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Naringenin Alleviates Acute Ethanol Exposure-induced Nerve Injury Through Regulating Neuroinflammation via PI3K/AKT Pathway-Mediated Modulation of Microglial M1/M2 Polarization

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ABSTRACT: Excessive alcohol consumption results in substantial neurotoxicity and contributes to the development of multiple neuropathological conditions. Regulation of neuroinflammatory processes is considered a critical determinant in mitigating alcohol-induced neuronal damage. Naringenin, the bioactive aglycone of naturally occurring citrus flavanone glycosides such as naringin, has been widely reported to possess potent antioxidant and anti-inflammatory activities. In the present study, we investigated whether naringenin exerts neuroprotective effects against acute ethanol-induced nerve injury. Naringenin treatment attenuated ethanol-induced intoxication, oxidative stress, apoptosis, and cellular stress, while maintaining neuronal morphology and functional integrity. Network pharmacology analysis further identified microglia as key cellular mediators underlying the anti-neuroinflammatory effects of naringenin. Mechanistically, naringenin markedly suppressed microglial activation and ethanol-induced morphological abnormalities *in vivo*, promoted a phenotypic shift toward the anti-inflammatory M2 subtype, and regulated the balance between pro- and anti-inflammatory cytokine production. Prophylactic intervention further indicated that these regulatory effects are mediated through activation of the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) signaling pathway. Additionally, co-culture experiments demonstrated that naringenin-induced suppression of pro-inflammatory cytokine release in microglia effectively preserved neuronal activity. These findings not only elucidate the regulatory role of naringenin in microglial polarization during acute ethanol exposure but also reveal its underlying neuroprotective mechanisms, thereby providing novel insights into the therapeutic potential of naringenin as a protective agent against acute ethanol-induced nerve injury.

Keywords: Naringenin; Ethanol; Neuroinflammation; Microglia; M1/M2 Polarization; PI3K-AKT pathway.

1. Introduction

Ethanol is among the most widely abused psychoactive substances worldwide [1]. An estimated 2.3 billion individuals consume alcohol, and harmful alcohol use accounts for approximately 1.3% of annual global mortality, rendering it a major contributor to worldwide disease burden [2,3]. Ethanol exhibits systemic toxicity, with the brain being one of the most vulnerable target organs. It readily crosses the blood-brain

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barrier, eliciting pro-inflammatory responses and neurodegeneration that impair cognitive processes such as learning and memory [4]. Nevertheless, the cellular and molecular mechanisms underlying ethanol-induced central nervous system (CNS) dysfunction remain incompletely elucidated and appear to vary across brain regions [5]. Although several therapeutic strategies exist, most interventions primarily target later stages of ethanol-induced pathology [6]. Therefore, there is an urgent need to develop effective therapeutic strategies for acute ethanol-induced nerve injury.

Ethanol influences multiple components of the immune response, including the activation of inflammatory signaling pathways, upregulation of cytokine expression, and recruitment of immune cells [7]. Both clinical and preclinical studies consistently demonstrated that ethanol exposure increased the expression of immune-related genes and elevated pro-inflammatory cytokine levels in humans and mice [8-11]. Consequently, ethanol-induced neuroinflammation is recognized as a central driver of secondary neuropathological processes such as neuronal apoptosis [3,12].

Microglia and astrocytes are the key resident glial cells of the CNS and play essential roles in both homeostasis and neuroinflammation. Microglial activation can persist for extended periods following excessive alcohol exposure [13]. In their activated (M1) state, microglia release pro-inflammatory cytokines such as interleukin (IL)-1 β , IL-6, tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ), which subsequently promotes astrocytic polarization toward a pro-inflammatory (A1) phenotype [14]. A1 astrocytes contribute to neuronal and oligodendrocyte loss and exhibit decreased synaptic support and phagocytic capacity [15]. Therefore, therapeutic strategies that regulate microglial activation and target neuroinflammatory signaling pathways represent a promising approach for preventing and treating ethanol-induced neurological injury.

