

Antidepressant-like effect of 5-O-methylvisammioside targeting SRC is accompanied by hippocampal neuroinflammation reduction in lipopolysaccharide-challenged mice

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Research Article

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Abstract

5-O-methylvisammioside (MeV), a phenolic compound found in the underground part (radix) *Saposhnikovia divaricate* (Turcz. ex Ledeb.) Schischk. (Apiaceae) in the early stage, has also been detected in the extract of *Eranthis longistipitata* (Ranunculaceae) in recent literature. In previous studies, it was discovered to have anti-inflammatory and neuroprotective activities. However, the potential mechanisms of MeV antidepressants remain unclear. In the present study, Network pharmacology was adopted to conduct drug-target networks following obtaining the shared targets between MeV and major depressive disorder (MDD) in multiple databases. The binding ability of the compound to the core target was verified through molecular docking. In addition, the biological processes and signaling pathways involved in the antidepressant effect of MeV by animal experiments. Our results were as follows: i) There were 85 shared targets of MeV & MDD in multiple public databases. The target protein with a higher degree in the protein-protein interaction (PPI) network was Proto-oncogene tyrosine-protein kinase Src (SRC). ii) Gene Ontology analysis showed that MeV treatment of MDD mainly involves the positive regulation of phosphorylation, the response to lipopolysaccharide, and other biological processes. It was closely related to the nuclear factor kappa B (NF- κ B) signaling pathway. iii) MeV administration significantly alleviated depression-like behaviors and reduced microgliosis in mice by inhibiting SRC phosphorylation. The antidepressant effects of MeV may be attributed to the NF- κ B signaling pathway. The results may contribute to demonstrating the effectiveness of MeV against MDD and facilitate the development of new traditional Chinese medicine ingredients.

Introduction

Major depressive disorder (MDD) is widely recognized as one of the most prevalent and pressing mental health issues[1]. Over the past few decades, the number of cases has increased dramatically globally, and individuals of all ages have been currently afflicted[2, 3]. According to numerous studies, depression may be an emotional or mental disease instigated by the accumulation of multiple factors with overlapping causal pathways, including the interaction between genetic, pathogenic factors and environmental factors[4, 5]. Physical illness is usually accompanied by biochemical abnormalities, such as the lack of neurotransmitters in the central nervous system directly affecting brain function or brain chemical imbalance affecting neuroimmune and neuroendocrine processes leading to emotional disorders[6]. The current treatment focuses on the brain monoamine hypothesis and traditional antidepressants, which inhibit the reuptake of monoamine neurotransmitters, thus increasing the concentration of synaptic monoamine and interacting with postsynaptic receptors. However, many patients with MDD fail to respond to routine treatment and even suffer from severe physical and mental torture[7].

Concerns about the limited efficacy of therapeutic options for depression have led to a search for new, non-monoamine-based targets and potential novel antidepressants[8]. As a complementary and alternative medicine for the treatment of depression, the antidepressant mechanism of traditional Chinese medicine is becoming of increasingly great concern[9]. *Saposhnikovia divaricate* is one of the

valuable and famous traditional Chinese medicine plants listed in several pharmaceutical records, such as the Chinese Pharmacopoeia and Shen Nong's Materia Medica[10]. In recent years, studies investigating the chemical constituents of *S. divaricata* showed that the main active components are chromones, coumarins, and polyacetylenes[11], among which the main chromones 5-O-methylvisammioside (MeV) were most associated with anti-inflammatory, analgesic, and anti-oxidative damage effects[12–14]. However, little information is available about MeV treating spirit high-risk psychological disorders such as depression. Although a study has shown that MeV has relatively definite neuroprotective effects, such as regulating brain-derived neurotrophic factor in mouse brain and via NF- κ B/I κ B α pathway reducing the content of inflammatory factors, improving oxidative stress and effectively reversing the depressive behaviors[15], the specific chemical basis and mechanism of antidepressant are still unclear.

Usually, traditional Chinese medicines or prescriptions exert therapeutic effects on multiple targets and pathways in the body through complex active ingredients, which are difficult to pinpoint through conventional detection methods[16]. Recently, network pharmacology is a branching technique of bioinformatic and systems biology, which links the intervention mechanism of chemical compounds, drug targets, and diseases[17]. Visualization in the form of "network targets" can effectively decipher the multi-molecular mechanisms of drug therapy effects on disease at the molecular level, which is expected to provide a scientific basis for understanding how drugs intervene in disease systematically and comprehensively[18]. The predicted results of network pharmacology are expected to provide new ideas and directions for exploring the antidepressant effect of MeV. This study is based on applying network pharmacology to find the crucial target and related pathway for MeV to play a role in MDD. Then, the prediction results of network pharmacology were verified by *in vivo* experiments and molecular docking methods, and the mechanism of action of MeV on MDD was initially discussed.

Materials and methods

Network pharmacology-based analysis

Screening of MeV-related disease targets

Molecular formula, molecular weight, 2D structure, 3D structures, and canonical SMILES information (<https://pubchem.ncbi.nlm.nih.gov/compound/21670038>)[19] of MeV were collected from the PubChem database. All targets of MeV were collected from the SwissTargetPrediction databases (<http://www.Swisstargetprediction.ch/>)[20] and PharmMapper databases (<http://www.lilab-ecust.cn/pharmmapper/>)[21–23]. The canonical SMILES of MeV was uploaded to the PharmMapper database (<http://www.lilab-ecust.cn/pharmmapper/>) to determine the MeV-related predictive targets. The parameters settings were default. The results of targets with z-score > 0 as the screening criteria were retained for further analysis. The results from 2 databases were integrated and duplicated output molecules were removed.

Identification of Major depressive disorder-Associated Targets

The keyword “Major depressive disorder” was used to search Drugbank (<https://www.drugbank.ca/>)[24], DisGeNET (<https://www.disgenet.org/>)[25, 26] and Genecards (<https://www.genecards.org/>)[27] databases for MDD-associated targets that have been experimentally verified. Intersections of 3 disease database targets were subjected to deduplication, and their UniProt[28] (<https://www.uniprot.org/>) numbers were retrieved.

Network Construction

The Venn diagram was drawn from the predicted MeV action target and assumed MDD-related target with VENNY2.1 online tool (<https://bioinfogp.cnb.csic.es/tools/venny/>) to identify potential MeV therapeutic targets in MDD. These common targets were extracted and submitted to the STRING database[29] (<https://string-db.org>) to obtain the protein-protein interaction (PPI) diagram. The screening condition of the organism is set to “Homo sapiens”, the protein interaction confidence score threshold was set to 0.9, and the rest are set to default values. Disconnected nodes were hidden in the network representation. The PPI network was constructed for the candidate targets of MeV-treated MDD using Cytoscape 3.7.2[30] (<https://cytoscape.org/>). Core targets of the PPI network of MeV against MDD were calculated by the Network Analyzer of Cytoscape. Considering that the above parameters can analyze the properties of nodes in the interactive network, we select the target nodes with high degree values in the PPI network and predict the potential core targets for further research.

GO enrichment and KEGG pathway analysis

To explore the core mechanism and pathway of MeV anti-MDD in gene function and signaling pathways, the Metascape[31] (<http://www.metascape.org/>) database was used to conduct Gene ontology (GO) analysis and the Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment. 85 gene symbols of drug-disease intersection targets were pasted on the Metascape webpage, and the species is limited to “H. sapiens”. Terms with a p -value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 were collected and grouped into clusters based on their membership similarities. The enrichment analysis includes the biological process (BP), cell composition (CC), molecular function (MF), and KEGG pathways.

Molecular docking validation

According to the degrees of screening key targets and their relevance in the etiology of depression, we confirmed key targets to perform molecular docking. The molecular 3D structure of MeV and the target protein were downloaded from PubChem[32] (<https://pubchem.ncbi.nlm.nih.gov/>) and the RCSB PDB[33] (<https://www.rcsb.org/>), respectively. Water molecules and original ligands were removed from the target protein through PyMol[34]. Later, docking validation was performed after hydrogenation and dehydration through PDBQT files generated by AutoDockTools (ADT)[35]. Since the grid box needs to cover the entire protein domain and accommodate free molecular motion, the grid box was set to the

maximum value ($126 \times 126 \times 126$). Finally, the molecular docking patterns were visualized with PyMOL software.

Experimental validation

Animals

Male ICR mice (4–6 weeks of age, 20 ± 2 g) were obtained from Jilin University (Changchun, China). The mice were housed separately on a 12 hr/12 hr light/dark schedule with lights on at 7 A.M. (D 0) and handled for 14 days. Animals were allowed to habituate for 3 days before the start of experiments. All studies were conducted in accordance with the Laboratory Animal Guideline for ethical review of animal welfare.

