.05, and present in at least 33.3% of the total sample (≥5). Gene ontology (GO) annotations were searched against the DAVID database [23]. Pathway and protein-protein interaction were assessed using the STRING database

[24].

2.6. RNA isolation and quantitative RT-PCR analysis

Total RNA was extracted from the hippocampus using the RNeasy® Lipid Tissue Mini Kit (QIAGEN, Valencia, CA, USA). RT-PCR was performed on biosystems QuantStudio7 Flex (Thermo Fisher Scientific, Waltham, MA, USA) using SYBR Green PCR Master Mix (QIAGEN) according to the manufacturer's protocol. The GAPDH gene was utilized as an internal reference to normalize sample differences and was used to calculate the relative expression levels of mRNAs using the $2-\Delta\Delta Ct$ technique. The primers that were amplified are listed in Supplementary Table S1.

2.7. Western blotting validation

Protein extraction and Western blotting were performed as described [20]. The primary antibodies were the following: NRAS

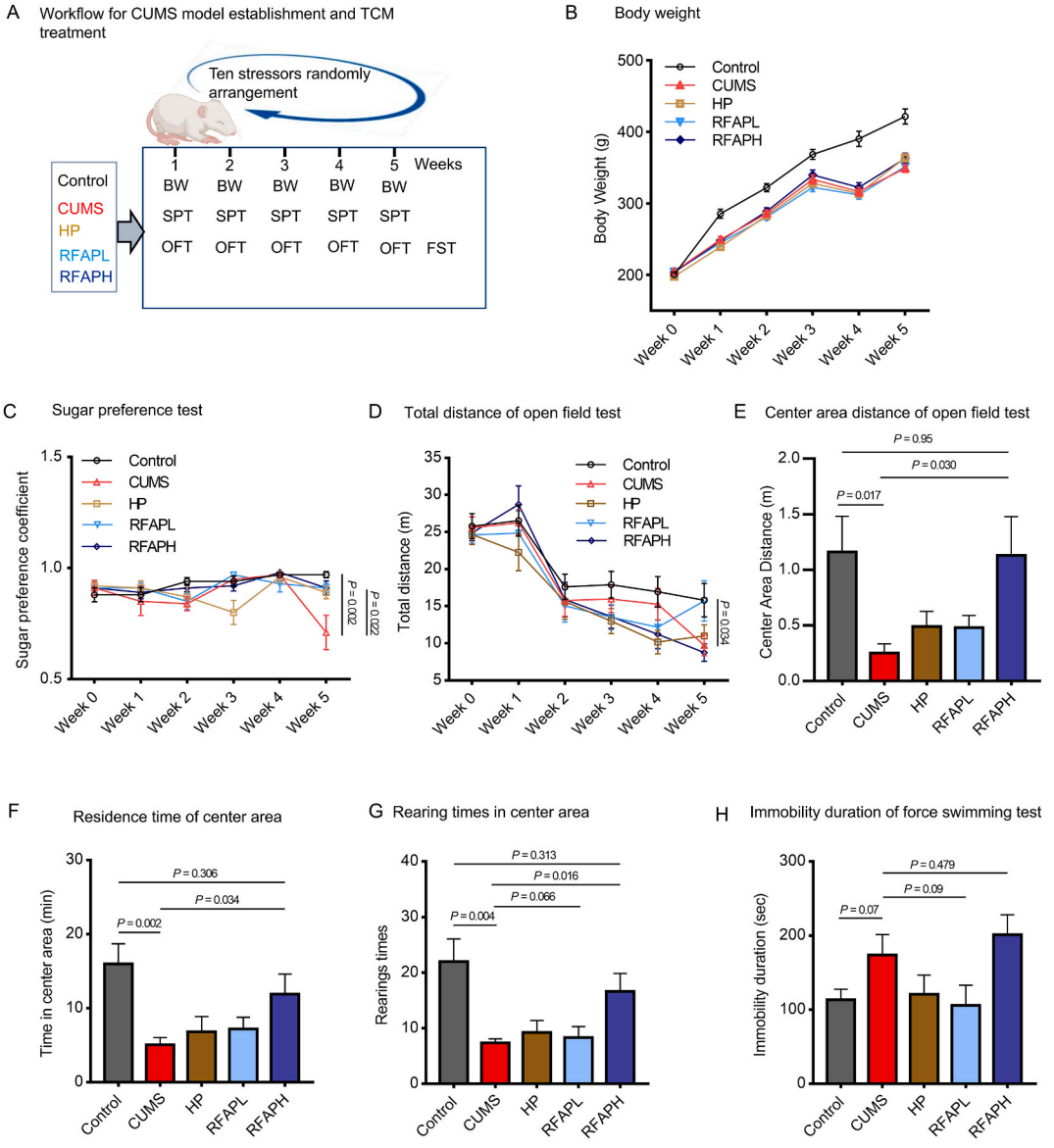


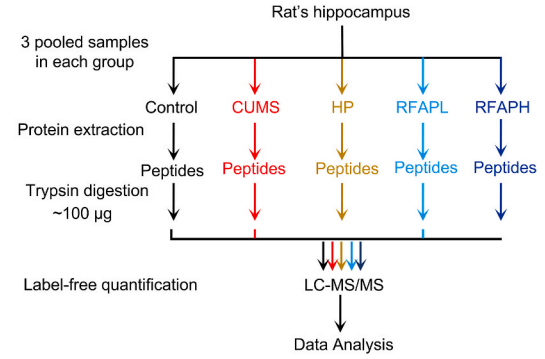
Fig. 1. CUMS model rat establishment and behavior tests with TCM treatments. (A) Schematic illustration of CUMS rat behavior tests and TCM treatment (five groups, n = 12 for each group). (B) Body weight measurement. (C) Sugar preference test. (D) Total distance of open field test. (E) Center area distance of open field test. (F) Residence time of center area. (G) Rearing times in center area. (H) Immobility duration of force swimming test. Data are expressed as mean ± SE. $P < 0.05$ as significant difference.