Recently, an increasing number of studies have been conducted to identify novel therapeutic agents derived from natural products [16]. Flavonoids, one of the most abundant classes of phenolic compounds, have been shown to exhibit potent antioxidant activity and neuroprotective potential [17]. Naringenin (4', 5, 7-trihydroxyflavanone) is the aglycone and bioactive metabolite of citrus flavanone glycosides, particularly naringin, which are abundant in citrus fruits [18,19]. In nature, it occurs in both aglycone and glycosylated forms, including naringin, narirutin, and naringenin-7-O-glucoside, with their relative proportions varying depending on the source [20]. Beyond their dietary occurrence, both naringenin and naringin have attracted considerable interest as functional bioactive compounds because of their diverse biological activities [21]. Notably, naringenin differs from naringin in its pharmacokinetic properties. Previous study has shown that the aglycone form exhibits more favorable bioavailability than the glycosylated forms [22], and comparative pharmacokinetic analyses further demonstrated that naringin is less efficiently absorbed than naringenin [23]. Compared with naringin, naringenin has been reported to demonstrate superior antioxidant capacity, together with enhanced hydroxyl and superoxide radical-scavenging abilities [24]. Therefore, naringenin was selected in the present study as the intervention compound to directly evaluate the potential neuroprotective effects. Pharmacological studies have further shown that naringenin possesses anti-inflammatory properties,

favorable oral safety, improved bioavailability, and multi-organ protective activity [25–28]. Preclinical studies have shown that oral naringenin mitigated dyslipidemia and hyperinsulinemia, alleviated hepatic steatosis, and thereby induced a significant reduction in atherosclerosis in high-fat diet-fed *Ldlr*^{−/−} mice [29]. It also attenuates liver fibrosis by modulating TGF- β /Smad3 and JNK pathways in CCl₄-treated mice [30]. Collectively, these findings suggest that naringenin represents not only a dietary component but also a promising nutraceutical and lead compound for drug development.

Previous studies have shown that naringenin, one of the major bioactive compounds in Citrus, provides neuroprotection by inhibiting the c-Jun N-terminal kinase (JNK)/c-Jun pathway, thereby preventing neuronal loss in a mouse model of optic nerve injury [31,32]. Another study reported that naringenin increased dopamine levels and protected against dopaminergic neuron depletion in a Parkinson's disease rat model [33]. In acute ethanol exposure models, naringenin has been shown to alleviate behavioral abnormalities, developmental defects, lipid accumulation, and oxidative stress in zebrafish larvae [34,35]. However, all these neuroprotective effects of naringenin are based on studies of neurodevelopmental or neurodegenerative diseases. The protective effects and molecular mechanisms of naringenin in acute ethanol-induced nerve injury remain unclear.

In the present study, we established both *in vivo* and *in vitro* models of acute ethanol exposure to investigate the effects of naringenin, focusing on the cortex and hippocampus, which are functionally associated with cognition. We examined the pathological alterations induced by acute ethanol exposure and evaluated the neuroprotective effects of naringenin. Furthermore, we identified potential molecular targets and signaling mechanisms responsible for these effects. Our findings provide novel insights into the neuroprotective function of naringenin and highlight its therapeutic potential for acute ethanol-induced nerve injury.

2. Material and Methods

2.1 Reagents

Naringenin and 5,7-Dimethoxycoumarin were purchased from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China). β -glucuronidase (Type L-II, 1351017 units/g, from limpets) and sulfatase (Type H-1, 30567 units/g, from *Helix pomatia*, containing 3190293 units/g of β -glucuronidase) were purchased from Sigma Chemical Co., Ltd (St. Louis, USA). HPLC-grade acetonitrile, methanol, and ethyl acetate were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). L(+)-Ascorbic acid was obtained from Yeasen Biotechnology Co., Ltd. (Shanghai, China). All other chemicals and reagents were of analytical grade.