Chemicals and reagents

LPS (L2880–10MG) was purchased from Sigma, and PP2 (HY – 13805) was purchased from MedChemExpress. 5-O-methylvisammioside (RS00021020) is the product of Nature-standard (Shanghai, China). Glycine (1275GR500), Sodium dodecyl sulfate (SDS) (3250GR500), and Tris-base (1115GR500) were purchased from Biofroxx. The antibodies against the TLR4, IBA1, and IL-1 β were purchased from Immunoway. The antibodies of SRC and Phospho-SRC were collected from Cell Signaling Technology. Anti-NF- κ B antibody and anti-IK β a antibody were purchased from Beyotime (Shanghai, China) and Bioss (Beijing, China), respectively. Other general reagents were purchased from commercial suppliers.

Experiment I: effect of MeV on LPS-induced depression

The experimental male ICR mice were divided into four groups (each group = 10 animals): control group (normal saline-treated), LPS (1 mg/kg/day) group, MeV (4 mg/kg/day) + LPS group, and fluoxetine (10 mg/kg/day) + LPS group. MeV was diluted in 0.9 % normal saline solution. The experimental procedure and drug treatment schedule are shown in Fig. 2A. MeV or fluoxetine was administered intraperitoneally 1 hour before LPS administration for the last three days, respectively. Mice were intraperitoneally administered with MeV, fluoxetine, or 0.9% saline (0.1 ml/10 g mice) daily (10 a.m.) for 14 days. Finally, the mice were sacrificed, and serum was collected at the end of the experiment. Brains were immediately removed, and the hippocampus was collected and quickly stored at -80 °C until further use.

Experiment II: Target verification of drug reversal of depression-like behavior

To further explore the core target of MeV in reversing depression-like behavior, we designed a new experiment and used the target SRC (Proto-oncogene tyrosine-protein kinase Src) inhibitor PP2. PP2 powder was dissolved in 0.9% saline and prepared into a working concentration. The mice were divided into the following 5 groups: control group, LPS group, MeV + LPS group, PP2 (2.5 mg/kg/day) + MeV group, and PP2 + MeV + LPS group. The drug treatment schedule was similar to that described above Experiment I (Fig. 4A). PP2/MeV is administered once a day, seven days a week, for two weeks. Mice in the control group were treated with vehicle solvent in the same way as mice in the administration group.

Behavioral assays

All behavioral tests were conducted in a separate room with the collection equipment connected to a digital camera recording system. Within 24 hours of the last administration, mice underwent open field testing (OFT), tail suspension testing (TST), forced swimming testing (FST), and sucrose preference testing (SPT) to evaluate depressive behaviors.

OFT: The open-field apparatus was a basin-shaped arena (48.8 cm diameter, 16 cm high) with a floor of 16 equal squares, which was placed inside a separate room with dim illumination. Each mouse was placed individually in the central area of the arena and video-recorded free travel for 8 min. Horizontal locomotor activity (the number of grid lines crossing traversed) and vertical locomotor activity (the number of times the mouse stood with both forepaws off the ground) of the mice in the last 5 minutes were analyzed separately. The arena was cleaned with 75% alcohol each time the mice were placed.

TST: Mice were fixed on the beam at 1–2 cm from the tip of the tail with tape/clip in an inverted state, and the head was over 20 cm from the ground. Each test lasted 8 minutes, and the accumulated immobility time (mice stopped struggling and lacked limb movement) within the last 5 minutes was counted.

FST: Mice were placed individually into a plastic cylindrical container (diameter: 11 cm; height: 25 cm) containing 12 cm of water maintained at $25 \pm 1^\circ\text{C}$. FST lasted 7 minutes, and then the mice were returned to their home cage. The first 2 minutes of the test were considered adaptation time, and the total time spent immobile state was recorded in the last 5 minutes. Mice were considered immobile when they floated or only moved slightly to keep their bodies balanced or heads above water. The water and bucket were changed and refreshed following each test.

SPT: The SPT was performed from 7:00 p.m. to 7:00 a.m. This procedure was based on a modified version of the test previously described by Liu[36] et al. On the 11th day of administration, three 12-hour sessions of sucrose consumption training were performed on mice for optimal sucrose preference. Next, mice were deprived of fluid for 12 hours before testing. On test day, the tap water and 2% sucrose (w/v) were placed in each cage, and the positions of the two bottles were switched six hours later. Fluid consumption (g) was calculated by weighing the bottles before and after the experiment. The sucrose preference rate was calculated as follows:

Sugar water preference value = $\text{sucrose consumption (g)} / [\text{sucrose consumption (g)} + \text{water consumption (g)}] \times 100\%$.

Western blot assay

Tissues were lysed using RIPA lysis buffer with protease inhibitors. Centrifugally extracted sample supernatants were mixed with protein loading buffer and boiled for 10 min. Equal amounts of protein samples were separated by 10% or 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5%

non-fat milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T), the membrane was probed with primary antibodies at 4°C overnight. The following primary antibodies were used: TLR4, IBA1, and IL-1 β (1:1000, Immunoway, China), SRC and Phospho-SRC (1:1000, Cell Signaling Technology, USA), NF- κ B (1:1000, Beyotime, China), IKB α (1:1000, Bioss, China). The next day, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, ZSGB-BIO, China). Finally, ECL reagents (PerkinElmer, Waltham, MA, USA) were used on a gel imaging system to screen the antigen–antibody complex (Tanon 5200; Shanghai, China). Band density analysis was performed with Image J software (National Institutes of Health, USA) and normalized to Housekeeping proteins.

Serum albumin detection

The serum albumin (ALB) concentration is usually measured using the bromocresol green (BCG) dye-binding method[37]. In this experiment, blood was collected by mice retroorbital bleeding. The serum was separated immediately after room temperature clotting by low-speed centrifugation at 2500 rpm for 15 min. Then, samples were transferred to 0.2 ml microtubes and refrigerated (-80°C) until analysis was performed. None of the serums used had any visible hemolysis or clot abnormalities. Data of ALB concentrations were determined in a full-wavelength multifunctional enzyme-labeling instrument (Thermo Scientific, Varioskan Flash) using commercial albumin assay kits.

Immunofluorescence analysis

All brain tissues were perfused with 4% paraformaldehyde and immersed in 10%, 20%, and 30% sucrose to gradient dehydrating successively. After optimal cutting temperature (OCT) compound embedding, tissues were sliced (20 μ m) with a freezing microtome. The staining process was as follows: washed with PBS for 3 $\times$ 5 min, the frozen section was blocked with 10% donkey serum at room temperature, and incubated with anti-IBA1, anti-MAP2 antibodies overnight at 4°C. Afterward, slides were stained with fluorescent secondary antibodies and further counterstained with 4'-6-Diamidino-2-phenylindole (DAPI) solution. Immunofluorescent images were obtained under the microscope (Olympus).

Statistical analyses

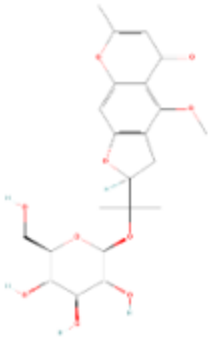
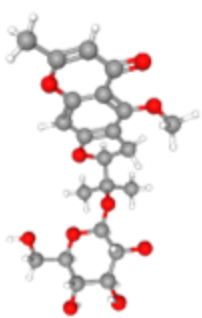
The statistical details of the experimental results were described in the corresponding figure legends. SPSS 25 was used to analyze the data. Data was expressed as the mean $\pm$ standard error of the mean (SEM), median, or n (%). The sample size (n) represents data from a distinct mouse in a representative experiment unless otherwise stated. Multiple comparisons and post hoc statistical analyses were compared by ANOVA followed by Tukey's test, for parametric variables, or by Kruskal-Wallis followed by Bonferroni correction, for non-parametric variables. Differences were considered statistically significant when $p < 0.05$.

Results

Predicted target screening of MeV

The chemical structure and parameters of MeV are shown in Table 1. 298 predictive targets were identified from the PharmMapper database, 201 official symbols of MeV-related targets for limiting to “Homo sapiens” were obtained in the UniProtKB database after filtering by z-score > 0. In addition, 100 putative protein targets were identified in the Swiss Target prediction database. Among these results, 29 targets with probability > 0 were incorporated into the drug target. Ultimately, a total of 222 drug targets were obtained from the database after removing the duplication.

Table 1 Chemical information of 5-O-methylvisammioside (MeV) and its parameters.

Molecular Name	Molecular Formula	Molecular Weight	2D Structure	3D Structure
5-O-methylvisammioside	C ₂₂ H ₂₈ O ₁₀	452.5 g/mol		

Acquisition of MDD-related targets

We obtained 10792, 413 and 1236 MDD-related targets from the GeneCards, Drugbank and DisGeNET databases, respectively. For the data from Genecards database, the higher the score value is the closer the relationship with the disease. Because there was too much data, the target with a score greater than the median was regarded as an MDD-related target. The maximum score of the target was 116.88, the minimum score was 0.24, and the median was 11.92. Subsequently, 1349 targets were obtained through three rounds of median screening. Then, summarizing the targets of three databases and deleting the duplicate values, 2194 target proteins related to MDD were obtained.

