ORIGINAL ARTICLE

Rec. Nat. Prod. 19:6 (2025) 702-716

Pearl Powder Exerts Antidepressant Effects by Modulating the GluN2A/BDNF/PSD-95 Signaling Axis

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(Received June 18, 2025; Revised August 28, 2025; Accepted September 01, 2025)

Abstract: To evaluate the antidepressant-like effect of pearl powder in rats and explore its potential mechanism based on network pharmacology analysis and experimental validation. The chronic multiple mild stress (CMMS) rat model was applied to evaluate the effects of pearl powder on depression-like behaviors in rats through the sucrose preference test, forced swimming test, and tail suspension test. In addition, based on the identification of the major amino acid components of pearl powder, a network pharmacology analysis was performed to identify potential molecular targets of pearl powder. Subsequently, we compared the identified targets of pearl powder with depression-related targets by a Venn diagram and obtained the intersecting key genes. These genes were further used to construct PPI network and KEGG pathway analysis. Finally, Western blotting assay was applied to verify the protein levels of the relevant targets regulated by pearl powder to explore the underlying mechanisms. The behavioral tests showed that different doses of pearl powder (16.7 mg/kg, 83.3 mg/kg, and 166.7 mg/kg, administered once daily for one week by gavage) significantly improved the sucrose preference and alleviated depression-like behaviors in CMMS rats (FST: immobility time decreased by 12.34% ± 2.34%; TST: immobility time decreased by 12.03% ± 7.75%). After pretreatment of pearl powder with hydrochloric acid, the major amino acids in pearl powder were detected by an automatic amino acid analyzer, with top 5 (Gly, Ala, Glu, Asp, and Arg) accounting for more than 80% of the total amount. Network pharmacology analysis revealed that the top 5 active amino acids in pearl powder could regulate several targets such as CASP3, BDNF, MTOR, GluN2B, and MAPK1. The 84 key genes were also obtained after compared with depression-related target genes. In the following GO enrichment and KEGG pathway analysis, the top 10 items in the bubble plot indicated that pearl powder might exert anti-depression effects by regulating neuronal synaptic membranes and signal transmission. The next Western blot analysis verified that pearl powder could upregulate GluN2Aand synaptic-related proteins PSD-95 and BDNF, thereby improving the synaptic structure and function in the hippocampus. This study first clearly demonstrated that pearl powder significantly improved depressive-like behavior in CMMS rats. By combining network pharmacology and confirmatory experiments, it elucidated that the major active amino acids in pearl powder might participate in the repair of synaptic damage by regulating the expression of synaptic-related proteins, ultimately exerting its pharmacological activity against depression.

Keywords: CMMS rats; depressive-like behavior; pearl powder; synaptic damage; NMDAR.

1. Introduction

The rapid transformation of the global economic structure and the social pressures had led to a continuous increase in mental disorders. Neuropsychiatric disorders ranked first in the total disease burden in China, with depression exhibiting a particularly high prevalence rate. Over the past three decades, the number of global cases surged by nearly 50%, and more than

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The article was published by ACG Publications
http://www.acgpubs.org/journal/records-of-natural-products November-December 2025 EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.551.2506.3569
Available online: September 09, 2025

264 million people across all age groups currently suffered from depression [1]. Depression is a common neuropsychiatric disorder characterized by high prevalence, high suicide rates, high recurrence rate, and significant disability [2]. Its primary clinical manifestations included persistent low mood, diminished interest, and anhedonia. It was often accompanied by cognitive dysfunction, memory impairment, and suicidal behaviors [3, 4]. The World Health Organization predicts that by 2030, depression will become the leading cause of disability and mortality worldwide.

Current evidence elucidates that the pathogenesis of depression arises from a complex interplay of factors. These include aberrant neural circuit dynamics, imbalances predicted by the monoamine hypothesis of neurotransmission, compromised neuroplasticity, and the dysregulation of neurotrophic factors, with brain-derived neurotrophic factor (BDNF) being of particular note. Furthermore, hypotheses implicating neuroimmune interactions and cytokine signaling pathways contribute to this multifactorial understanding [5-9]. Collectively, these theoretical frameworks are of paramount importance in advancing our comprehension of the intricate pathophysiological mechanisms and the progressive course of depressive disorders.

Unfortunately, modern medicine still has limited pharmacological options for depression treatment. Common antidepressants mainly included monoamine oxidase inhibitors, selective serotonin reuptake inhibitors, selective serotonin and norepinephrine reuptake enhancers, and dopamine reuptake inhibitors [10]. However, these drugs have several issues, such as significant individual differences in effective dosage, severe side effects, and difficulty in improving the concomitant insomnia and anxiety [11, 12]. In addition, antidepressants were prone to withdrawal reactions when discontinuing or switching medications, which might lead to symptom exacerbation [13]. Therefore, it is especially important to seek new effective active substances from traditional Chinese medicine and aquatic organisms [14, 15].

Pearl powder belongs to the category of sedatives and tranquilizers in traditional Chinese medicine, with pharmacological effects of calming the mind, settling agitation, and benefiting the intellect. Current experimental studies showed that pearl powder could improve sleep quality and alleviate negative emotions such as depression and irritability [16, 17]. Proteomics studies indicated that pearl powder improved cognitive impairment induced by sleep deprivation in rats by scavenging oxidative free radicals and inhibiting oxidative phosphorylation [18-20]. Clinical studies also demonstrated that the peptide components in pearl powder extract possessed anti-oxidative damage, anti-analgesic activity and anti-depression. The aim of this study is to clarify the pharmacological mechanisms of pearl powder in combating depression, providing a theoretical basis and foundation for future clinical observational trials.