(1:800, ptg, 10724-1-AP), MARK1 (1:1000, ptg 21552-1-AP), PPP2R2D (1:1000, ab181071), SMARCE1 (1:1000, ptg, 10814-1-AP), β-actin (proteintech66009-1-lg), and GAPDH (1:5000, ab8245)) The membranes were conjugated with secondary antibody (1:10 000, goat anti-rabbit IgG, ZDR 5119, goat anti-mouse IgG, ZDR 5006) for 1 h at room temperature. The ChemiDoc™ MP Imaging System (Bio-Rad Co., USA) was used to quantify protein bands, and Image Lab software was used to evaluate the results (Bio-Rad Co., USA).

2.8. Statistical analysis

In the statistical analysis, the student *t* test, one-way ANOVA, and two-way ANOVA were applied. One-way ANOVA and two-way ANOVA were used for within-group comparisons of single or two variables. All data are expressed as mean ± SD, and $p \leq 0.05$ was considered statistically significant. Data analysis and visualization were performed by R (version 4.0.3).

A Workflow of quantitative proteomic analysis



B Proteome identification and quantification

Proteomics parameters	Results
Peptides spectra match	401280
Identified peptides	21812
Identified proteins	2344
Quantified proteins (Q-value<1.0)	2287
Quantified proteins (Q-value<1.0, unique peptides ≥2)	2155
Quantified proteins (Q-value<1.0, unique peptides ≥2, identified ≥33.3%)	2149

C Ratio distribution of protein profile in each group

Groups	Up-regulated		Down-regulated	
	Mean	SD	Mean	SD
CUMS	2.81*	0.35	0.64*	0.02
HP	0.84#	0.02	1.80#	0.22
RFAPL	0.68#	0.03	1.69#	0.11
RFAPH	0.86#	0.02	1.44#	0.08

*compared with Control; #compared with Model.

D Upregulated and downregulated proteins *vs.* Control

	Differentially expressed proteins	Upregulated	Downregulated
CUMS	270*	107*	163*
HP	158#	84#	74#
RFAPL	256#	110#	146#
RFAPH	186#	77#	109#

Cutoff: unique peptide≥2, *p*-value≤0.05, proteins identified at least 33.3% of total(≥ 5).
*compared with Control; #compared with CUMS.

E PCA analysis

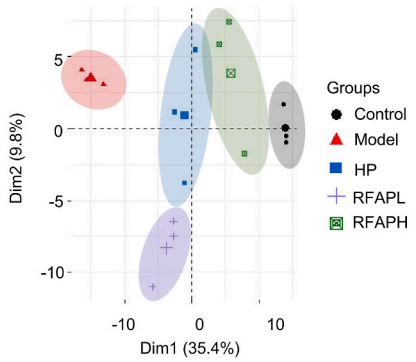


Fig. 2. Proteome profiling identification and quantification. (A) Workflow of label-free proteomic analysis. (B) Proteome identification and quantification. (C) Ratio distribution of protein profile in each group. *Compared with Control; #compared with Model. (D) Up-regulated and down-regulated proteins vs. Control. Cutoff: unique peptide ≥ 2, p-value ≤ 0.05, proteins identified at least 33.3% of total (≥5). *Compared with Control; #compared with CUMS. (E) PCA analysis.