PPI network construction and analysis

From the comparative analysis between 2194 MDD-related and 222 MeV putative targets, Venn diagram results show that there were 85 predicted as MeV targets for the treatment of MDD (Fig. 1a). The details are provided in Supplementary Table S1. Based on the STRING PPI analysis, the network consisted of 61 nodes and 118 edges, the average node degree was 2.78, the local clustering coefficient was 0.489, and the enrichment *p*-value < 1.0e-16 (Fig. 1b). In the visualization results of Cytascape, the color and size of each node were correlated with its "degree" values; the brighter and larger nodes had a higher "degree" value (Fig. 1c). The details are provided in Supplementary Table S2. The above results indicate the presence of the core target between MeV and MDD. The core gene SRC (Proto-oncogene tyrosine-protein kinase Src) was speculated to play a significant role in MeV-treated MDD.

GO and KEGG pathway enrichment analysis of the putative shared targets between MeV and MDD

Enrichment analysis of 85 potential therapeutic target genes was performed using the Metascape database (version: v3.5.20230101). GO enrichment is beneficial to deepen the understanding of the gene function and biological significance of the presumed target of MeV therapy for MDD at the systematic level. Here, there were 1113 items of GO enrichment analysis results, including 947 biological processes, 64 cellular components, and 102 molecular functions. The top 20 terms with significant enrichment in BP, MF, and CC categories were displayed according to p -values < 0.01 . The results showed that the most significant molecular function included serine-type endopeptidase activity, kinase binding, signaling receptor activator activity, and so on (Fig. 1d). While in the biological process category, target proteins were mainly involved in response to hormones, response to hypoxia, response to xenobiotic stimulus, response to peptides, response to nutrient levels, response to lipopolysaccharide, positive regulation of phosphorylation, positive regulation of cell migration, positive regulation of cytokine production, as well as negative regulation of response to external stimuli (Fig. 1e). In addition, intersection genes were mainly enriched in the vesicle lumen, collagen-containing extracellular matrix, external encapsulating structure, dendrite, neuron-to-neuron synapse, and so on in the cellular component analysis (Fig. 1f).

KEGG enrichment analysis showed that the anti-MDD mechanism of MeV mainly involved 147 signaling pathways ($p < 0.01$), and the top 20 enrichment pathways were visualized by the bubble chart (Fig. 1g). The results showed that the genes shared by MeV and MDD were mainly enriched in the cAMP signaling pathway (hsa04024), NF-kappa B signaling pathway (hsa04064), and Serotonergic synapse (hsa04726), excluding other disease pathways. Moreover, by importing the top 20 KEGG pathways and associated target proteins into the Cytoscape software, the target-pathway network of anti-depression effects of MeV was generated (Fig. 1h). Nodes in the network represent MDD, targets, pathways, and MeV. The principle of interconnection between nodes is that if the target of MeV for MDD treatment is the same as the target of the signal pathway, the two are connected by an edge. A network was constructed to study the role of MeV in MDD treatment from multiple targets and pathways. The network intuitively reflects the potential target basis and action mechanism.

MeV interacts structurally with SRC

Based on the network pharmacology results, we screened the protein, SRC (PDB ID: 1A07), and performed molecular docking with MeV. The binding poses and interactions of MeV with the protein were obtained with Autodock 4, and the binding energy of the corresponding interaction was generated. The docking results were presented as molecular surface and cartoon representation, which can effectively represent the topical details of binding sites. The binding between ligands and target proteins was mainly mediated by hydrophobic interactions, especially hydrogen bonds (shown as dashed lines). The results showed that MeV could interact with SRC. The figure shows that MeV forms one hydrogen bond with GLY239 and TYR205 in SRC and two hydrogen bonds with LYS203. Surface representation of the crystal structures of SRC and MeV also is shown in Fig. 1i. The absolute binding affinities of each

docking mode were more than 5 kcal/mol, indicating the combination of the ligand and protein is stable. The binding energy of SRC and MeV docking was -5.907 kcal/mol. Based on the above results, we predicted that the core target SRC might play a pivotal role in MeV treatment for MDD.

MeV reversed depressive-like behaviors induced with LPS

To verify the results of network pharmacology, we used LPS-induced model mice to investigate the anti-depression effect of MeV. In previous studies, MeV of 4 mg/kg was an effective dose to relieve LPS-induced neuroinflammation[15]. Therefore, 4 mg/kg was selected to conduct this experiment (Fig. 2a). Our results showed that LPS treatment significantly reduced animal body weight compared to the control group, and MeV or fluoxetine reversed the dramatic weight loss ($F_{3,24} = 16.531$, $p < 0.001$, Fig. 2b). For OFT, there was no difference between the experimental groups in locomotor activity ($F_{3,24} = 1.445$, $p > 0.05$, Fig. 2c) or rearing ($F_{3,24} = 1.253$, $p > 0.05$, Fig. 2d). Additionally, the immobile time during the TST ($F_{3,24} = 4.750$, $p < 0.05$, Fig. 2e) and FST ($F_{3,24} = 6.114$, $p < 0.01$, Fig. 2f) was longer for LPS-treated mice than saline-treated mice, which was reversed by MeV treatment. The above results suggested that MeV attenuated LPS-induced significant depressive-like behaviors in mice.

Effects of MeV on the NF- κ B pathways in LPS-induced depression mice

LPS, a proinflammatory agent, has been widely used to establish mouse models of depression, which mimics neuroinflammation-related clinical manifestations in patients with mood disorders[38]. The pattern recognition receptor TLR4 is expressed in microglia and participates in mediating some components of stress-induced inflammation, including NF- κ B and IL-1 β [39]. During the inflammatory process, the nuclear translocation of the NF- κ B heterodimer is closely related to microglial activation[40]. Moreover, SRC has also been extensively explored in the context of neuroinflammation. Studies in microglia show that the nuclear accumulation of NF- κ B was specifically regulated by SRC. It is a crucial molecule for regulating microglia proinflammatory stimulation[41]. To determine whether the relief of depression-like behaviors in MeV-treated mice was mediated by the NF- κ B signaling pathway, western blot analysis was performed to detect levels of these proteins in the hippocampus (Fig. 3a). As compared to control mice, TLR4 ($F_{3,12} = 5.544$, $p < 0.05$, Fig. 3b), NF- κ B ($F_{3,8} = 7.858$, $p < 0.01$, Fig. 3c), I κ B α ($F_{3,16} = 8.469$, $p < 0.01$, Fig. 3d), IL-1 β ($F_{3,12} = 6.103$, $p < 0.01$, Fig. 3e), and IBA1 ($F_{3,12} = 13.110$, $p < 0.001$, Fig. 3f) were significantly upregulated in LPS-treated mice, and MeV or Flu decreased the levels of these proteins. These data suggest that LPS induces activation of the microglial and the NF- κ B pathway, which may mediate the antidepressant effects of MeV. Significantly, however, upregulated P-SRC in the hippocampus of LPS group mice was down-regulated by MeV rather than fluoxetine (SRC: $F_{3,12} = 0.677$, $p > 0.05$; P-SRC: $F_{3,24} = 7.673$, $p < 0.01$; P-SRC/SRC: $F_{3,20} = 11.268$, $p < 0.001$, Fig. 3g, h, i). Thus, the antidepressant mechanism of MeV may be different from fluoxetine and is closely related to P-SRC.

Blockage of SRC abolished the effect of LPS on depression-like behaviors in mice

Activation of Src-family kinases (SFKs) has been reported to be involved in regulating innate immune cell function and inflammatory response. Among them, the canonical roles of Lyn[42], Fyn[43] and Hck[44], member of SFKs, in modulating external irritant-mediated immune cell response has been established by several studies. However, as the first discovered SFK member, the regulatory contribution of SRC in the above process is sometimes overlooked[45]. Therefore, a question as to whether the specific role of central nervous system microglia in regulating inflammatory depressive behavior is assigned to SRC has yet to be solved. More specifically, we aim to explore whether SRC is a crucial target for MeV regulating NF- κ B signaling pathway to reverse inflammatory depression-like behaviors.