2. Materials and Methods

2.1. Preparation of Crude Protein from Pearl Powder (CPPP)

Approximately 10.0 g of pearl powder was put into a 500 mL beaker, mixed with 10 mL of water to maintain a state of infiltration. Hydrochloric acid (10 mL, 6 mol/L) was gradually added in four increments. The mixture was stirred thoroughly to allow full reaction between the acid and calcium salts. Once the foam had subsided naturally, the mixture was filtered through suction filtration by using filter paper, rinsing thoroughly with double distilled water. When filtration is nearly dry, hydrochloric acid was added to immerse the solid, agitated gently for 10 minutes to ensure complete reaction, then the mixture was filtered again. The acid soaking and filtration process was repeated until no bubbles formed. Finally, the filtrate was rinsed with water until reached neutral pH, yielding orange or orange-tinted crude protein.

2.2. Animals and Reagents

SD male rats (180-200g, aged 4-6 weeks) were purchased from the Animal Experiment Center of Ningbo University. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, 1978 revision) and approved by the Animal Ethics Committee of Ningbo University (approval number 2020-149). This study also strictly adhered to the ARRIVE guidelines for reporting animal research. Sucrose was purchased from Solarbio (No.: S8271, Beijing); saline was purchased from Solarbio (No: IN9000, Beijing); pearl powder, sourced from freshwater pearls, was purchased from Ningbo Dachang Pharmaceutical (No.: 2022005, Ningbo, Zhejiang) with ≥98% purity, meeting Chinese Pharmacopoeia (2020) standards. The CPPP was prepared as suspension by dispersing in saline for intragastric administration. One week before the experiment began, the rats were housed in a temperature- and humidity-controlled, relatively enclosed environment (temperature 22 ± 1°C, humidity 60% ± 5%, 12-hour light/dark cycle), with free access to food and water, to acclimate the environment.

2.3. Establishment of the Chronic Multiple Mild Stress (CMMS) Model

A "chronic multiple mild stress depressive-like" rat model was established by using a multisensory stress simulation device [21]. Rats were randomly divided into five subgroups(n=5): normal control group (no stress simulation or treatment applied), CMMS group (stress simulation + saline), CMMS subgroups with different dose of pearl powder (16.7 mg/kg, 83.3 mg/kg and 166.7 mg/kg). As we mentioned before, rats in CMMS subgroups were placed in the multisensory stress simulation device, where they were exposed to various stress factors such as temperature (30±2°C), humidity (80±5%), a restricted animal cage with 1.5 times space size of the rat itself, flashing lights (5 Hz), and noise (80±10 dB) [21]. The whole model-building period in the multisensory stress simulation device lasted for four weeks, with normal access to water and food. At the beginning of the fourth week, each CMMS rat received 0.5 ml of CPPP or saline, administered once daily for one week. At the end of the fourth week, behavioral tests were performed (Sucrose Preference Test (SPT), Tail Suspension Test (TST), Forced Swimming Test (FST)), and hippocampal tissue was collected for western blot assays (specific procedure shown in Figure 1A).

2.4. Sucrose Preference Test

Each rat was housed individually in a cage and executed 48-hoursadaptive training. During this period, two water bottles were placed in each cage: one containing a 1% sucrose solution and the other containing pure water. The positions of the bottles containing water and sucrose solution were swapped every 24 hours. Subsequently, food and water deprivation was performed for 24 hours, and the formal experiment began. The amount of water and sucrose consumed by each rat in the next 24 hours was measured (the consumption was measured by weighing the bottles). The sucrose preference rate was calculated using the following formula: sucrose consumption / (water consumption + sucrose consumption) × 100%.

2.5. Forced Swimming Test

On day 29, prior to the experiment, the rats were placed in a 25°C testing room for 1 hour to acclimate, then placed into a circular water container with a diameter of 20 cm (with a black background panel at the rear). The rats were allowed to swim freely for 6 minutes, and the immobility time during the last 4 minutes was recorded (immobility was defined as the rat floating with its head up on the water surface and making small, necessary movements to keep its head above water, such as swimming with one hind limb).

2.6. Tail Suspension Test

On day 30, prior to the experiment, the rats were placed in the testing room for 1 hour to acclimate. The tail of each rat was fixed with medical tape about 2 cm from the tip, and the other end of the tape was attached to the top of the tail suspension apparatus. The head of rat was 15 cm above the ground. The rat was suspended for 7 minutes, and the immobility time during the last 6 minutes was recorded (immobility was defined as the rat being passively suspended with completely motionless).

2.7. Measurement of Active Amino Acid Components in Pearl Powder and Targets Screening of Network Pharmacology

Pearl powder was dissolved in 10% hydrochloric acid to simulate the digestion and degradation of pearl proteins in the gastric acid environment. The amino acid mixture after acidic pretreatment was analyzed using an automatic amino acid analyzer (Hitachi, Japan, L-8900) to detect the amino acid components and their content. Based on the quantified data, the main amino acid components of pearl powder were identified. The corresponding amino acid SMILES files were obtained from PubChem and uploaded to the Swiss Target Prediction and Superpred databases. The target genes were obtained and merged.

2.8. Identification of Depression Prediction Targets

By searching the GeneCards database (https://www.genecards.org) and OMIM (https://www.omim.org) database, the keyword "Depression" was entered to obtain related disease targets. The target genes from GeneCards (Relevance score≥2) and OMIM databases were then merged, and duplicates were removed.

2.9. PPI Network Construction

To facilitate visualization, a Venn diagram analysis was performed to compare the target genes of the active amino acids in pearl powder with the genes associated with depression, identifying the intersection, which represented the potential therapeutic targets of pearl powder. The overlapping target genes were input into the String database (https://cn.string-db.org) and the resulting data from String database were imported into Cytoscape 3.10.1 to construct the corresponding PPI network. Each target is represented by a node, and the relationships between the targets are represented by connecting lines.