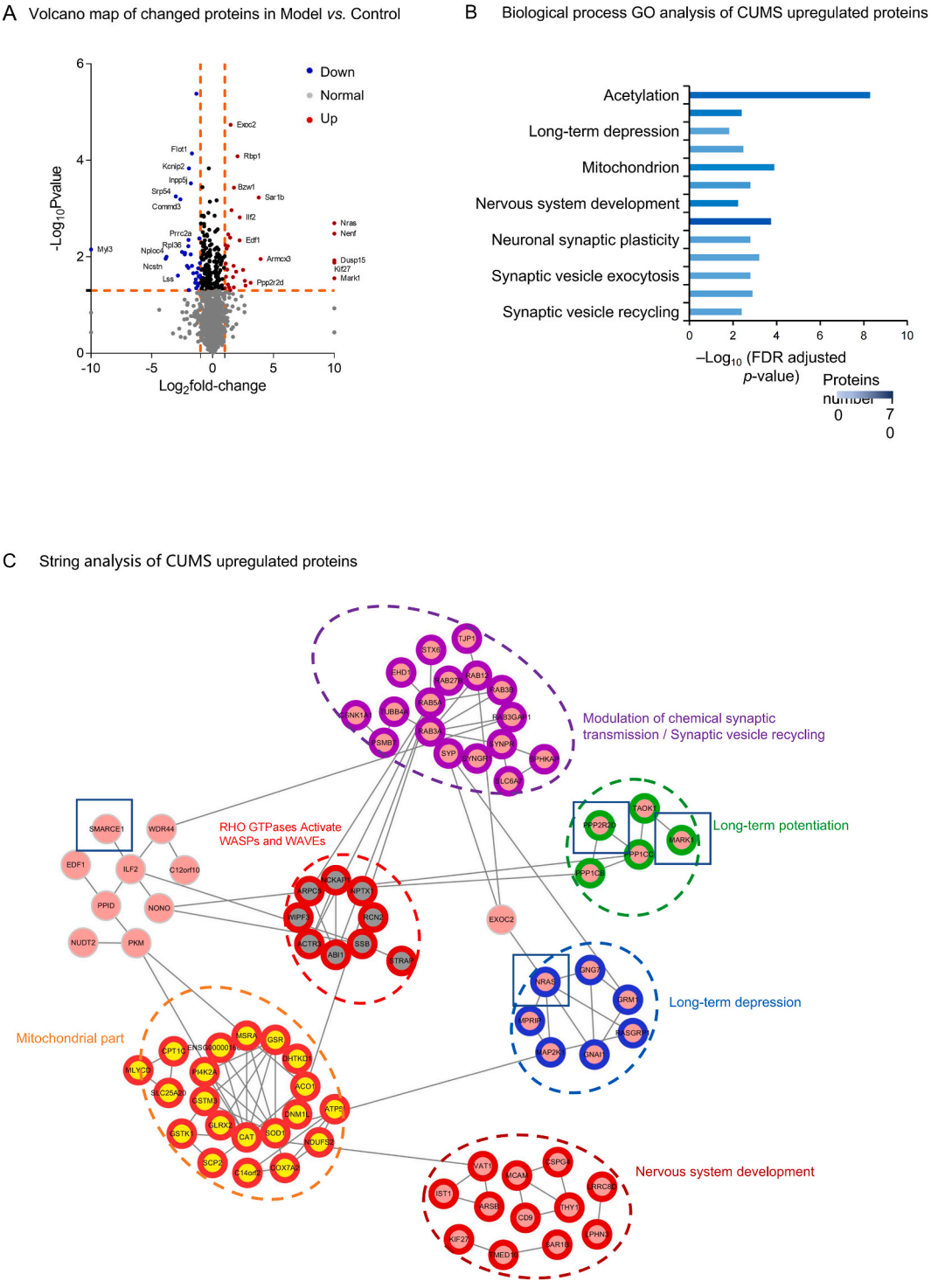


Fig. 3. Characteristics of CUMS up-regulated proteins. (A) Different expressed proteins were identified by the volcano map. A list of overexpressed proteins (red) with log-rank P-value < 0.05 and log2 (CUMS/Control ratio) > 1.5 and a list of down-regulated proteins (blue) with log-rank P-value < 0.05 and log2 (CUMS/Control ratio) < -1.5. (B) GO analysis of CUMS up-regulated proteins in CUMS/Control. (C) String analysis of CUMS up-regulated proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