To confirm the role of SRC in the NF- κ B signaling pathway, the inhibitor PP2 can inhibit the phosphorylation of SRC, and it was intraperitoneally injected into mice and the behaviors were evaluated (Fig. 4a). Given that PP2 inhibitors have been developed for cancer treatment and that there is limited data on their use in depression studies, in this case, we reference the doses applied in Parkinson's disease model mice. Results of the study showed that 2.5 mg/kg PP2 was not associated with adverse effects in the short term (12 days) and played a positive role in motor dysfunction in model mice[46]. Therefore, the working concentration of PP2 in this experiment is still 2.5 mg/kg. In OFT (Fig. 4b, c), the four groups of mice injected with LPS had slightly reduced locomotor activity ($F_{4,45} = 1.081, p > 0.05$) or rearing ($F_{4,35} = 0.769, p > 0.05$) compared to the saline-treated mice, but no difference between the groups. In TST (Fig. 4d), co-administration of PP2 significantly blocked the effect of MeV on the immobility time of mice, while there were significant differences in immobility time among the other four groups: the immobility time of the LPS group was significantly higher than that of the Control group, but the immobility time of the LPS + MeV group and the LPS + PP2 group was markedly lower than that of the LPS group ($F_{4,45} = 4.158, p < 0.01$). Additionally, the immobility time during the FST (Fig. 4e) was significantly longer for LPS-treated mice than for other groups of mice unless the LPS + MeV + PP2 group ($F_{4,40} = 7.110, p < 0.001$). Among them, MeV or PP2 treatment significantly improved the immobility time. Then, the sucrose preference test (SPT) was performed to further evaluate depression-like behaviors. The results of the SPT (Fig. 4f) showed that the sucrose consumption of the Control group and the LPS + MeV group was comparable. The sucrose intake of the LPS group was significantly lower than that of other groups except the LPS + MeV + PP2 group ($F_{4,45} = 7.029, p < 0.001$). Behavioral results showed that PP2 counteracted the effect of MeV in model mice. Thus, SRC is critical to the role of MeV in model mice, and SRC signaling is blocked enough to alleviate depression-like behavior in LPS-induced mice.

ALB is one of the biomarkers to diagnose malnutrition. Meanwhile, inflammatory diseases and other acute or chronic diseases cause the albumin levels of organisms to decline by inhibiting synthesis, increasing catabolism/vascular permeability, or a combination of these processes[47]. One of the signs of activation of the inflammatory response system in patients with MDD is lowered ALB

concentrations[48]. A total of 36 serum samples were included in our experiment for statistical analysis. Serum ALB concentrations of mice in the LPS group were significantly lower than those in all other groups with the exception of the PP2 injection group ($H = 29.042$, $p < 0.001$ for Kruskal-Wallis tests). These results demonstrated that MeV or Flu pretreatment successfully upregulated the reduction of ALB triggered by LPS injection in mice, but SRC may not be involved in this process (Fig. 4g).

Western blots analysis (Fig. 5a) showed that PP2 administration did not reverse the upregulation of TLR4 ($F_{4,25} = 4.084$, $p < 0.05$, Fig. 5b) protein levels by LPS. However, the LPS-induced upregulations of NF- κ B ($F_{4,20} = 4.341$, $p < 0.05$, Fig. 5c), I κ B α ($F_{4,20} = 4.417$, $p < 0.05$, Fig. 5d), IL-1 β ($F_{4,10} = 7.081$, $p < 0.01$, Fig. 5e), and phosphorylated SRC ($F_{4,20} = 6.995$, $p < 0.01$, Fig. 5f) were suppressed by PP2, which is consistent with the results of MeV. In addition, the protein levels of phosphorylated SRC, rather than TLR4, NF- κ B, and I κ B α , were significantly inhibited by the co-administration of MeV together with PP2 (Fig. 5f-h). These results suggested that the anti-depression effect of MeV is possibly dependent on reduced expression of NF- κ B and its downstream signaling proteins containing I κ B α and IL-1 β by the blockade of phosphorylated-SRC.

SRC inhibition abolished the effect of LPS on microglial in the hippocampus

To examine the effect of SRC on LPS-induced activation of microglia, the expression of ionized calcium-binding adaptor molecule (IBA1) was detected by Western blot and immunofluorescence. With western blot assay (Fig. 6a, b), PP2 or MeV also blocked the effects of LPS on the protein levels of IBA1 in the hippocampus of model mice ($F_{4,25} = 4.320$, $p < 0.01$). As illustrated in Fig. 6c-f, the quantitative results of immunofluorescence staining depicted that the exposure to LPS led to higher fluorescence intensity of IBA1 in the CA1/CA3 region of the hippocampus compared with that in the control group. However, the expression of IBA1 in the hippocampal CA1/CA3 region was restored to a similar control group level following treatment with MeV compared to mice in the LPS group. Moreover, the change in increased IBA1 expression in hippocampal microglia of LPS-treated mice was similarly alleviated by PP2 administration (CA1: $F_{4,10} = 8.619$, $p < 0.01$, Fig. 6c, e; CA3: $F_{4,10} = 10.189$, $p < 0.01$, Fig. 6d, f). These results indicated that the SRC is indeed involved in the regulatory effect of MeV on microglia.

Discussion

The high heterogeneity of MDD in symptoms and etiology makes the pathogenesis elusive[49]. At present, reducing the severity and duration of current depression and the possibility of recurrence mainly depends on the treatment of antidepressants. While antidepressants provide efficacy, they come with side effects such as low receptor specificity, a wide range of adverse reactions, and high toxicity[50]. It is well known that many components of Chinese herbal medicine have been neglected in experimental and clinical studies because of their physicochemical properties, such as poor water solubility or oral availability. Based on data mining, network pharmacology helps us find valuable information from big data, which is especially suitable for the multi-target and multi-pathway characteristics of traditional

chinese medicine and then guides us to conduct meaningful research[51]. Currently, MeV is extracted mainly from the dried root of *Radix Saposhnikoviae*, which is commonly used as a Chinese herbal remedy for immune, nervous, and respiratory diseases[11]. Due to the low oral availability, when screening the effective ingredients of *Saposhnikovia divaricata* (Turcz.) Schischk. by DL (drug-likeness) and OB (oral bioavailability) values in the Traditional Chinese Medicine systems pharmacology database and analysis platform (TCMSP) database, they were overlooked. Here, we take MeV as an independent research object to further explore the mechanism of action on MDD. Previous studies have confirmed that MeV is a novel histone H3 phosphorylation epigenetic suppressor, which plays a neuroprotective role by attenuating inflammation and cleaved caspase-3- and -9a-related apoptosis on focal cerebral ischemia in rats[14]. In addition, Sun et al. found that MeV inhibited the over-activation of BV-2 microglia stimulated by LPS, reduced the content of inflammatory factors by the NF- κ B/I κ B α pathway, and reversed the depression-like behavior induced by LPS in mice[15]. Therefore, the above findings suggest that the MeV may be vital for the therapeutic effect of MDD, which is worthy of further exploration. The main research idea of this study was to evaluate the antidepressant effect of MeV and the potential mechanism based on the perspective of disease-target-drug. Three LPS exposures were employed to establish significant depressive symptoms accompanied by microglial activation in mice. MeV administration significantly alleviated depression-like behaviors and reduced microgliosis in mice by inhibiting SRC phosphorylation. MeV has an antidepressant effect that may be related to the SRC-mediated NF- κ B signaling pathway (Fig. 7). The results provide references for further study of the pathogenesis and treatment of depression.

After searching public databases, 85 potential MeV and MDD intersection targets were identified, which were screened by the value of "degree" to acquire pivotal targets. Then, to annotate the functions of 85 validated targets and related pathways by the GO and KEGG enrichment, the results showed that the target genes had been implicated in various biological processes (such as the positive regulation of hormone response, hypoxia response, lipopolysaccharide response, and phosphorylation) and signaling pathways. Comorbid depression is common actually, such as endocrine diseases (thyroid hormone[52]), metabolic diseases (diabetes[53]), visceral diseases (alcoholic liver disease[54]), vascular diseases[55] and the pessimism of cancer patients[56]. Here, we mainly discuss the results of enrichment analysis excluding other diseases. From the pathogenesis perspective, MDD has also been considered a disease of microglia or a disease caused by changes in neuroplasticity[57, 58]. Microglia are the primary resident innate immune cells in the central nervous system and actively regulate microenvironmental changes in healthy and disordered brains[59]. Such cellular activation regulates inflammation, synaptic thinning or pruning, and neuronal connections[60]. Polarized microglia-mediated the inflammatory response of the central nervous system mainly through the NF- κ B signaling pathway[61]. Specifically, NF- κ B enters the nucleus when activated, promotes the expression of inflammatory genes, destroys neuronal synapses, leads to structural disruption and dysfunction of neural networks, and increases the risk of depression. Exposure to psychological stress can induce the activation of damage (or danger)-associated molecular patterns, and affect the production of cytokines, neurotransmitters, neuropeptides, and centrally-acting peripheral hormones[58]. TLR4, as a pattern recognition receptor, plays a central part in innate immunity.