2.10. GO and KEGG Pathway Analysis

To further investigate the biological functions of potential therapeutic targets in depression treatment, GO and KEGG enrichment analyses were performed using the DAVID database (https://david.ncifcrf.gov/). GO analysis mainly included the screening of biological processes (BP), cellular components (CC), and molecular functions (MF). KEGG enrichment analysis was also applied to identify important signaling pathways involved in biological processes. Subsequently, the GO and KEGG data were uploaded to a bioinformatics platform (bioinformatics.com.cn) for visualization analysis.

2.11. Western Blot Assay

Hippocampal tissue preparation: Hippocampal tissue was mixed with protein lysis buffer RIPA (NCM, WB3100) at a ratio of 1 mg:100 μL and completed proteolytic cleavage by ultrasonication. After an ice bath for 20-30 minutes to allow sufficient protein dissolution, the sample was centrifuged at 13,200 rpm for 30 minutes. The supernatant was mixed with loading buffer (NCM, WB2001) at a

1:4 (v/v) ratio, and the mixture was heated at 100°C for 10 minutes. The prepared loading sample was then stored at -80°C. Protein concentration in the supernatant was measured using the BCA kit (CWBio, CW0014S). The amount of loading sample was 60 μg, with the following sample order from left to right: control group, depression group, medium-dose pearl powder group, and high-dose pearl powder group. All samples were loaded into 7.5% or 10% SDS-PAGE gels and underwent electrophoresis for 1.5 hours at 150 V to separate the proteins. The gel was then placed in rapid transfer buffer（NCM, WB4600）, and proteins in the gel were transferred to PVDF membranes using a current of 400 mA for 30 minutes. After blocking the PVDF membrane with blocking buffer (NCM, P30500) for 10 minutes, the membrane was incubated with various primary antibodies respectively (including rabbit anti-PSD-95 (1:1000, Beyotime, AF1096), rabbit anti-GluN2A (1:1000, ABclonal, A0924), rabbit anti-GluN2B (1:1000, Proteintech, AF7029), rabbit anti-BDNF (1:1000, ABclonal, A16299), rabbit anti-Beta Actin (1:5000, Proteintech, 81115-1-RR)) overnight at 4°C (approximately 18 hours). The membrane was washed three times with 1×TBST (Solarbio, T1085), 10 minutes for each time. The membrane was then incubated with goat anti-rabbit IgG H&L secondary antibody (1:10,000, Abcam, ab175773) for 1 hour at room temperature. After removing the secondary antibody, the membrane was washed three times with 1×TBST, 10 minutes for each time. Finally, detection was performed by using an Odyssey fluorescence scanner (LI-COR Biosciences, Odyssey 9120), and semi-quantitative analysis was carried out through grayscale quantification by using ImageJ software (NIH, V1.53).

2.12. Data Analysis

In this study, statistical analysis was performed using GraphPad Prism 9.0.0 software. Statistical differences were analyzed using one-way ANOVA, and data were presented as mean ± SD. One-way ANOVA was conducted for comparisons among multiple groups, followed by Tukey's post-hoc test for multiple comparisons. A P-value less than 0.05 was considered as statistically significant.

3. Results and Discussion

3.1. Behavioral Tests

The dosage of CPPP was confirmed by both reference and its maximum solubility. In behavioral pre-tests, we examed the lower dose such as 1 mg/kg and 10 mg/kg. However, the anti-depression effect was not prominent. Thus, we selected the dose range between 16.7 mg/kg and166.7 mg/kg in subsequent experiments. The results showed that compared to the normal rats, CMMS rats exhibited a significant decrease in sucrose preference, indicating anhedonia (Figure 1B). However, after CPPP intervention, the sucrose preference in CMMS rats was restored by 17.89% ± 4.14%, especially the oral administration of high-dose CPPP (P= 0.0421). In the FST and TST, CMMS rats exhibited significantly longer immobility times compared to normal rats, indicating a more pronounced tendency towards hopeless behavior (FST: P= 0.0028; TST: P=0.0035; Figure 1C-D). CPPP treatment also reduced immobility time of CMMS rats in both FST and TST (FST: 12.34% ± 2.34%; TST: 12.03% ± 7.75%). These findings suggested that CMMS rats displayed significant depressive-like behaviors, and CPPP treatment could alleviate these behaviors to some extent, in the dose-dependent manner.