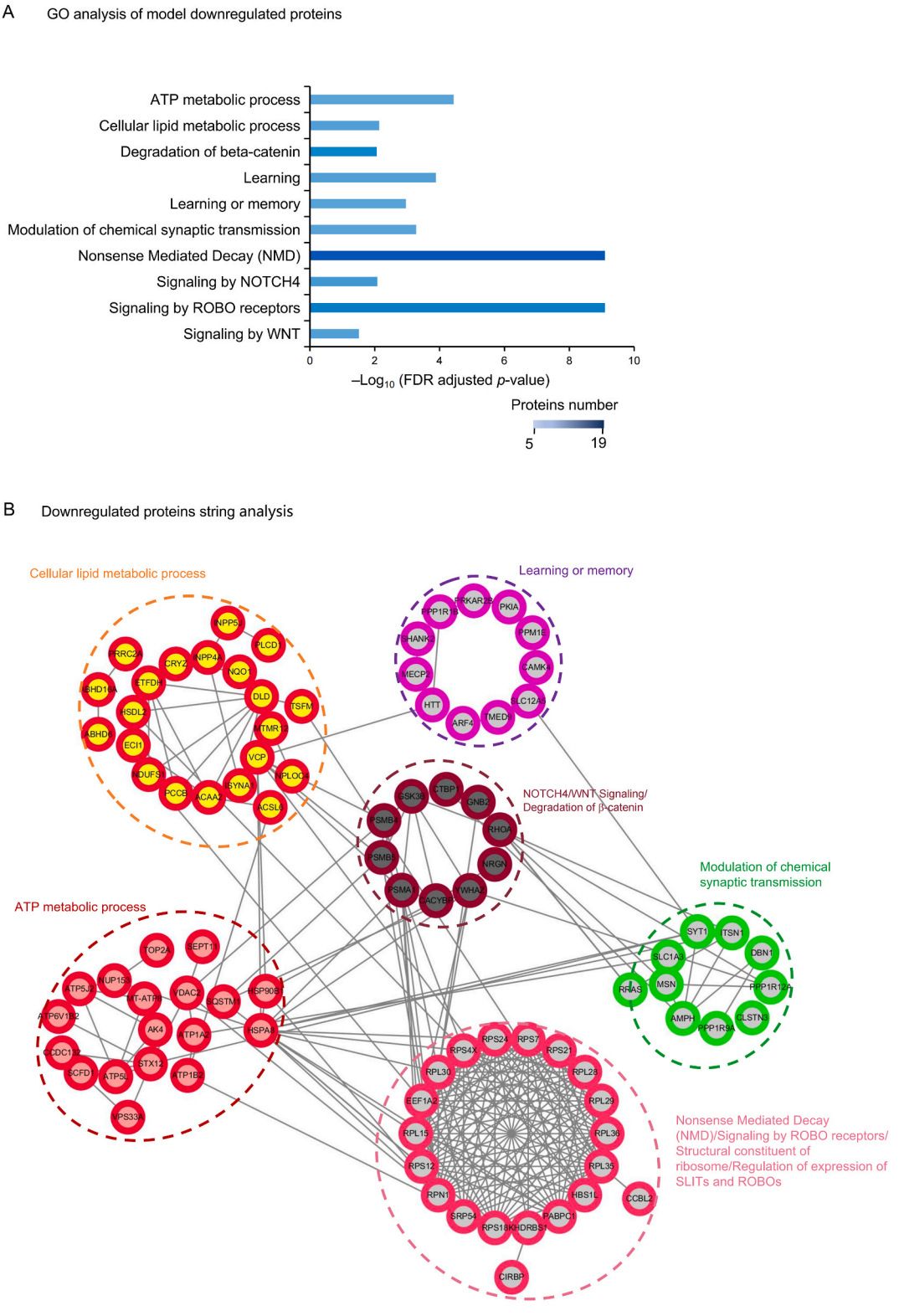


Fig. 4. Characteristics of CUMS down-regulated proteins. (A) GO analysis of CUMS up-regulated proteins in CUMS/Control. (B) String analysis of CUMS up-regulated proteins.

3. Results

3.1. TCM treatment and behavioral observation

The workflow for CUMS model establishment and TCM treatment were shown in Fig. 1A. After 5 weeks of CUMS, there was a decrease in weight in administered groups and model group ($p < 0.0001$) (Fig. 1B), indicating CUMS direct interference with body

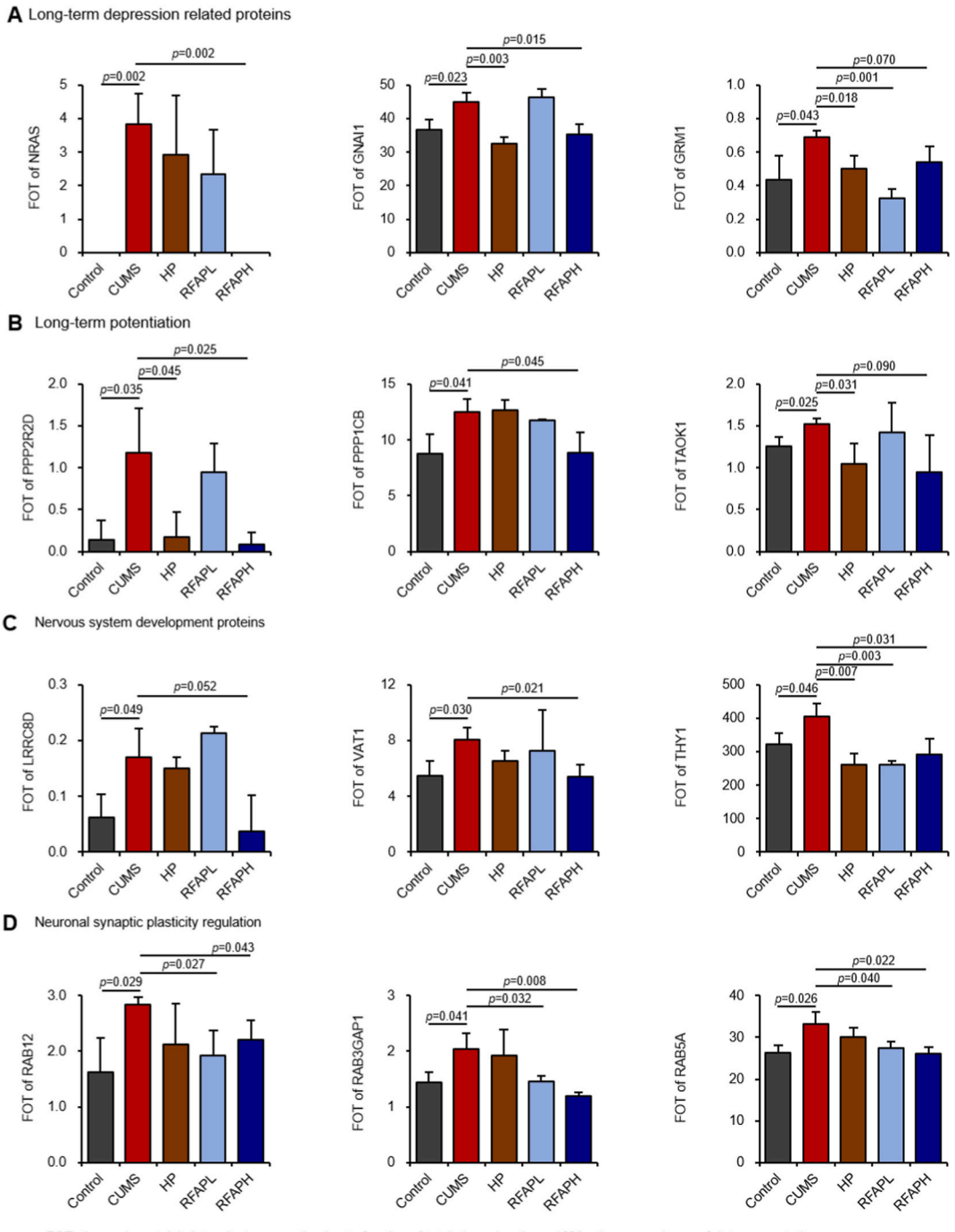


Fig. 5. Evaluation of up-regulated and their role in depression. (A) Level of long-term depression related proteins in each group. (B) Level of long-term potentiation related proteins in each group. (C) Level of nervous system development proteins in each group. (D) Level of neuronal synaptic plasticity regulation related proteins in each group. Data are expressed as mean ± SD (A–D, pooled samples, n = 3).