TLR4 recognizes LPS, which triggers activation of c-SRC-kinase responses mediated by $[Ca^{2+}]_i$ increase and NF- κ B signaling pathway activation, mediating cell proliferation and motility[62, 63]. TLR4, when located on the surface of microglia, activates various signaling pathways mediating the production of inflammatory cytokines under the stimulation of LPS in the central nervous system. As an "integration factor" of endogenous and exogenous stimulators, SRC participates in numerous cellular processes, such as proliferation, survival, adhesion, migration[64]. Therefore, to maintain normal cellular processes, SRC activity is tightly regulated by phosphorylation sites. Phosphorylated SRC-Tyr416 is considered a marker for c-SRC activation[65]. In parallel, a study has shown that the Src function is necessary and sufficient for triggering microglial cell activation[66]. Our results showed that multiple LPS stimulation up-regulated the expression levels of TLR4, PSRC, NF- κ B, I κ B α , and IL-1 β proteins in the hippocampus, also activated microglia. MeV eliminated these effects, while the SRC inhibitor PP2 plays a similar role except TLR4. Meanwhile, when PP2 and MeV coexist, the regulatory effect of MeV on NF- κ B pathway proteins and microglia is inhibited. Previous study has shown that PP2 treatment significantly reduced PSRC expression and LPS-induced microglial neuroinflammation triggered by LPS in mice[67], which is consistent with our results. Therefore, these results, and other previously reported results, strongly support that SRC may act as a major direct target involved in the MeV-mediated regulation of the NF- κ B signaling pathway in depression.

However, there still are some limitations in our current research. On the one hand, although network pharmacology provides a predictable approach based on biochemistry, pharmacology, and network biology, the target data of drugs and diseases are collectively obtained from multiple public databases, especially the open database has little target data of MeV, which is insufficient to provide an objective and comprehensive analysis of the drug's intervention outcomes for MDD. On the other hand, numerous studies have shown that the hippocampus[68, 69], anterior cingulate cortex[70], and lateral habenula[71] participate in regulating neuroinflammatory depression. We focus our research on the hippocampus, as impairment of this area may be a core factor in the pathophysiology of depression. Although we had explored the effects of MeV in the hippocampus, we could not completely rule out the role of other brain regions, such as the anterior cingulate cortex and lateral habenula, during the period of MeV affecting LPS-induced depressive symptoms. These mechanisms should be validated in subsequent research.

Conclusion

We combined network pharmacology and experimental validation to explore and verify the potential mechanism of MEV in the treatment of MDD. MeV alleviates LPS-induced depression-like behaviors in mice via appropriately regulating neuroinflammation and microglial activation. This might be related to the activities of the SRC-mediated NF- κ B pathway. These findings provide preliminary evidence that MeV may be proposed as one of the potential therapeutic agents to prevent neuroinflammatory MDD.

Abbreviations

ALB	serum albumin
ANOVA	analysis of variance
BP	biological process
CC	cell composition
DAPI	4'-6-Diamidino-2-phenylindole
FST	forced swimming test
GO	gene ontology
IBA1	Ionized calcium-binding adapter molecule 1
IKB α	I kappa B alpha
IL-1 β	interleukin-1 β
KEGG	kyoto encyclopedia of genes and genomes
LPS	Lipopolysaccharide
MDD	major depressive disorder
MeV	5-O-methylvisammioside
MF	molecular function
NF- κ B	nuclear factor-kappa B
OFT	open field test
PPI	protein-protein interaction
PP2	4-Amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine
P-SRC	Phosphorylated- Proto-oncogene tyrosine-protein kinase Src
SEM	standard error of the mean
SMILES	simplified molecular input line entry system
SPT	sucrose preference test
SRC	Proto-oncogene tyrosine-protein kinase Src
TLR4	Toll-like receptor 4
TST	tail suspension test

Declarations

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Author contributions Conceived and designed the experiments: WZ and BL. Acquired data: WZ. Analyzed and interpreted the data: RC and WZ. Wrote the manuscript: WZ. All authors have read and approved the final manuscript, contributed to the article, and approved the submitted version.

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Data Availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

This article does not contain any studies with human participants performed by any of the authors.

Ethical Approval All animals were treated according to the guidelines for the Care and Use of Laboratory Animals and the experimental procedures were approved by the Ethics Committee for Animal Use of Jilin University.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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Figures

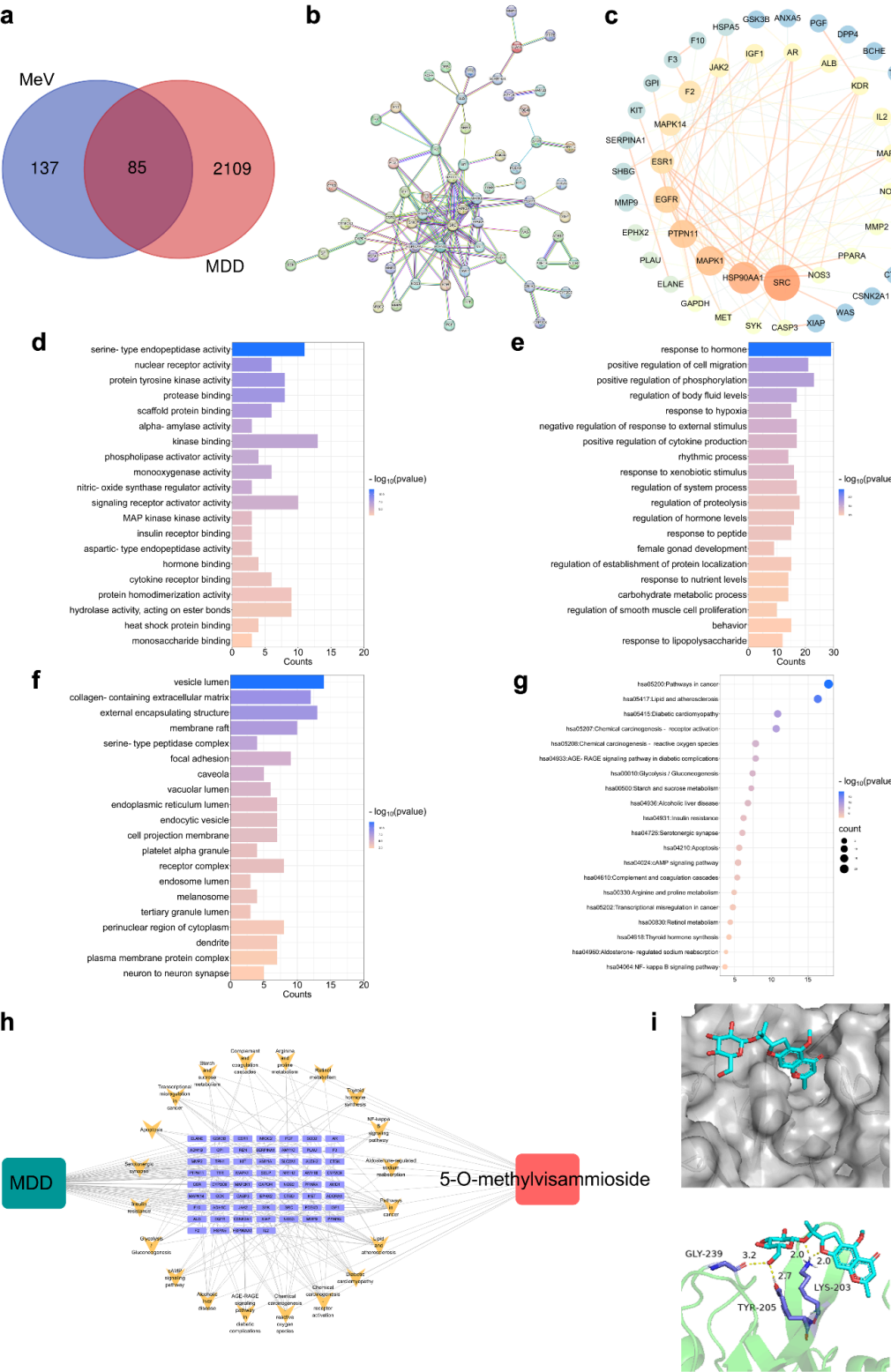


Figure 1

Network pharmacological analysis. **a** The Venn diagram of 5-O-methylvisammioside (MeV) and MDD-associated targets. **b** The protein-protein interaction (PPI) network of 85 targets according to the STRING database. Hiding free proteins that do non-interacting, there were 61 shared proteins in 85 disease and drug intersection targets. **c** The PPI network of MeV and MDD targets (Delete stray genes). Nodes represent proteins (The colors from bright to dark the size from large to small represent the degree of binding between proteins). Edge represents the protein-protein association. Gene ontology (GO) analysis of genes encoding proteins targeted by MeV. **d** GO - Molecular function analysis. **e** GO - biological process analysis. **f** GO - cellular component analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. **g** KEGG pathway analysis. The top 20 pathways were selected according to the arrangement rules of the Metascape tool. Specifically, the top 20 clusters and their representative enriched items (one per cluster) were selected. **h** The Target - pathway network showed the potential mechanism of MeV to treat MDD. The yellow triangle represents the pathway, and the purple square represents the target. Large green and red squares represent diseases and drugs, respectively. **i** Molecular docking results of MeV binding to its predicted protein targets.