2.2 Animals

Eight-week-old male C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). The mice were housed in a specific-pathogen-free environment under controlled conditions, including a 12 h light/dark cycle at 24 ± 2 °C and 45–55% relative humidity. The mice

had free access to water and food. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3 Binge Ethanol Feeding Model and Drug Treatments

After a 1-week acclimatization period, mice were randomly categorized into four groups ($n = 5$ per group): control group, binge ethanol feeding (EtOH) group, low-dose naringenin treatment (EtOH+Nar-L, 50 mg/kg) group, and high-dose naringenin treatment (EtOH+Nar-H, 100 mg/kg) group. Mice in the EtOH, EtOH+Nar-L, and EtOH+Nar-H groups received a single oral dose of ethanol (5 g/kg), whereas mice in the control group received an equivalent volume of saline. After ethanol exposure, the naringenin or corn oil was administered via intraperitoneal injection. Twelve hours after treatment, the mice were anesthetized with an appropriate dose of tribromoethanol (approximately 20 $\mu\text{L/g}$ body weight, intraperitoneal injection) and subsequently euthanized. Serum and brain tissues were collected for subsequent study.

The oral ethanol dose used in this study has been widely applied in established binge ethanol feeding mouse models, and previous reports using this regimen have documented peak blood alcohol concentrations of approximately 250–400 mg/dL, consistent with severe intoxication in humans [36–42]. The sample size was estimated a priori based on alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) activity measurements using G*Power software (v3.1.9.7, Heinrich-Heine-Universität Düsseldorf, Germany) [43,44]. A minimum of three animals per group was calculated to achieve 80% power at a significance level of 0.05 for a medium effect size (ANOVA, $f = 0.25$). To increase statistical reliability and account for biological variability among individual animals, we ultimately included five animals per group.

To further explore the mechanism of naringenin, the PI3K inhibitor LY294002 (MedChemExpress, State of New Jersey, USA) was administered intranasally 1 hour before naringenin treatment. The dosing regimen and administration procedure were based on previously described methods [45,46]. Briefly, 15 μL of LY294002 (0.2 mg/mL) was slowly delivered into one nasal cavity over approximately 15 s using a pipette, and the same procedure was repeated in the contralateral nostril after a 2–3 min interval. In addition, a second PI3K-AKT pathway inhibitor, wortmannin (15 $\mu\text{g/kg}$; MedChemExpress, New Jersey, USA), was injected intravenously 1.5 h before naringenin treatment, as described previously [47–49].

Furthermore, to evaluate the feasibility of naringenin for oral administration, mice were administered naringenin either by intragastric gavage or intraperitoneal injection. Oral gavage was performed as previously described [50,51]. Briefly, naringenin was suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na) and administered by intragastric gavage at a dose of 100 mg/kg. Mice in the other group received an equivalent volume of 0.5% CMC-Na via oral gavage.

2.4 Loss of Righting Reflex (LORR) Test

After ethanol administration, mice were placed in a supine position in V-shaped plastic troughs. The time to LORR was defined as the interval from ethanol administration until the mice were unable to right themselves three times within 30 s [52]. After the onset of ataxia, the LORR duration was defined as the time between loss and recovery of the righting reflex.

2.5 Rotarod Test

A rotarod apparatus (ENV-575A, Med Associates, USA) was used to assess motor coordination following ethanol and naringenin treatment. One day prior to testing, mice were trained to remain on the rotating rod for at least 100 s at a constant speed of 5 rpm. During the test, the rotation speed was accelerated from 5 to 40 rpm over a 300 s period. The latency to fall was recorded for each mouse, and three trials were conducted per animal.

2.6 CatWalk Gait Analysis

Gait analysis was performed using the CatWalk system (Noldus Information Technology, Netherlands) as previously described [53]. Mice were trained to cross the glass walkway for two consecutive days prior to testing. On the third day, only runs in which the mice crossed the walkway smoothly without pausing were collected, and at least five valid runs were recorded for each mouse. Footprints were detected by light transmitted through the glass plate and captured using a high-speed camera positioned beneath the walkway. Stride length and swing speed were automatically quantified using CatWalk XT 9.0 software (Noldus Information Technology, Netherlands).

2.7 Cell Culture and Treatment

The BV2 microglial cell line and HT22 mouse hippocampal neuronal cell line were cultured in DMEM (Hyclone, South Logan, USA) supplemented with 10 % fetal bovine serum (FBS, Gibco, Grand Island, USA) and 1% Penicillin-Streptomycin (Invitrogen, Waltham, USA) at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were planted in 96-well plates (1.0×10^4 cells/well), 24-well plates (2.5×10^5 cells/well), and 6-well plates (1.0×10^6 cells/well). Upon reaching approximately 80-90% confluence, cells were treated with a series of concentrations of ethanol (0-800 mM) and naringenin (0-100 μ M) for 24 h.