weight would be efficacious during the experimental period. Sugar preference tests showed in Fig. 1C. The sucrose preference index of the CUMS group decreased to 0.7 compared with the control group ($p = 0.002$). However, the decrease was significantly rescued in CUMS-RFAPL group and CUMS-RFAPH group ($p = 0.022$). These results suggest that rats with chronic mild unpredictable stress developed anhedonia and the absence of positive affect symptoms. As shown in Fig. 1D–G, the total distance, center area distance, the time in the center area and rearing times of the OFT, respectively. After 5 weeks of CUMS, the total distance, center area distance, the time in the center area, and rearing times of the model group were significantly decreased than that of normal control, respectively ($p = 0.03$, $p = 0.017$, $p = 0.002$, $p = 0.004$). However, the total distance, the total distance, center area distance, the time in the center area, and rearing times were significantly elevated following the 5 weeks of pharmaceutical intervention, even close to the normal

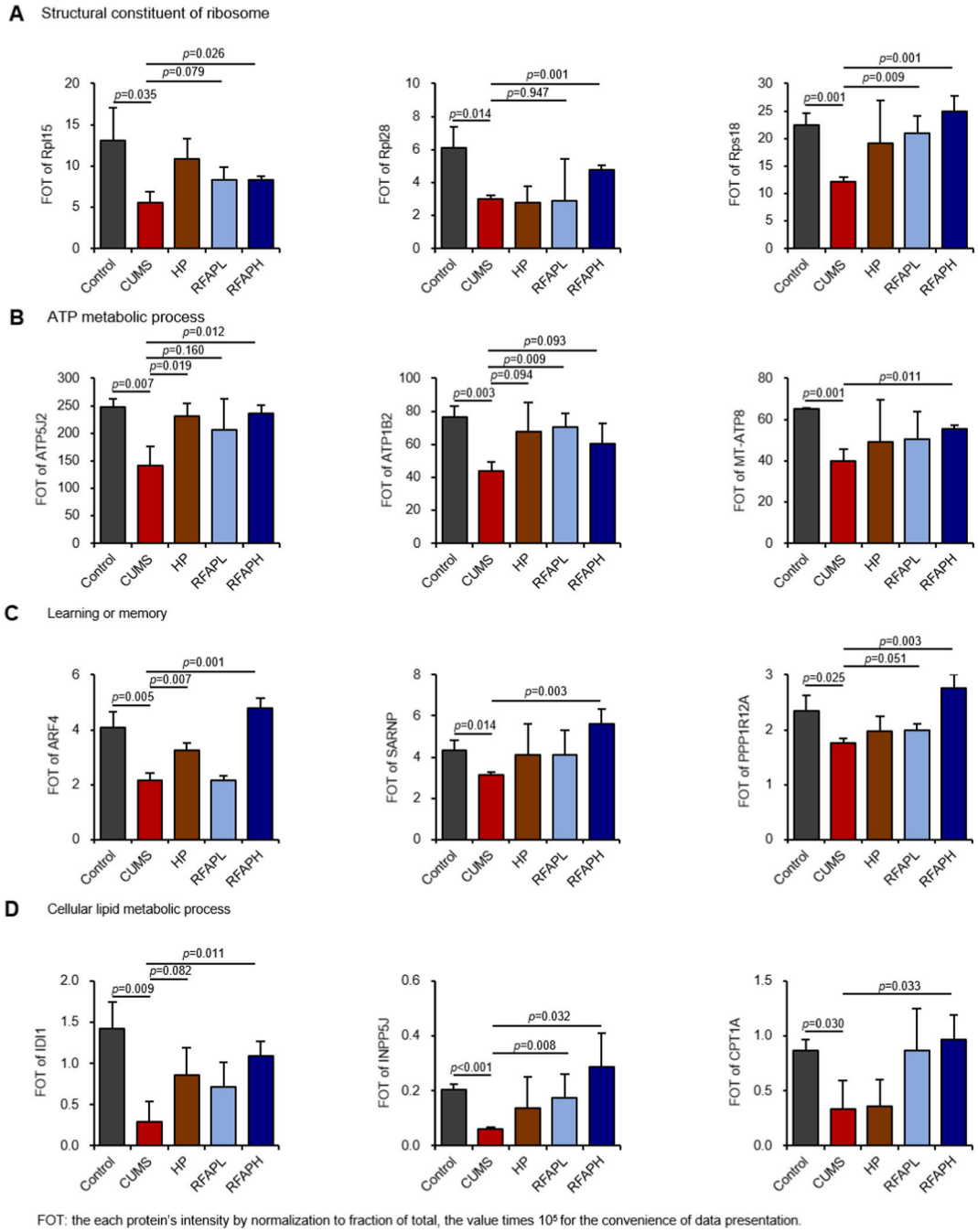


Figure 6. Evaluation of down-regulated and their role in depression. (A) Level of structural constituent of ribosome related proteins in each group. (B) Level of ATP metabolic process related proteins in each group. (C) Level of learning or memory related proteins in each group. (D) Level of cellular lipid metabolic process related proteins in each group. Data are expressed as mean ± SD (A–D, pooled samples, n = 3).

control level ($p = 0.034$, $p = 0.03$, $p = 0.034$, $p = 0.016$), indicating that CUMS have effects on potential anxiety-like behavior and the exploratory and exercise behavior of rats. Moreover, the results showed that the exploratory activity decreased, and the interest in the outside environment showed a significant decline in CUMS rats. However, RFAPH could have a significant in resolution of symptoms.