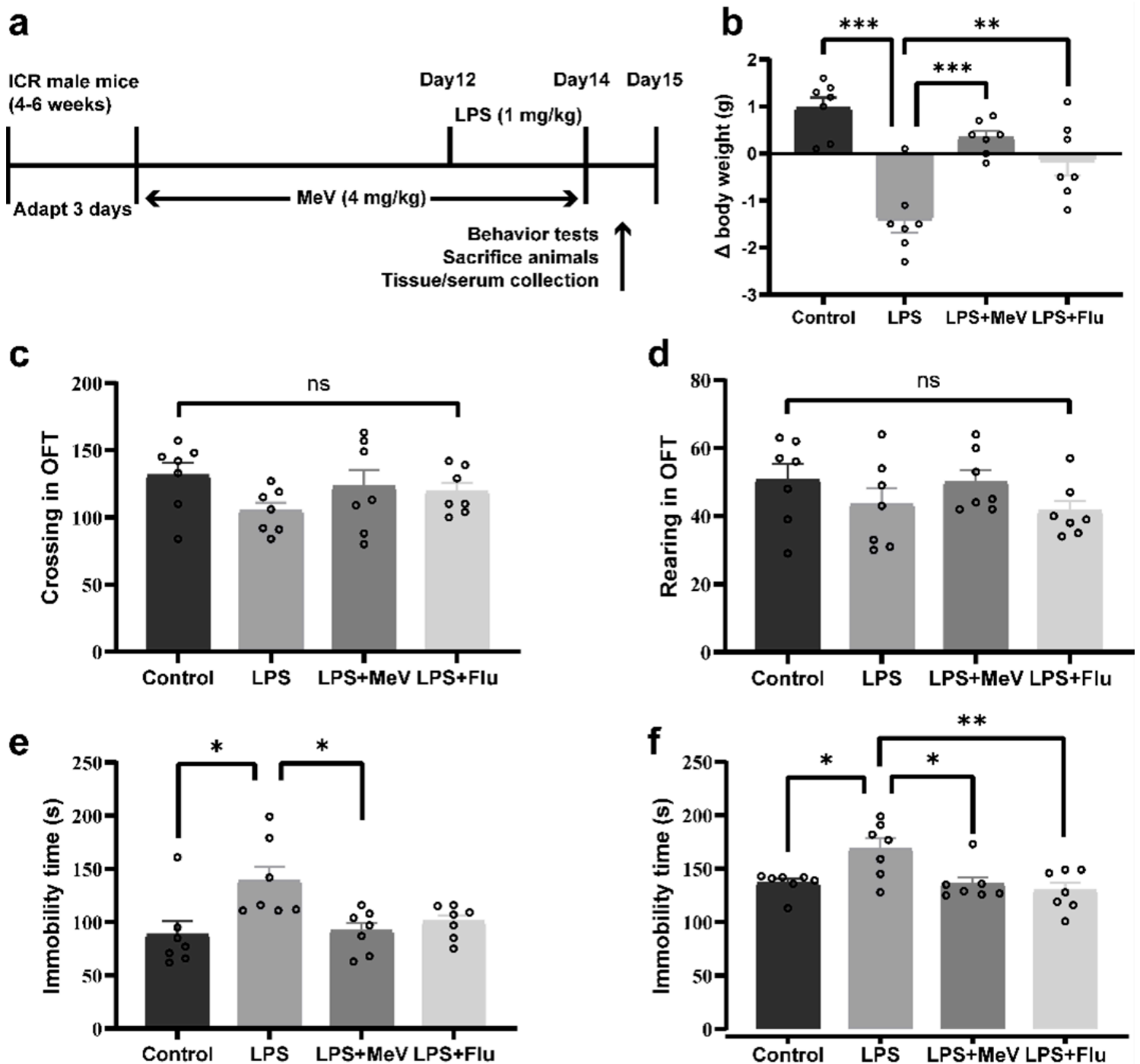


Figure 2

Effects of MeV on LPS-induced depressive behaviors. **a** Drugs treatment schedule (animals were treated for 14 days with MeV and 3 days (last) for LPS). **b** MeV reversed the body weight loss induced by LPS in depressed mice. **c, d** In the open field test (OFT), no significant difference was observed between locomotor activity and rearing in any group. **e** MeV alleviated the increase in immobility time of LPS model mice in the tail suspension test (TST). **f** MeV alleviated the increased immobility time of LPS-induced mice in the forced swimming test (FST). Data were presented as mean $\pm$ SEM ($n = 7$ per group). ANOVA followed by Tukey's test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

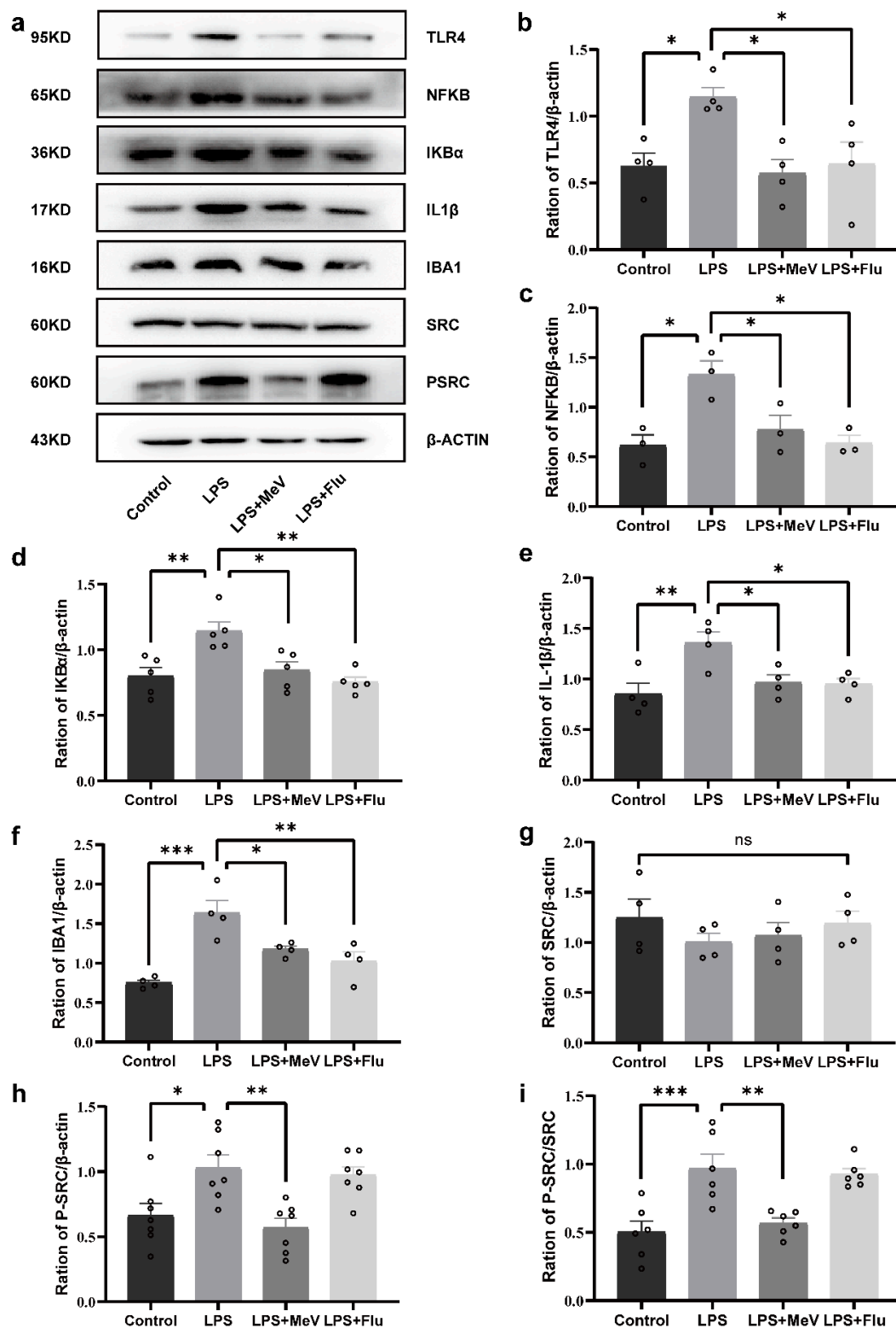


Figure 3

MeV reversed the up-regulation of TLR4, NF-κB, IκBα, and IL-1β in the hippocampus of model mice. **a** Representative Western blot images of proteins. **b-f, h, i** MeV markedly prevented the up-regulation of **b** TLR4, **c** NF-κB, **d** IκBα, **e** IL-1β, **f** IBA1, **h** P-SRC, and **i** P-SRC/SRC in the hippocampus of model mice, while there was no significant difference in the relative protein expression of **g** SRC. The quantified results

were normalized to β -ACTIN. All values were presented as the mean $\pm$ SEM ($n = 3-7$ per group). ANOVA followed by Tukey's test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