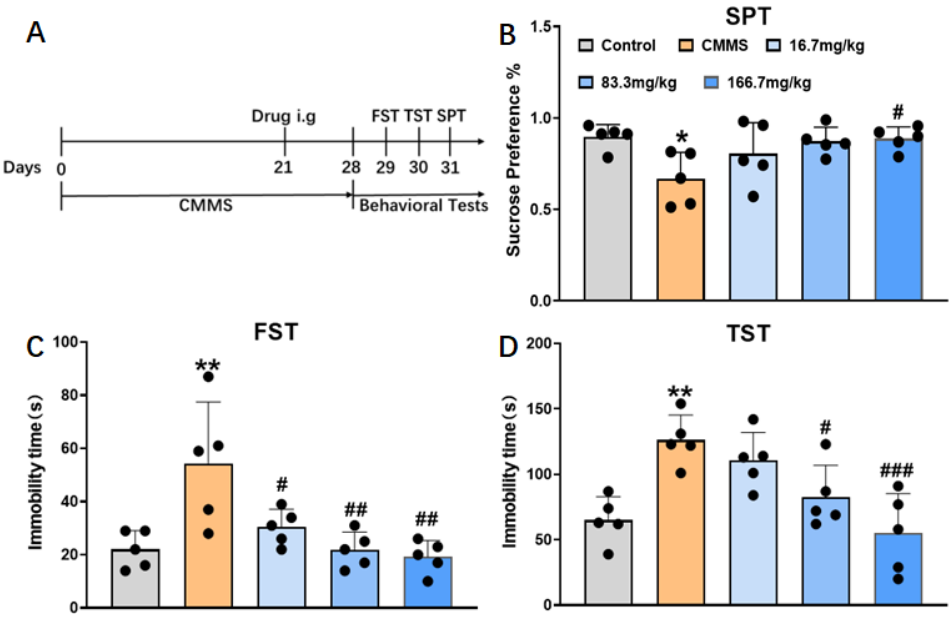
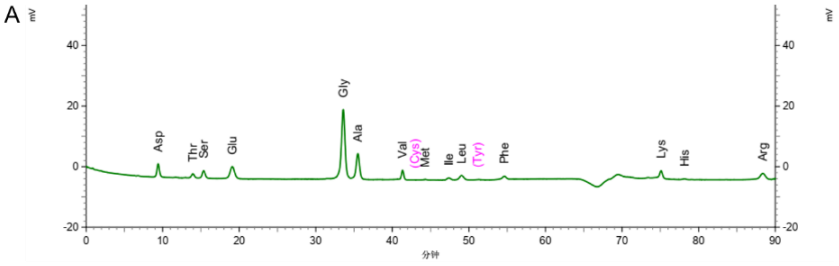


Figure 1. CPPP significantly improved depressive-like behavior in CMMS rats. (A) Experimental schedule: Construction of the CMMS rat model (a total of 4 weeks) and the pearl powder treatment period (from the third to the fourth week), followed by behavioral testing after the fourth week. (B-D) The effect of pearl powder on depressive-like behavior in CMMS rats through the SPT, FST, and TST experiments. Data are presented as mean ± SD, with statistical analysis conducted using One-way ANOVA for multiple group comparisons, followed by Tukey's post-hoc test; n=5; *P<0.05 and **P<0.01 indicated a statistically significant difference compared to the control group; #P<0.05, ##P<0.01 and ###P<0.001 indicated a statistically significant difference compared to the model group.

3.2. Amino Acid and Network Pharmacology Analysis

Pearl powder was dissolved in 10% hydrochloric acid to simulate the gastric acid environment, and the acidified mixture was detected by using an automatic amino acid analyzer (Hitachi, Japan, L-8900), to obtain the quantitative data of the amino acid components and their content (Figure 2A-B). The SMILES codes of top 5 and top 10 amino acids in pearl powder were input into the SuperPred and SwissTargetPrediction databases. It was found that the top 5 and top 10 amino acids had 193 and 213 potential targets respectively, indicating that bioactivities of pearl powder were primarily represented by the top 5 amino acids. In addition, since the top 5 amino acids (Gly, Ala, Glu, Asp, Arg) accounted for more than 80% of the total content, we ultimately chose the 193 potential target genes corresponding to these top 5 amino acids for network pharmacology research. These molecular targets were imported into Cytoscape 3.10.1 to construct a network topology diagram with 183 nodes and 1081 edges (Figure 3A). The diagram revealed that the hub genes mainly included CASP3, HIF1A, MAPK1, MTOR, CXCR4, STAT1, PIK3R1, BDNF and others. These target genes are primarily associated with cell proliferation and apoptosis, inflammation, neurotrophin regulation, and immune modulation functions.

A



B

No. of peak	Time/min	Name	Molar concentration (nmol/mg)	Pecentage	Mass concentration (ng/mg)
1	9.4	Asp	7.05	5.9	938.13
2	13.9	Thr	2.41	2.0	287.44
3	15.3	Ser	4.73	4.0	496.85
4	19.1	Glu	11.60	9.7	1706.34
5	33.6	Gly	52.43	43.8	3935.85
6	35.5	Ala	18.38	15.3	1637.73
7	41.3	Val	4.06	3.4	474.97
8	44.3	Met	0.18	0.2	27.12
9	47.4	Ile	1.38	1.2	181.48
10	49.0	Leu	3.63	3.0	476.11
11	54.6	Phe	2.82	2.4	465.67
12	75.1	Lys	4.06	3.4	593.70
13	78.2	His	0.39	0.3	60.96
14	88.4	Arg	6.62	5.5	1152.92

Figure 2. Determination of amino acid content and quantified Data after acid hydrolysis of pearl powder. (A) Amino acid chromatogram of the acid hydrolysate of pearl powder, showing the separation of different amino acids, with the x-axis representing time (minutes) and the y-axis representing signal intensity (mV). Major amino acid peaks are labeled. (B) Table detailing the identified amino acids and their contents in pearl powder, listing their respective peak numbers, retention times (minutes), names, molar concentrations (nmol/mg), percentage contents (%), and mass concentrations (ng/mg). A total of 14 amino acids were detected and quantified. The top five amino acids by percentage content are: Glycine (Gly, 43.8%), Alanine (Ala, 15.3%), Glutamic acid (Glu, 9.7%), Aspartic acid (Asp, 5.9%), and Arginine (Arg, 5.5%).

Additionally, the depression-related target genes from the GeneCards (Relevance score ≥ 2.0) and OMIM databases were also integrated, and 2,618 related target genes obtained. The 193 potential target genes of the top 5 amino acids in pearl powder were intersected with the depression-related target genes, resulting in 84 intersecting genes. These targets were selected as key genes for treating depression with pearl powder (Figure 3B). To explore the mechanism of pearl powder in treating depression, GO enrichment and KEGG pathway analyses were conducted on these 84 key genes. The GO enrichment analysis identified 1,569 items: 1,328 biological processes (BP), 150 molecular functions (MF), and 91 cellular components (CC). The top 10 items from the bubble chart suggested that the top 5 amino acids in pearl powder might participate in the treatment of depression by modulating synaptic membranes and signal transmission between synapses in neurons (Figure 4A-C).