BV2 cells were treated with phosphate buffered saline (PBS, Hyclone, South Logan, USA) or 400 mM ethanol for 12 h. The supernatant was collected, and cell debris was removed by centrifugation at $5000 \times g$. The resulting supernatant was used as conditioned medium (CM) and ethanol-stimulated conditioned medium (E-CM), respectively. After HT22 cells had attached, they were treated with equal volumes of CM or E-CM for 24 h. For calcium imaging experiments, the BV2:HT22 ratio was set at 1:1.

Primary hippocampal neurons were cultured from postnatal (day 0) mouse pups. Hippocampal tissues were dissected and enzymatically digested with 0.25% trypsin (Gibco, Grand Island, USA) for 15 minutes at 37°C. Digestion was halted by adding an equal volume of DMEM/F-12 medium (Gibco, Grand Island, USA) containing 10% FBS. The cell suspension was then centrifuged at $500 \times g$ for 2 minutes, and the resulting dissociated cells were resuspended in DMEM/F-12 medium containing 10% FBS. Cells were plated on poly-D-lysine-coated coverslips and maintained in a 37°C incubator with 5% CO₂. After 30 min, Neurobasal medium (Gibco, Grand Island, USA) supplemented with 1% Penicillin-Streptomycin, B27 (Gibco, Grand Island, USA), and GlutaMAX (Thermo Fisher Scientific, Waltham, USA) was added. Half of the medium was replaced with fresh medium every 3-4 days.

2.8 Cell Viability Assay

Cell viability was assessed using the MTS-based CellTiter 96® AQueous one solution cell proliferation assay kits (Promega, Madison, USA). BV2 and HT22 cells were seeded in 96-well plates and exposed to varying concentrations of ethanol and naringenin as described above. The MTS reagent was added to each well and incubated for 1 h. The absorbance, which is directly proportional to the number of viable cells, was measured at 490 nm using a microplate photometer (Thermo Fisher Scientific, Waltham, USA). The results are expressed as the percentage of living cells (normalization by control). Each concentration was evaluated with 6 replicates to ensure statistical robustness.

2.9 Nitric Oxide (NO) Assay

BV2 were planted in a 96-well plate. After treatment with ethanol and naringenin for 24 h, the cell culture supernatant was harvested and mixed with an equal volume of Griess reagent (Beyotime, Shanghai, China). The absorbance was measured at 540 nm using a microplate photometer. Sodium nitrite was used as a standard to calculate the NO concentration, and each concentration was evaluated with 6 replicates.

2.10 Calcium Imaging *in vitro*

HT22 cells were divided into four groups: CM group (co-cultured with CM), CM+Nar group (co-cultured with CM and 6.25 μ M naringenin), E-CM group (co-cultured with E-CM), and E-CM+Nar group (co-cultured with E-CM and 6.25 μ M naringenin). After 24 h of culture, cells were incubated with Fluo-4 (Thermo Fisher Scientific, Waltham, USA) and imaged at a rate of 0.5 Hz using a Nikon A1R confocal microscope (Nikon, Melville, USA). Cells were maintained in Hanks' Balanced Salt Solution (HBSS, Gibco, Grand Island, USA) containing 1.26 mM Ca^{2+} . After establishing a 1 min baseline (F_0), 5 or 20 μ M ATP was added, and imaging was continued for an additional 4 min. Fluorescence intensity was normalized by subtracting background signals and using the average baseline values prior to ATP addition. Single cells were segmented using Cellpose 3.0.10 [54]. The peak and peak time were defined as the maximum intensity value and the time to reach this maximum after ATP addition, respectively. The power was calculated as the mean square deviation of intensity values after ATP addition, reflecting the extent of intracellular Ca^{2+} fluctuations [55]. The rising and descent rates represented the rates of Ca^{2+} influx and efflux, respectively.