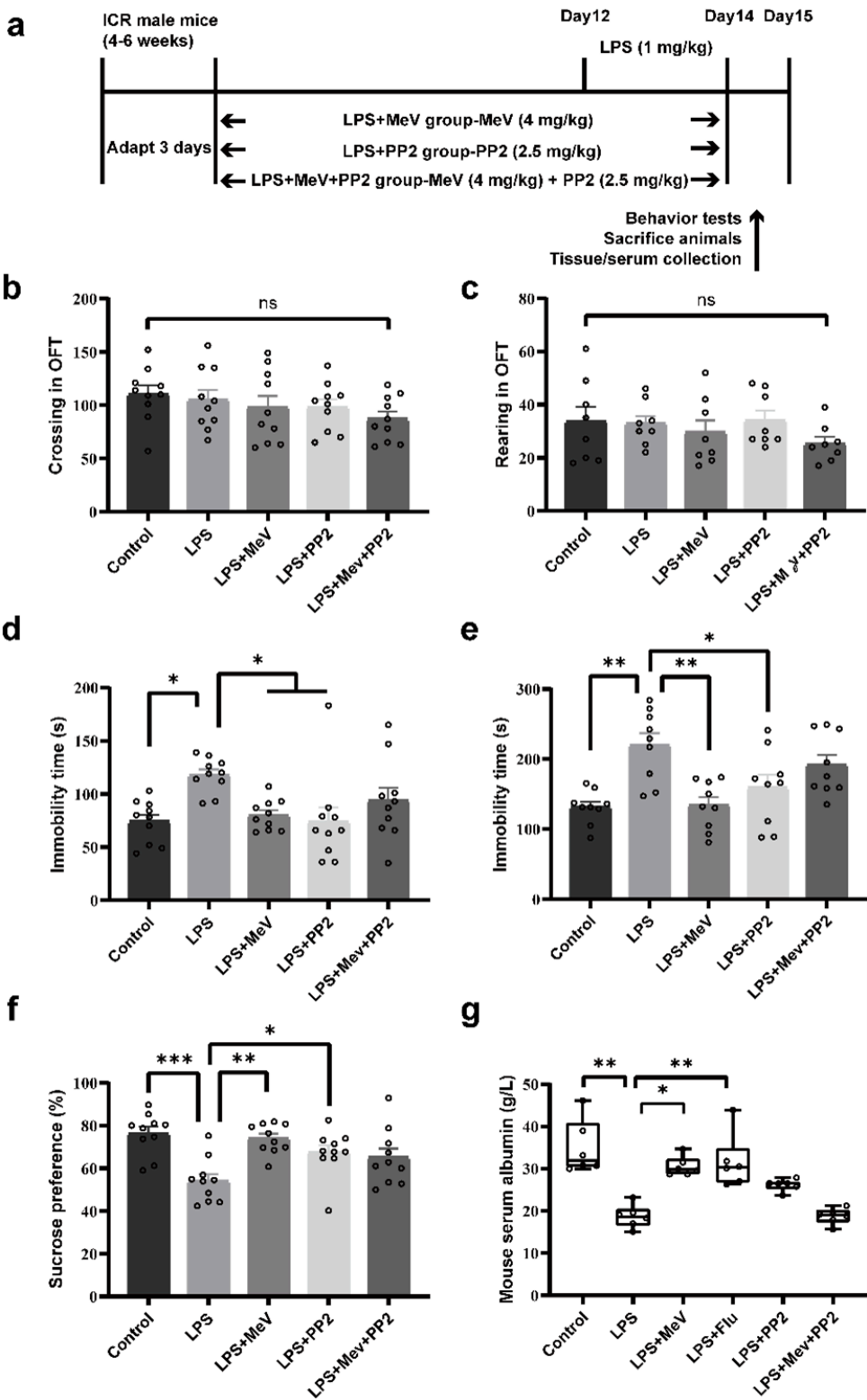


Figure 4

Inhibition of SRC activity ameliorates LPS-induced mice depression-like behaviors. **a** MeV and/or PP2 were continuously injected for 14 days and behavioral experiments were completed on the 15th day. **b, c** In the OFT, administration with MeV (4 mg/kg), PP2 (2.5 mg/kg), LPS (1 mg/kg) had no effect on either

locomotor activity or rearing. **d, e** MeV, PP2 treatment significantly improved the immobility time of LPS-induced mice in TST, FST. **f** MeV, PP2 reversed the decrease in the percentage of sucrose consumption in the LPS-induced depression mice in the sucrose preference test (SPT). The above data were presented as mean $\pm$ SEM (n = 8-10 per group). ANOVA followed by Tukey's test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **g** MeV pretreatment upregulated the LPS-induced decrease of mouse ALB, but SRC may not be involved in this process. The data was expressed as the median (n = 6 per group) and the Kruskal-Wallis test was used for pairwise comparison. * $p < 0.05$, ** $p < 0.01$.

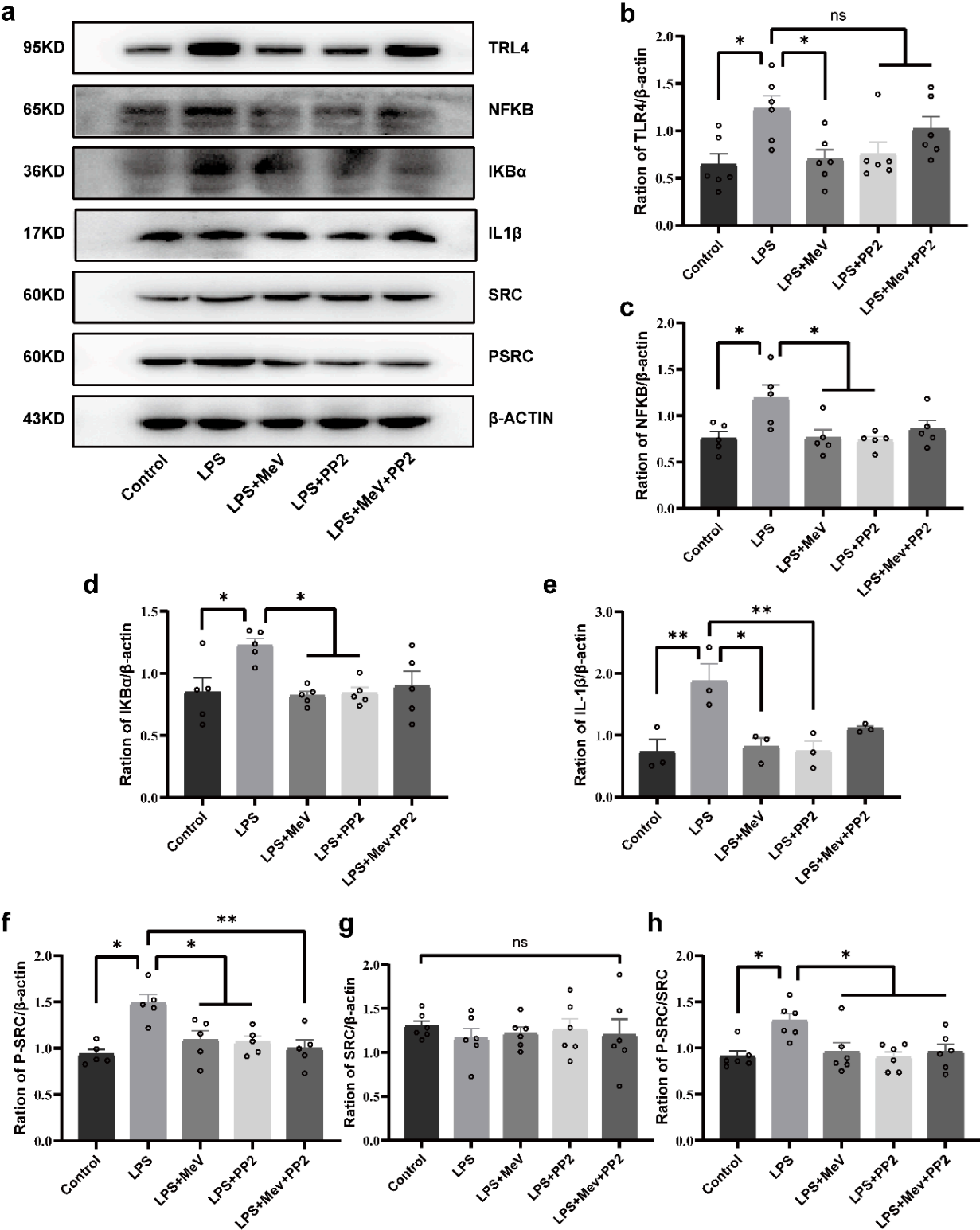


Figure 5

SRC plays a pivotal role in the NF- κ B pathway in the hippocampus of LPS-induced model mice. **a** Representative Western blot images of NF- κ B pathway associated protein levels in the hippocampus from the different treatment groups. **b-h** Quantification of expression levels of **b** TLR4, **c** NF- κ B, **d** IKB α , **e** IL-1 β , **f** P-SRC, **g** SRC, and **h** P-SRC/SRC; β -Actin was used as the loading control. Values expressed as mean $\pm$ SEM (n = 3-6 per group). ANOVA followed by Tukey's test. * $p < 0.05$, ** $p < 0.01$.