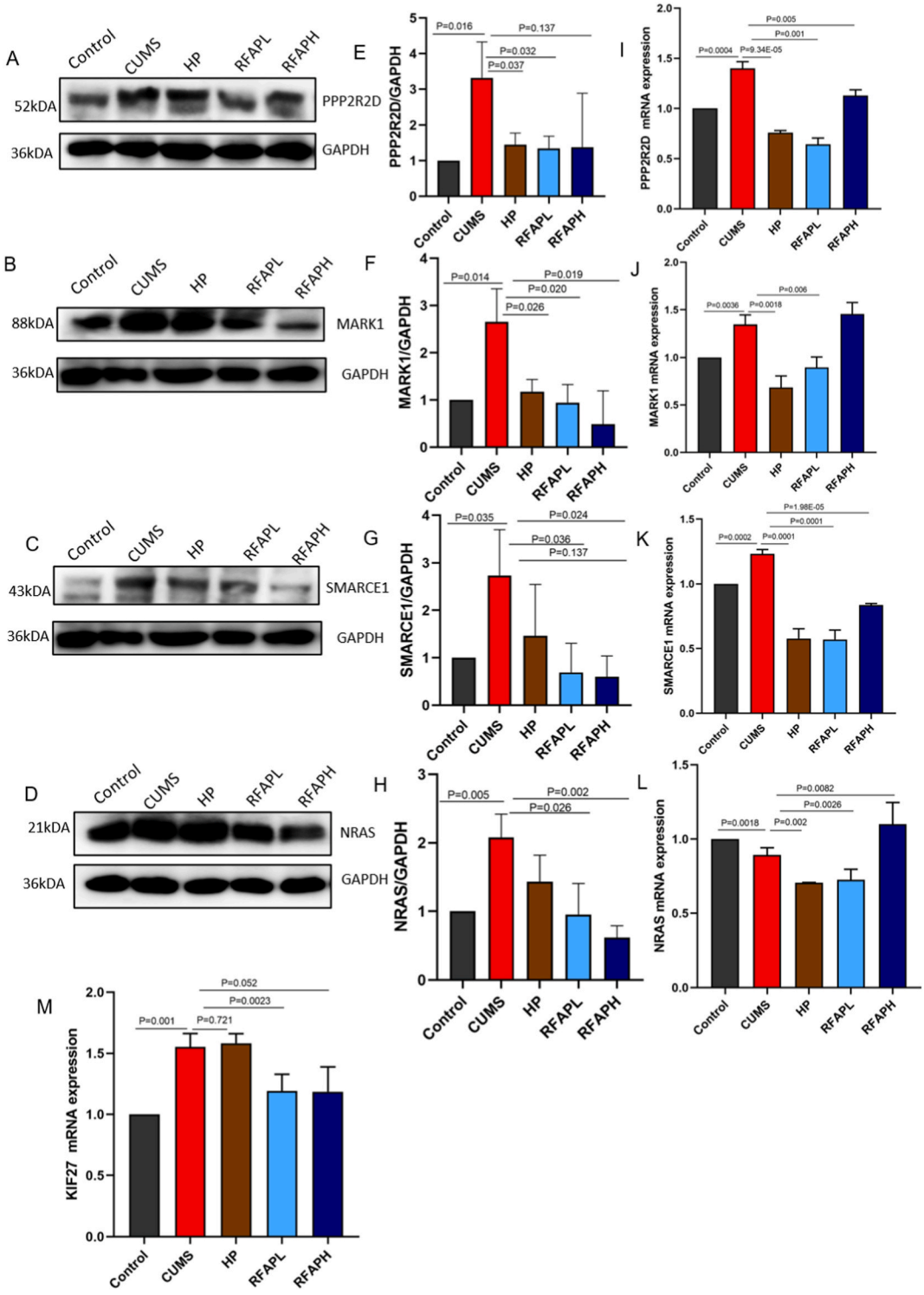


Fig. 7. Quantification of key proteins of western blotting and RT-PCR in depression. (A) WB verified the change proteins (A–H). B. RT-PCR verified the change proteins (I–M). Note: Data are expressed as mean ± SD (A–M, pooling samples, n = 3).

Another piece of evidence from the immobility duration of the FST also showed that the suspension and immobility time of the model group was significantly longer than that of the normal control ($p = 0.07$) (Fig. 1H). After the drug intervention, CUMS-RFAPL showed a trend of reduction in the suspension and immobility time of rats. The results suggested that rats with CUMS had typical behavioral despair.

3.2. General features of the depression brain proteome

The differentially expressed proteins in five depressant groups were identified using mass spectrometry. The workflow is shown in Fig. 2A. The number of identified peptides and proteins were 21,812 and 2344, respectively. Eventually, a total of 2287 quantified proteins were identified (unique peptides $\geq$2, identified in at least eight samples) (Fig. 2B). The ratio distribution of up-regulated and down-regulated proteins in different groups in the CUMS model are shown in Fig. 2C. The changing trends in different groups (CUMS model vs. control, drug treatments vs. CUMS model) are opposite. The number of changed proteins are shown in Fig. 2D). According to the principal component analysis (PCA), the variances within groups were significantly smaller than that between groups. The PCA results revealed that the control, HP, and RFAPLH groups were clustered, whereas the CUMS group was dispersed among the other clusters. It indicated the potential protective effect of HP and RFAPLH (Fig. 2E).