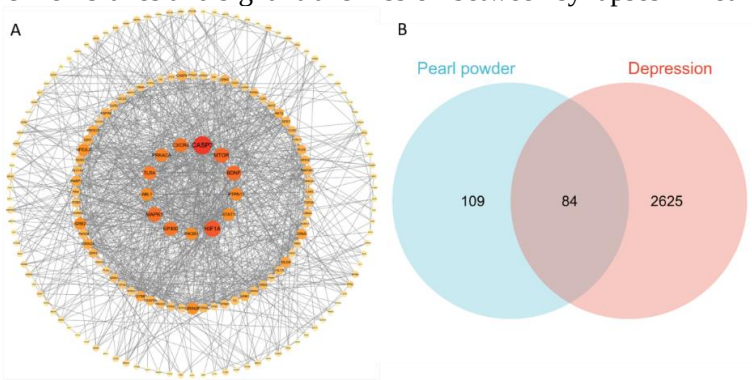


Figure 3. PPI Network of top 5 Amino Acids from Pearl Powder. A. Network topology of the top 5 amino acids in pearl powder. Node characteristics reflect their network centrality: deeper

color and larger size indicate higher degree value. B. Venn diagram of potential intersecting genes between the top 5 amino acids in pearl powder and depression-related targets.

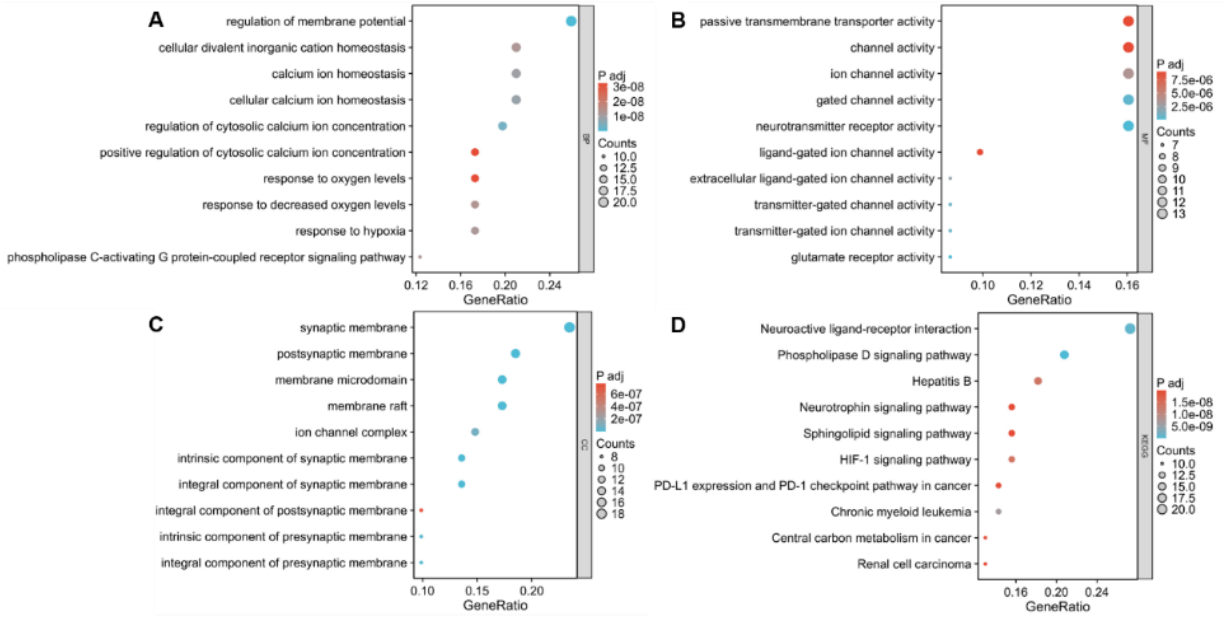


Figure 4. Results of GO and KEGG Enrichment Analysis based on 84 key genes intersected from potential target genes of the top 5 amino acids in pearl powder and depression-related targets. (A-C) Bubble chart of the top 10 biological processes (BP), molecular functions (MF) and cellular components (CC) in the GO enrichment analysis. (D) Bubble chart of the top 10 pathways in the KEGG enrichment analysis. The X-axis and Y-axis represented the gene ratio and the full name of the process, respectively. The color and size of each bubble represented the P-value and the number of genes, respectively.