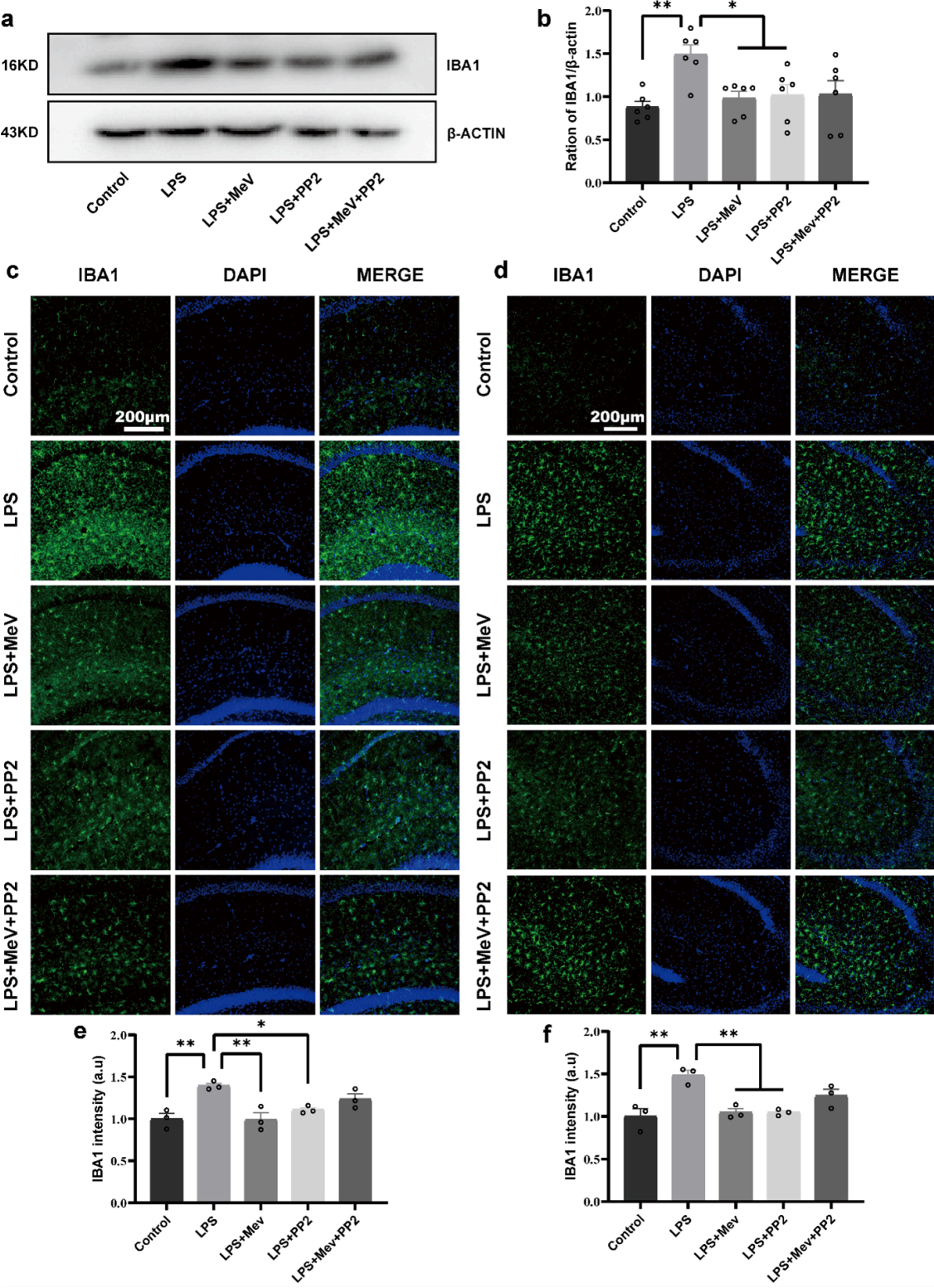


Figure 6

MeV pretreatment inhibited the hippocampal expressions of IBA1 in LPS-induced brain inflammation. **a**, **b** The hippocampus lysates were subjected to immunoblot analysis with antibodies against IBA1. (n = 6 per group). **c**, **d** IBA1 immunofluorescence staining of hippocampal CA1/CA3 region (n = 3 per group). IBA1 was used as a marker of microglia activation. **e**, **f** Expressions were quantified by densitometry. Values expressed as mean $\pm$ SEM. ANOVA followed by Tukey's test. * $p < 0.05$, ** $p < 0.01$.

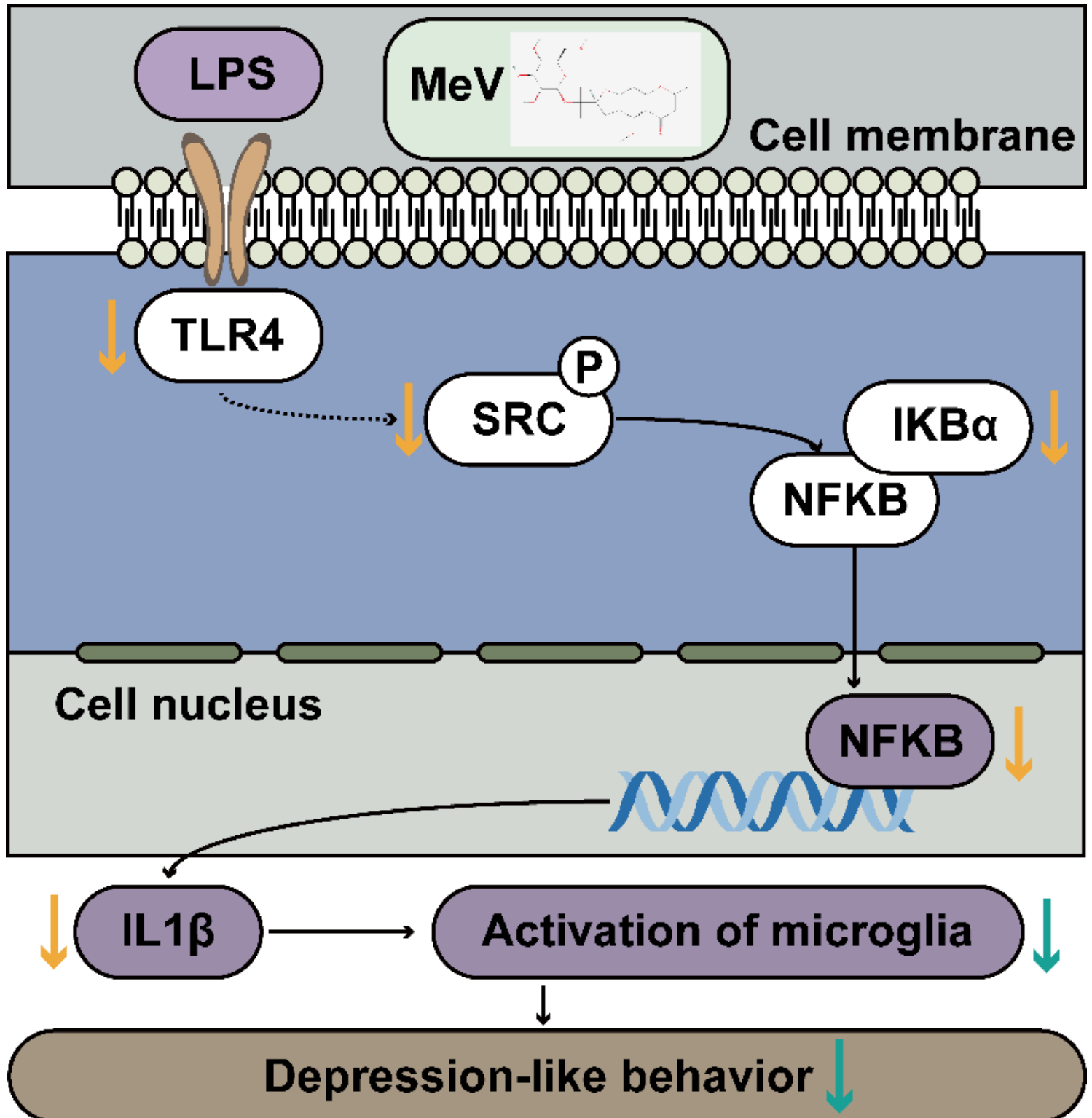


Figure 7

Mechanism of depression-like behavior of MeV in LPS-induced model mice. Regulation of pathway proteins/cell or animal behaviors by MeV is shown by orange/green arrows, respectively, with down-regulation indicated by down arrows.

Supplementary Files

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