3.3. GO and string analysis of up-regulated proteins in CUMS model

The volcano map of CUMS model changed proteins (CUMS vs. control) are shown in Fig. 3A. The significantly up-regulated proteins included EXOC2, RBP1, BZW1, SAR1B, IIF2, EDF1, etc. The significantly down-regulated proteins included FLOT1, KCNIP2, INPP5J, SRP54, COMMD3, PRRC2D, etc. GO and STRING analysis of CUMS up-regulated proteins involved biological function related to acetylation, immune system process, long-term depression, long-term potentiation, mitochondrion, nervous system development, neuronal synaptic plasticity, short-term neuronal synaptic plasticity, synaptic vesicle exocytosis et al. (Fig. 3B and C).

3.4. GO and string analysis of down-regulated proteins in CUMS model

The biological procedure GO and STRING analysis revealed that the down-regulated proteins in the CUMS model were involved in the pathway related to learning or memory, modulation of chemical synaptic transmission, ATP metabolic process, cellular lipid metabolic process, recombinant notch homolog 4(NOTCH4)/wingless/integrated (WNT) signaling/degradation of β-catenin, nonsense mediated decay (NMD), and ROBO receptors (Fig. 4A and B).

3.5. Depression related proteins were up-regulated in CUMS and RFAPH attenuated the change

The expression of LTD-related proteins, such as NRAS, GNAI1, and GRM1, was significantly up-regulated in the CUMS group and was restored by HP and RFAPH administration. (Fig. 5A). The long-term potentiation (LTP) is widely recognized as one of the major cellular mechanisms that underlie learning and memory [25]. LTP proteins, such as PPP2R2D, PPP1CB, and TAOK1 were significantly up-regulated in the CUMS group and restored after RFAPH administration (Fig. 5B). Nervous system development proteins (LRRC8D, VAT1, and THY1), as well as Neuronal synaptic plasticity regulation proteins (RAB12, RAB3GAP1 and RAB5A) were also up-regulated in the CUMS group and restored in the RFAPH groups (Fig. 5C and D).

3.6. Structural constituents of the ribosome, learning or memory, ATP, and cellular lipid metabolic process related proteins were down-regulated in CUMS and RFAPH rescued the change

The structural components of ribosome-associated proteins were significantly down-regulated in the CUMS group. This effect was reversed with the treatment of RFAPH. These proteins included RPL15, RPL28, RPS18, etc. (Fig. 6A). Of these, RPL28 and RPS18 were participated in the repressive regulation of the Structural constituent of the ribosome pathway. ATP metabolic process, including ATP5J2, ATP1B2, MT-ATP8, etc., were significantly down-regulated in the CUMS group. ATP5J2 and MT-ATP8 were restored by RFAPH administration (Fig. 6B). Learning or memory-related proteins (ARF4, SARNP, PPP1R12A, etc.) were significantly down-regulated in the CUMS group, and this effect was reversed by RFAPH administration (Fig. 6C). ARF4, SARNP, and PPP1R12A were also participated in the repressive regulation of the Structural constituent of ribosome pathway. Cellular lipid metabolic process, including IDI1, INPP5J, CPT1A, etc., were significantly down-regulated in the CUMS group and returned after RFAPH administration (Fig. 6D).

3.7. Quantification of key proteins of western blotting and RT-PCR

Most of the up-regulated proteins were related to long-term depression, long-term potentiation, nervous system development proteins and neuronal synaptic plasticity regulation. Some of them has post-translation modification, such as phosphoprotein and Acetylation proteins. Quantification of western blotting and RT-PCR showed in Fig. 7. The results of RT-PCR and Western blot were consistent with the results of proteomic analysis. Both gene and protein levels of PPP2R2D and MARK1, which are associated with the long-term dementia pathway, were significantly upregulated in the CUMS group but decreased to near control levels in the HP and RFAPH groups. (Fig. 7A, B, E, F, I, G). SMARCE1 gene and protein levels of RHO GTPases Activate WASPs and WAVEs pathway were

substantially elevated in the CUMS group. In the HP and RFAPH groups, it dropped to nearly control group levels. (Fig. 7C, G, K). NRAS gene and protein levels of long-term depression pathway were substantially elevated in the CUMS group. It dropped to nearly control group levels in the HP and RFAPH groups (Fig. 7D, H, L). KIF27 gene levels of the nervous system development pathway were substantially elevated in the CUMS group. It dropped to nearly control group levels in the RFAPL and RFAPH groups (Fig. 7M).

4. Discussion

TCM has been used to treat depression related diseases for a long history. The exact antidepressant efficacy effects are well established. The current study has proven a significant pharmacological effect of the antidepressant effect of RFAP. Due to its holistic, multidrug, multitarget nature, the exact mechanisms of RFAP exerted antidepressant properties via the signaling pathway still remain to be fully understood. Thus, the present study, for the first time, provides the biological mechanism of RFAP efficacy via proteomics and molecular biology.