2.11 Alcohol Metabolizing Enzymes Assay

Collected brain tissue was immediately homogenized on ice to prepare a 10% tissue homogenate. The homogenate was separated by centrifugation according to the manufacturer's instructions and the supernatant was collected. Samples were stored at -80°C until further analysis. ADH and ALDH activities in brain tissue homogenate were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.12 Brain Oxidative Stress Assay

Malondialdehyde (MDA) level and the activities of catalase (CAT), glutathione peroxidase (GSH), and superoxide dismutase (SOD) in brain tissue homogenate were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.13 Enzyme-linked Immunosorbent Assay (ELISA)

The brain pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α and the anti-inflammatory cytokine IL-10 were measured with ELISA kits (Solarbio, Beijing, China) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate photometer, and the cytokine concentration was detected based on standard curve.

2.14 Pharmacokinetic Analysis

Mouse serum and brain tissues were collected at 0, 0.5, 1, 2, 4, 8, and 12 h after ethanol and naringenin treatment. Quantification of naringenin followed previously described methods [56-60]. Brain tissues were homogenized in normal saline (300 μ L/g tissue weight) and deproteinized with three volumes of methanol. After centrifugation, the supernatant was evaporated to dryness under vacuum and reconstituted in 0.5 mL of pH 5 acetate buffer to obtain tissue extracts.

For quantification of naringenin glucuronides, 100 μ L of tissue extract or serum was mixed with 100 μ L of β -glucuronidase (1000 units/mL in pH 5 buffer) and 100 μ L of ascorbic acid (150 mg/mL), followed by incubation at 37°C for 2 h. For quantification of naringenin sulfates, 100 μ L of tissue extract or serum was incubated with 100 μ L of sulfatase (1000 units/mL in pH 5 buffer) and 100 μ L of ascorbic acid at 37 °C for 1 h. Following enzymatic hydrolysis, samples were acidified with 100 μ L of 100 μ M HCl and extracted with 500 μ L of ethyl acetate containing 5,7-dimethoxycoumarin (2.0 μ g/mL) as the internal standard. The ethyl acetate layer was evaporated to dryness under N₂ gas, and the residue was reconstituted in acetonitrile; 20 μ L of each sample was subjected to UPLC-MS/MS analysis. Free form naringenin was quantified using the same procedure, except that pH 5 buffer was added instead of enzyme. Calibration standards were prepared by spiking blank serum or tissue extracts with known concentrations of naringenin and processed identically to the samples. Calibration curves were constructed by plotting the peak area ratio of naringenin to the internal standard against the corresponding concentrations using linear regression.

Sample analysis was performed using a UPLC-MS/MS system (Agilent Technologies, Santa Clara, USA). Chromatographic separation was conducted on an ACQUITY UPLC BEH C18 (2.1 $\times$ 100 mm, 1.7 μ m) maintained at 30 °C and eluted at a flow rate of 0.3 mL/min. The mobile phase was composed of solvent A (0.1% aqueous formic acid) and solvent B (100% acetonitrile). The gradient elution program was as follows: linear gradient from 5% to 100% B (0-4 min), then from 100% to 5% B (8-10 min). Mass spectrometry detection was carried out in electrospray ionization (ESI) positive and negative ion mode. Pharmacokinetic parameters following intraperitoneal administration were analyzed using the non-compartmental analysis via Phoenix WinNonlin (version 8.3.5, Certara, Delaware, USA).

2.15 Quantitative Real-Time PCR (qRT-PCR)

Following the manufacturer's instructions, an RNAsimple Total RNA Kit (TianGen, Beijing, China) was used to prepare the RNA samples. And 2 µg total RNA from each sample was reversed transcribed into cDNA using SuperScript III (Thermo Fisher Scientific, Waltham, USA). The reaction system was prepared according to the instructions of the SYBR Premix Ex Taq™ kit (TaKaRa, Kusatsu, Japan) and performed on a LightCycler R 480 System (Roche, Basel, Swiss). The PCR conditions were as follows: initial activation at 95 °C for 30 s, followed by 40 cycles with denaturation at 95 °C for 5 s, and annealing and extension at 60 °C for 30 s. The mRNA levels of genes were calculated by normalization with the β-actin expression level. All gene primer pairs are reported in Table 1.

Table 1. Primer sequences used for qRT-PCR.