KEGG pathway analysis (with a significance level of $P < 0.05$) revealed that these 84 common targets were highly enriched in 157 pathways. Based on the gene ratio and P-value, the top 20 major pathways were displayed (Figure 4D), including neuroactive ligand-receptor interaction, Alzheimer's disease, phospholipase D signaling pathway, sphingolipid signaling pathway, apoptosis, neurotrophin signaling pathway, etc. The phospholipase D signaling pathway plays a role in phospholipid metabolism and cell growth, while the sphingolipid signaling pathway is important in neuronal cell signaling [22]. Therefore, KEGG pathway analysis also suggested the potential neuroregulatory functions of pearl powder. We then imported these 84 common targets into the STRING database to construct a PPI network topology (Figure 5), including 82 nodes and 393 edges. The hub genes included Caspase-3 (CASP3), Brain-Derived Neurotrophic Factor (BDNF), Mechanistic/Mammalian Target of Rapamycin (MTOR), Glutamate Ionotropic Receptor NMDA Type Subunit 2B (GluN2B), Mitogen-Activated Protein Kinase 1 (MAPK1), etc. These hub genes were closely associated with cell proliferation and apoptosis, as well as neurotrophic and neuroregulatory functions, playing critical roles in the pathological mechanisms of depression. For instance, CASP3, as an apoptosis-executing protein, likely exacerbated neurodegenerative damage in depression by regulating neuronal apoptosis [23]. BDNF was linked to the efficacy of antidepressant treatments through its promotion of synaptic plasticity and neurogenesis [24]. The mTOR signaling pathway was pivotal in rapid antidepressant mechanisms, as exemplified by ketamine, which improved synaptic function by activating mTOR [25]; GluN2B, involved in glutamatergic signaling, likely contributed to synaptic dysfunction in depression due to its aberrant expression [26]. MAPK1 influenced emotional regulation in depression by modulating neuroplasticity and inflammatory responses [27]. The high connectivity of these hub genes suggested that they collaborated through complex signaling networks to collectively regulate the neurobiological processes of depression.

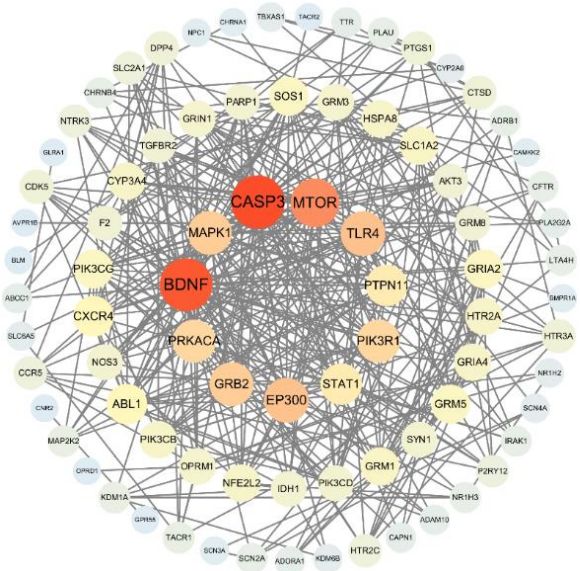


Figure 5. The Potential Molecular Target Network of 84 key genes. The protein-protein interaction network is visualized. Node characteristics reflect their network centrality: deeper color and larger size indicate higher degree value.

3.3. Western Blot Results

The hippocampus plays an important role in spatial cognition and memory, and is closed associated with the process of depression [28]. Considering the close relationship between N-methyl-D-aspartic acid receptor (NMDAR) and synaptic plasticity, this study examined changes of NMDAR subunits GluN2A, GluN2B, as well as synaptic-related protein PSD-95 and neurotrophic factor BDNF in the CMMS depression model. Meanwhile, the effects of CPPP on the expression of these synaptic proteins were also assessed.

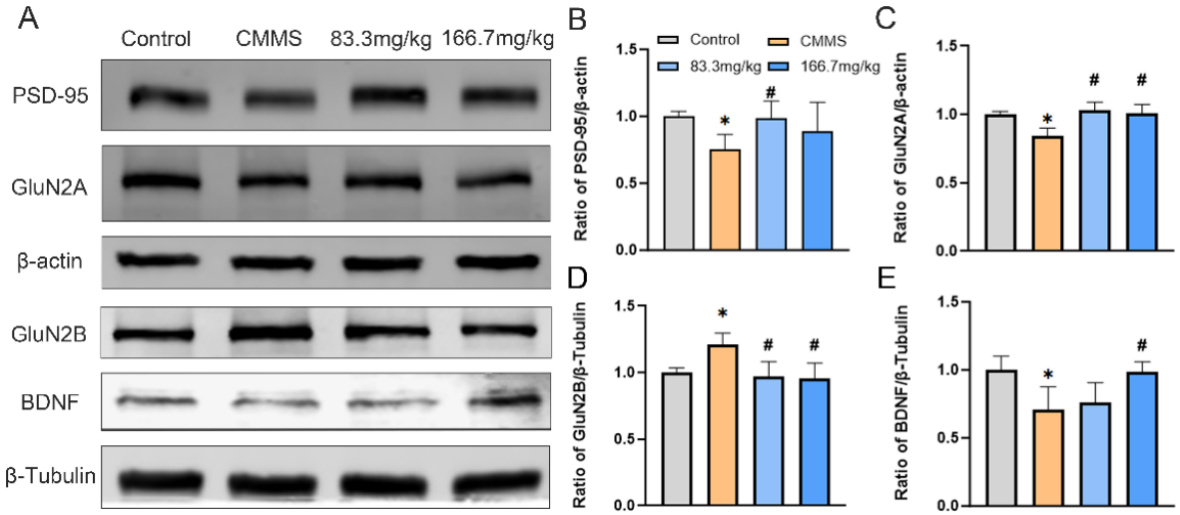


Figure 6. Western blot verification of the effect of CPPP on synaptic protein expression in the hippocampal tissue of CMMS rats. (A). Representative WB bands of hippocampal synaptic proteins in CMMS rats. (B-E). Quantification analysis of protein bands, including PSD-95, GluN2A, GluN2B, and BDNF. Data are presented as mean ± SD, with statistical analysis conducted using One-way ANOVA for multiple group comparisons, followed by Tukey's post-hoc test; n=3; *P<0.05 and **P<0.01 indicated a statistically significant difference compared to the control group; #P<0.05, ##P<0.01 and ###P<0.001 indicated a statistically significant difference compared to the model group.