We performed a systematic search of the literature. As reported in the previous literature, the eight main components of RFAP are the major antidepressant active components, including Paeoniflorin, albiflorin, Crocin-I, geniposide, gardenias, total flavonoids, Quercetin, and Paeonol. All of them presented potential pharmacological mechanisms. Of these, Paeoniflorin possibly plays a neuroprotective effect in improving chronic stress-induced depression and neuronal damage in rats through activation of the ERK-CREB pathway [26,27]. Studies have shown that albiflorin directs antidepressant role by the cytoplasmic phospholipase A2 (cPLA2) -protein kinase B (Akt1)-indoleamine 2,3-dioxidase 1(IDO1) regulatory loop [28]. Crocin-I has been found to relieve depression-like behaviors may by modulating “microbiota-gut-brain” axis in mice exposed to chronic restraint stress [29]. It was proclaimed that the geniposide might improve anxiety and depressive behavior in LPS-induced depression mice by modulating BTK/JAK2/STAT1 neuroprotective signaling [30]. Gardenias has antidepressant effects on PRS mice by inhibiting DNA methyltransferase 1 (DNMT1) and normalizing brain-derived neurotrophic factor (BDNF) expression through DNA demethylation in the hippocampus [31]. The total flavonoids isolated from Albizzia julibrissin extracts reverse behavioral alterations and serotonin dysfunction in chronically stressed rats, possibly by stimulating hippocampal neurogenesis [32]. Quercetin, a well-known flavonoid compound, has antidepressant effects by inhibiting the activation of PI3K/AKT/NF-κB inflammatory signaling and subsequent neuroplasticity improvement [33]. Paeonol may be involved in the BDNF-Rac1/RhoA pathway to reduce behavioral and neuronal damage caused by CUMS, which may represent a novel drug for depression [34]. The protective effect of RFAP on CUMS may be the result of the antidepressant effect of these compounds and the combined efficacy of natural herbs in the treatment of depression.

The number of identified proteins was 2344, and eventually, a total of 2287 quantified proteins were identified (unique peptides $\geq$2, identified in at least eight samples). The high coverage of quantified protein in the hippocampus of our study benefits in obtaining much more information for further pathway analysis. The protective effect of RFAP could be observed from changes in protein profiles in PCA (Fig. 2E). Proteins differently expressed in the CUMS group and, meanwhile, restored in the RFAPH groups were used to identify potential targets of RFAPH. The upregulation of LTD, LTP, nervous system development proteins and neuronal synaptic plasticity regulation proteins showed evidence of the successful establishment of the CUMS model.

LTP and LTD are the two main types of proteins related with long-term synaptic plasticity [35]. LTP and LTD characteristics of these synapse plasticity experiments may be applied to mimic the neuroplasticity of the human brain []. The changes in synaptic strength, including LTP and LTD, are considered the major cellular mechanisms that underlie learning and the encoding of memories across the brain [35]. The restoration of LTP, LTD, and nervous system related proteins after RFAPH administration showed the potential influenced pathways of RFAPH.

Several interesting phenomena were discovered in our study. Both gene and protein levels of PPP2R2D and MARK1, associated with the long-term dementia pathway, were significantly upregulated in the CUMS group. PPP2R2D, a regulatory subunit of PP2A. Previous studies have reported that PP2A inhibition may provide a novel treatment for depression characterized by lateral habenula hyperactivity [37]. In our study, a lot of PP2A family proteins up-regulated in CUMS model, but in the HP and RFAPH groups, it decreased to levels comparable to the control group, which is confidential with the previous report and thus confirmed the protective effect of RFAPH may through restoring the expression of PP2A family to rescue of GABAB and GIRK function. MARK1 as one of 12 kinases that are related to AMP-activated protein kinase (AMPK). Brain derived neurotrophic factor (BNF) and KCl-induced depolarization of nerve cells enhanced MARK1 activity, suggesting that MARK1 may be involved in regulating synaptic plasticity [38]. In our study, MARK1 proteins up-regulated in CUMS model but restored to near control group levels in the HP and RFAPH groups, indicating that RFAPH plays a protective role by restoring the expression of MARK1 to rescue and regulate the function of synaptic plasticity. The gene and protein levels of RHO GTPases Activate WASPs and WAVEs pathway-related SMARCE1 significantly increased in the CUMS group. SMARCE1 is a master transcription regulator to maintain the function of neuroplasticity. It belongs to the SNF/SWI family of chromatin remodeling protein [39]. In our study, SMARCE1 proteins up-regulated in CUMS model but decreased to near control group levels in the HP and RFAPH groups, indicating RFAPH may be a new interpret new target for a protective effect of regulation of transcription function. A previous study reported that changed proteins in amino acid, fatty acid, and glycerophospholipid metabolism, accompanied by alterations of metabolic enzymes in the hippocampus in CUMS rat model [40]. Our result indicated the change of ATP and cellular lipid metabolic process related proteins beyond the previous report. These hippocampal protein candidates may be linked to stress-induced susceptibility to depression, anxiety, and stress [41]. The key proteins of these pathways were validated via western blotting qualification. The proteomics research showed further validation that the negative role of LTD and LTP proteins in depression.

There are some limitations to our study. One of the limitations of this study is the insufficiency of mechanisms validation in the pathway interpretation. The detailed molecular mechanism deserves further investigation. A further limitation of the study is that by

using a CUMS rat model to estimate depressive-like behaviors, the evidence for the validation effect is insufficient. Further models (memory model and learning models) and behavioral assays should be introduced to confirm this conclusion. Additionally, the mechanisms for down-regulated proteins need to be more studied. In our study, we established the CUMS model and analyzed up-regulated proteins that were restored after RFAP treatment. As to down-regulated proteins with functional decline, it is hardly returned to normal. However, down-regulated proteins might be potential drug targets that will be worth diving into in future studies.

5. Conclusions

Our study provided proteomics-based evidence that RFAP demonstrated treatment effects in rat CUMS model via long-term potentiation and depression renovation.

Author contribution statement

Yang Wu, Ying Hao, Pengcheng Fan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Guohua Yu, Li Li, Shanglong Wang, Xin Li, Zengliang Zhang, Shengcan Zou: Contributed reagents, materials, analysis tools or data.

Zimin Liu, Yuanyuan Shi: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository with the dataset identifier PXD034278.

Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e13429.

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