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')	Annealing temperature (°C)	Length (bp)
<i>Il-6</i>	CTTCCATCCAGTTGCCTTCT	CTCCGACTTGTGAAGTGGTATAG	62.0	134
<i>p50</i>	GGATGACAGAGGCGTGATTAG	CCTTCTCTGTCTGTGAGTTG	62.0	114
<i>Tnf-α</i>	CGATGGGTTGTACCTTGTCTAC	GAGGTTGACTTTCTCCTGGTATG	62.0	121
<i>p52</i>	CCGCCCTATCACAAGATGAA	GTCTTCCACCAGAGGGTAATATG	62.0	120
<i>p65</i>	GCTCAAGATCTGCCGAGTAAA	GTCCCGTGAAATACACCTCAA	62.0	113
<i>Relb</i>	TGCCGAATCAACAAGGAGAG	TGCTGAACACCACGGATATG	62.0	100
<i>p53</i>	GCCATGGCCATCTACAAGAA	AATTTCCTTCCACCCGGATAAG	62.0	127
<i>Bdnf</i>	GTGACAGTATTAGCGAGTGGG	GGGATTACACTTGGTCTCGTAG	62.0	143
<i>Dlg4</i>	GTGACAACCAAGAAATACCGC	TTCACCTGCAACTCATATCCTG	62.0	146
<i>Bax</i>	TGCTACAGGGTTTCATCCAG	GACACTCGCTCAGCTTCTT	62.0	111
<i>Bcl-2</i>	GTGGATGACTGAGTACCTGAAC	GAGACAGCCAGGAGAAATCAA	62.0	125
<i>Hmox1</i>	ACACATCCAAGCCGAGAATG	TACAAGGAAGCCATCACCAG	62.0	93
<i>Nfe2l2</i>	TCCCATTTGTAGATGACCATGAG	CCATGTCCTGCTCTATGCTG	62.0	150
<i>Syp</i>	CCTGTCCGATGTGAAGATGG	AGGTTTCAGGAAGCCAAACAC	62.0	147
<i>Gap43</i>	AGAGGAGGAAAGGAGAGAAGG	GCCTTATGAGCCTTATCTTCCG	62.0	150

2.16 Western Blot Analysis

Western blotting was performed as described in our previous work [31]. The cell pellets and hippocampus were homogenated in RIPA Lysis Buffer (Beyotime, Shanghai, China) with protease and phosphatase inhibitors (Roche, Basel, Swiss) and stand on ice for 30 min. The supernatants were collected after the lysates were centrifuged at 4 °C at 12,000 rpm for 15 min at 4 °C. The supernatants were detecting the protein concentration used the Pierce™ BCA Protein Assay Kits (Thermo Fisher Scientific, Waltham, USA) and mixed with 5 × sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (Yeasten, Wuhan, China). Then the proteins were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, USA), which was then blocked with 5% milk or bovine serum albumin (BSA, NOVON, Beijing, China) for 1 hour at room temperature and immersed in the primary antibodies overnight at 4°C. The primary antibodies were as follows: rabbit anti-iNOS (1:1000, 13120, Cell Signaling Technology, Danvers, USA), rabbit anti-iNOS (1:1000, 18985-1-AP, Proteintech, Wuhan, China), rabbit anti-COX2 (1:1000, 12282, Cell Signaling Technology), rabbit anti-IBA1 (1:1000, 019-19741, Fujifilm, Osaka, Japan), rabbit anti-GFAP (1:10000, ab207165, Abcam, Cambridge, USA), rabbit anti-CD16 (1:2000, 83110-1-RR, Proteintech), rabbit anti-CD206 (1:1000, 18704, Proteintech), rabbit anti-ARG1 (1:5000, 16001-1-AP, Proteintech), rabbit anti-P53 (1:1000, T40060F, Abmart, Shanghai, China),

rabbit anti-BAX (1:1000, T40051, Abmart), rabbit anti-BCL-2 (1:1000, T40056, Abmart), rabbit anti-Phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) (1:1000, 4228, Cell Signaling Technology), rabbit anti-PI3 Kinase p85 (1:1000, 4292, Cell Signaling Technology), rabbit anti-Phospho-Akt (Ser 473) (1:2000, 4060, Cell Signaling Technology), and rabbit anti-Akt (pan) (C67E7) (1:1000, 4691, Cell Signaling Technology). Afterward, the PVDF membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at room temperature. Finally, protein bands were visualized using the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, USA). GAPDH and β -tubulin antibody was used as a loading control. The band intensities were quantified using ImageJ software (NIH, Bethesda, USA). Each experiment was evaluated with 3 replicates.