The results showed that PSD-95 protein expression was significantly downregulated in the hippocampus of CMMS rats (P=0.0360; Figure 6A-B), suggesting that depression might cause synaptic damage. However, CPPP (83.3 mg/kg) effectively increased PSD-95 expression, indicating its potential effect in synaptic repair. In addition, the expression of GluN2A was significantly reduced in the hippocampus of CMMS rats (P=0.0318), while GluN2B was significantly increased (P=0.0044) (Figure 6C-D). Since NMDAR played an essential role in synaptic transmission and plasticity, the significant changes in GluN2A and GluN2B expression in CMMS rats might have potential impacts on the morphology and function of hippocampal synapses. CPPP (83.3 mg/kg) effectively reversed the expression of GluN2A and GluN2B by 22.55% ±3.33% increment in GluN2A (P=0.0131) and 19.53% ±5.71% decrease in GluN2B (P=0.0162), compared to their expression in CMMS rats. BDNF is a neurotrophic factor that plays an important role in synaptic plasticity and dendritic spine growth [29]. CPPP (166.7 mg/kg) also partially alleviated the significant decrease of BDNF expression in CMMS rats (P=0.0408; Figure 6E), indicating that CPPP promoted neuronal repair and synaptic remodeling.

In this study, a multisensory stress simulation device was used to construct a CMMS rat model, with parameter settings based on previous studies [21], with a few adjustments made to the modeling duration. Considering that this study required one week of administration, we extended the total modeling duration from the original 21 days to 28 days (including 7 days of administration). Some studies had shown that although a 21-day stress stimulation period could effectively establish a depression model [30], but the stress stimulation time extended to 28 days helped the strengthen of this model with more stabilized depressive-like behaviors, including obvious anhedonia and anxiety behavior. SPT is a classical behavioral test to evaluate the degree of anhedonia in rodents [31], while the FST and TST were also effective in assessing the degree of behavioral despair in rodents [32, 33]. In this study, CMMS rats showed a lower sucrose preference in the SPT, and significantly increased immobility time in both FST and TST. However, the intervention of CPPP significantly improved sucrose preference and immobility time, exhibiting excellent antidepressant effects.

The hippocampus contains high levels of glucocorticoid receptors and glutamate, which are involved in regulating the hypothalamic-pituitary-adrenal (HPA) axis, making it more susceptible to stress and depression [28]. In animal models of depression, chronic stress was proved to impair hippocampus-dependent explicit memory [34], as well as leading to the reduction of dendritic branching in neurons and impaired synaptic plasticity in the hippocampus [35, 36].

Pearl powder is believed in traditional Chinese medicine theory to eliminate negative emotions such as depression and irritability. However, it is still unclear through which signaling pathways pearl powder directly modulates synaptic function and neurotrophic factors. This lack of mechanistic clarity impeded its scientifically validated application and further development within the framework of modern medicine. In conjunction with our findings in the network pharmacology, the active amino acids in pearl powder regulated target genes such as CASP3, BDNF, MTOR, GluN2B, and MAPK1, among which BDNF, GluN2B, and MAPK1 were closely related to synaptic plasticity. Extensive literatures revealed that the top 5 amino acids in pearl powder (Gly, Ala, Glu, Asp, and Arg) had potential neuroregulatory functions, acting as neurotransmitters or regulatory factors, promoting excitatory and inhibitory signaling in neural circuits [17]. Asp and Glu, as excitatory neurotransmitters, regulated synaptic transmission, plasticity, and learning/memory by activating NMDAR, AMPA, and kainate receptors, since Glu was the primary excitatory neurotransmitter in the central nervous system (CNS) while Asp was weak in specific brain regions (e.g., hippocampus) [37, 38]. Arg also regulated ion channels and receptor activity by synthesizing putrescine, spermidine, and spermine, thereby modulating neurotransmitter release and synaptic plasticity [39]. Additionally, Arg served as a substrate for nitric oxide synthase to produce nitric oxide (NO), a signaling molecule involved in synaptic plasticity, blood flow regulation, and neuroprotection [40, 41]. Alanine acted as a metabolic substrate for neurotransmitter synthesis, participating in the production of compounds such as Glu and GABA in neurons and astrocytes. It also supported neuronal energy demands through the glucose-alanine cycle in glia-neuron metabolic coupling, indirectly promoting neurotransmitter synthesis and memory formation [42, 43]. Gly, as a co-agonist of NMDAR, played an important regulatory role in excitatory synapses, while also serving as a major inhibitory neurotransmitter in the CNS, inhibiting neural signaling via glycine receptors (GlyR). Furthermore, glycine formed an excitatory-inhibitory balance with Glu through their synergistic action at NMDAR, maintaining neural network stability

[44-47]. Therefore, this study further explored the effects of CPPP on the expression of synaptic-related proteins in the hippocampus of CMMS rats to verify its antidepressant pharmacological mechanism.

In the central nervous system, NMDAR is an ion channel gated by the excitatory neurotransmitter glutamate [48], and plays an important role in neural signal transmission and synaptic plasticity [49]. GluN2A and GluN2B were widely distributed in the rodent cortex and hippocampus, especially in the CA1 and CA3 pyramidal cells [50, 51]. This study found an imbalance in CMMS rats, with downregulation of GluN2A expression (P=0.0318) and abnormal elevation of GluN2B (P=0.0044), which might lead to NMDAR-related dysfunction and ultimately affect synaptic plasticity. On the postsynaptic membrane, the GluN2A-NMDAR-mediated rapid Ca^{2+} influx not only promoted the expression of synaptic-related proteins like PSD-95 and BDNF, but also maintained electrophysiological signaling in the postsynaptic membrane, such as Long-term Potentiation and Long-term Depression [52, 53]. On the other hand, GluN2B-NMDAR, mainly located on the extrasynaptic membrane, induced persistent Ca^{2+} influx and neurotoxicity [54, 55] and inhibited the BDNF-TrkB pathway [56, 57]. The results suggested that pearl powder might regulate the expression of synaptic-related proteins such as PSD-95 and BDNF by upregulating GluN2A and inhibiting GluN2B, thereby significantly affecting synaptic functions. Furthermore, the investigation elucidated the improvement of pearl powder on pivotal pathways of synaptic plasticity, specifically the NMDAR subunits GluN2A and their downstream PSD-95/ BDNF signaling cascade in the hippocampal milieu of CMMS rats. This research unveiled a novel paradigm that the therapeutic potential of pearl powder against depression was realized through the amelioration of synaptic homeostasis.