2.17 Immunostaining

Primary neurons were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature and then permeabilized with PBST ($1 \times \text{PBS} + 0.1\%$ Tween-20) containing 0.1% Triton X-100 for 15 minutes at 4°C. After washing three times with PBST, the neurons were blocked with 5% BSA in PBST for 1 hour at room temperature. Neurons were then incubated overnight at 4°C with primary antibodies: chicken anti-MAP2 (1:10000, ab5392, Abcam) and rabbit anti-PSD95 (1:500, 20665-1-AP, Proteintech). The following day, neurons were washed three times for 5 minutes each with PBST and incubated at room temperature with the appropriate secondary antibody (1:500, Abcam) for 1 hour. They were subsequently stained with 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, Waltham, USA) for 10 minutes. Images were obtained from Nikon AXR confocal microscope (Nikon, Melville, USA).

2.18 Immunohistochemistry

Mice were anesthetized and then perfused with $1 \times \text{PBS}$ and 4% PFA. Fresh brains tissues were fixed in PFA overnight followed by a graded dehydration (10%, 20% and 30% sucrose solution) at 4 °C and frozen in OCT, sliced into 30 μm sections using a frozen slicer. The slices were dried at room temperature and washed in $1 \times \text{PBS}$ before incubated in the PBST ($1 \times \text{PBS} + 0.1\%$ Tween-20) containing 0.3% Triton X-100 for 15 min. Then the slices were blocked with 5% BSA in PBST for 2 h. The slices were incubated with primary antibodies in the blocking solution at 4 °C overnight, including rabbit anti-IBA1 (1:1000, 019-19741, Fujifilm), rabbit anti-GFAP (1:1000, ab7620, Abcam), rabbit anti-CD16 (1:200, 83110-1-RR, Proteintech), rabbit anti-CD206 (1:200, 81525-1-RR, Proteintech), mouse anti-CHOP (1:250, 2895S, Cell Signaling Technology), and rabbit anti-Phospho-JUN (1:200, 3270S, Cell Signaling Technology). On the second day, slices were washed three times for 10 min each with PBST, and at room temperature incubated with matched secondary antibody (1:500, Abcam) for 2 h and in DAPI for 15 min. Images were obtained from Nikon AXR confocal microscope.

2.19 Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling (TUNEL) Staining

For quantification of neuronal apoptosis, double staining of TUNEL (green) and rabbit anti-neuronal nuclei antigen (NeuN, 1:1000, 702022, Invitrogen, Waltham, USA, red) was performed using the TUNEL

BrightGreen Apoptosis Detection Kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. Images were obtained from Nikon AXR confocal microscope. The number of TUNEL-positive neurons was counted using ImageJ software, data was expressed as the ratio of TUNEL-positive neurons (%).

2.20 Sholl Analysis

DIV 12 neurons were transfected with a green fluorescent protein (GFP) plasmid. After 24 h, neurons were treated with ethanol and naringenin, and images were acquired at DIV14 using a Nikon AXR confocal microscope. Sholl analysis was performed using an ImageJ plugin [61]. Briefly, for each neuron, bitmap images were analyzed from the soma to the most distal dendrites using the Threshold and Simple Neurite Tracer plugins. Concentric circles with gradually increasing radii (10 μm increments) were generated, and the crossing points were automatically counted. For microglia, Sholl analysis was performed using the same procedure, except that concentric circles were applied at 5 μm intervals.