PSD-95, as a synaptic scaffold protein, showed downregulation in CMMS rats (P=0.0360), which directly led to synaptic structural disorganization and reduced signal transduction efficiency. However, CPPP could restore PSD-95 levels, potentially by stabilizing the spatial localization of NMDAR and TrkB receptors. This promoted the formation of the PSD-95-TrkB complex, and subsequently activated the downstream CREB signaling pathway. This process formed a positive feedback loop with the restoration of BDNF expression (P=0.0408): BDNF-TrkB binding not only promoted synaptic plasticity through the PI3K/Akt and MAPK/ERK pathways [58, 59], but also negatively regulated the abnormal activation of the PSD-95-nNOS signaling complex [60], blocking oxidative damage caused by NMDAR overactivation. This multi-node regulatory network might explain the significant effects of pearl powder in improving synaptic plasticity and neuronal survival (Figure 7).

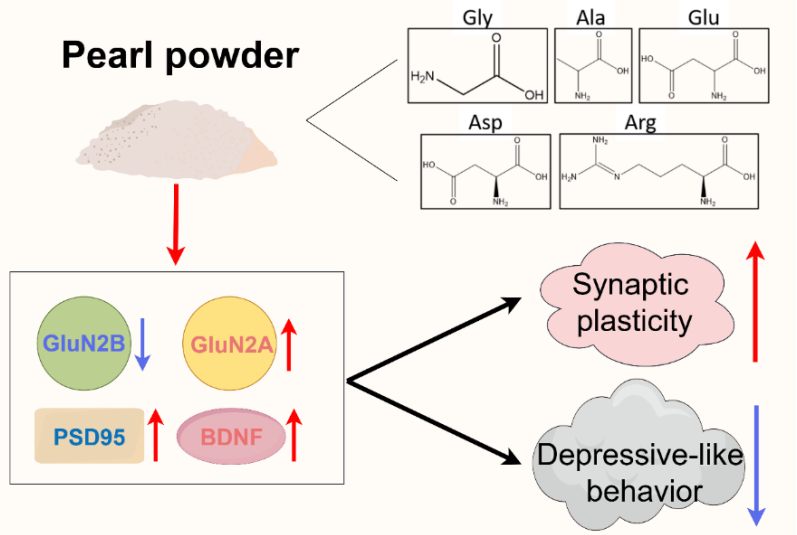


Figure 7. Potential Signaling Pathway of Pearl Powder in treating depressant. The top five amino acids in pearl powder (Gly, Ala, Glu, Asp and Arg) coordinately modulate NMDA receptor subunit composition and downstream synaptic proteins in the hippocampus. Specifically, these amino acids downregulate the expression of the GluN2B subunit, while upregulating GluN2A, PSD-95 and BDNF. Such molecular changes synergistically enhance synaptic plasticity and thereby suppress depressive-like behaviors. Red arrows indicate upregulation; blue arrows indicate downregulation.

There are several limitations in this study. First, the experiment focused on the hippocampal NMDAR-BDNF axis, but depression is a multi-brain region network disorder, and the synchronized changes in the prefrontal cortex-amygdala loop still need to be verified. Second, the functions of the GluN2A/GluN2B balance in regulating Ca^{2+} dynamics (such as the activation threshold differences of calcium/calmodulin-dependent kinase II) need to be further explored using in vivo calcium imaging techniques. In addition, this study only used a fixed dose of pearl powder in a single depression animal model and did not address some specific constituents to refine its antidepressant effect. Instead, we primarily attributed these effects to amino acid components, since most proteins would be digested and enzymatically hydrolyzed into amino acid monomers. Future investigations would also unambiguously identify the principal bioactive constituents and delineate their synergistic or antagonistic interplay by combining LC–MS/NMR structural analysis and HPLC-driven fractionation with in vitro and in vivo bioassays.

4. Conclusion

In culmination, this study revealed a pioneering antidepressant mechanism of pearl powder, which demonstrated its restorative efficacy via NMDAR subunit reconfiguration and activation of the PSD-95/BDNF pathway, through the twin lenses of synaptic plasticity and neurotrophic support. This finding not only supplied a pharmacological rationale for repurposing pearl powder in depression, but also established the theoretical foundation for devising integrative Chinese medicine strategies. Future endeavors were suggested to encompass: (1) clinical validation of the synergistic efficacy between pearl powder and SSRIs; (2) investigation of pearl powder's regulatory influence on the HPA axis–gut microbiota axis; and (3) AI-driven screening of pearl powder constituents to pinpoint lead compounds that finely tuned the GluN2A/GluN2B ratio.

Acknowledgments

Thanks to Laboratory Animal Center of Ningbo University for the technical support. This work was funded by Ningbo Natural Science Foundation Project (2024J276), Enterprise Technology Development Project (H2022000162) supported by Ningbo Dachang Pharmaceutical Co., Ltd and a grant from international Sci-tech Cooperation Projects under the "Innovation Yongjiang 2035" Key R&D Programme (2024H011).

Conflict of Interest

The authors declare that there is no conflict of interest.

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