2.21 Network Pharmacology and Molecular Docking

The 3D structure of naringenin was obtained by searching the PubChem database (<https://pubchem.ncbi.nlm.nih.gov>). And the naringenin-related targets were obtained using the ETCM (<http://www.tcmip.cn/ETCM/>), TCMSPP (<https://tcmsp.com/index.php>), Drug bank (<https://go.drugbank.com/>), Therapeutic Target Database (<https://db.idrblab.net/ttd/>), HIT 2.0 (<http://www.badd-cao.net:2345/>), TCM-Suite (<http://tcm-suite.aimicrobiome.cn/>), and PharmMapper (<https://lilab-ecust.cn/pharmmapper/index.html>) database. All potential targets were standardized to Gene names through the UniProt (<https://www.uniprot.org/>) database. Acute ethanol exposure-related targets were obtained from OMIM (<https://www.omim.org/>), GeneCards (<https://www.genecards.org/>), TTD (<https://db.idrblab.net/ttd/>), and ETCM (<http://www.tcmip.cn/ETCM2/front/#/>). Disease-related targets for neuroinflammation were obtained from three databases: OMIM, GeneCards, and DisGeNET (<https://www.disgenet.org/>). Then the obtained targets were combined and eliminated duplicates to identify potential disease targets associated with neuroinflammation. Differentially expressed genes (DEGs) were obtained from the GEO microarray dataset GSE20489 [62]. Genes with a $p\text{-value} \leq 0.05$ and $|\log_2\text{FoldChange}| \geq 0$ were considered differentially expressed. Venn diagrams were employed to obtain the potential targets among DEGs, disease genes, and potential targets of naringenin.

Gene Set Enrichment Analysis (GSEA) was performed using GSEA software (<https://www.broadinstitute.org/gsea/>) for the biological process subcollections in the human Molecular Signatures Database (MSigDB). Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the potential targets were performed through David database (<https://davidbioinformatics.nih.gov/>). Genes involved in the top five KEGG pathways were imported into the STRING platform to construct a protein-protein interaction (PPI) network, which was visualized using Cytoscape 3.9.0 software. Then the cytoHubba plugin was used to perform cluster analysis and identify hub genes. To predict potential correlations among hub genes, the random forest and the receiver

operating characteristic (ROC) curve analysis were employed to predict and calculate the gene with the highest contribution to sample grouping.

The top five proteins (NFKB1, EGFR, AKT1, TNF, and JUN) were downloaded from the RCSB PDB database (<https://www.rcsb.org/>), and protein structures were preprocessed by adding hydrogen atoms and removing water molecules. Molecular docking was performed using AutoDockSuite 4.2.6 and MGLTools 1.5.7. Docking results were evaluated based on binding energy; binding energies < -5.0 kcal/mol were considered indicative of feasible binding. For further analysis, the binding mode with the lowest binding energy was selected. The docking file was exported for visualization in PyMOL 3.0.0. Protein expression levels in different single-cell and single-nucleus clusters of brain tissue were obtained from the Human Protein Atlas database (<https://www.proteinatlas.org/>). Weighted score was calculated through the weighted average of $\log_2(\text{nTPM}+1)$.

2.22 Statistical Analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using Student's *t*-test for two-group comparisons, and one-way or two-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's *post-hoc* multiple comparison tests for multiple-group comparisons. Tukey's test was used to compare every mean with every other mean, whereas Dunnett's test was used to compare every mean to a control mean. *p* values were specified as follows: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

3. Results

3.1 Naringenin Exhibited Neuroprotective Effect in Binge Ethanol Feeding Mice

Acute ethanol exposure has been reported to induce loss of consciousness and impaired motor coordination [52]. To evaluate whether naringenin exerts a protective effect against acute ethanol intoxication, we established a binge ethanol feeding model in mice using a single oral dose of ethanol (5 g/kg). Immediately after ethanol administration, naringenin or corn oil was delivered via intraperitoneal injection (Figure 1A). To determine the effect of naringenin on ethanol-induced intoxication, we first examined the LORR test, a standard behavioral marker of inebriation. In both the EtOH and EtOH+Nar-L groups, mice lost their righting reflex within 20 min of ethanol gavage. However, high-dose naringenin treatment significantly extended the time of LORR to approximately 30 min compared with the EtOH group (Figure 1B). In addition, mice in the EtOH group required approximately 134-160 min longer to recover their righting reflex than those in the EtOH+Nar-L and EtOH+Nar-H groups (Figure 1C).

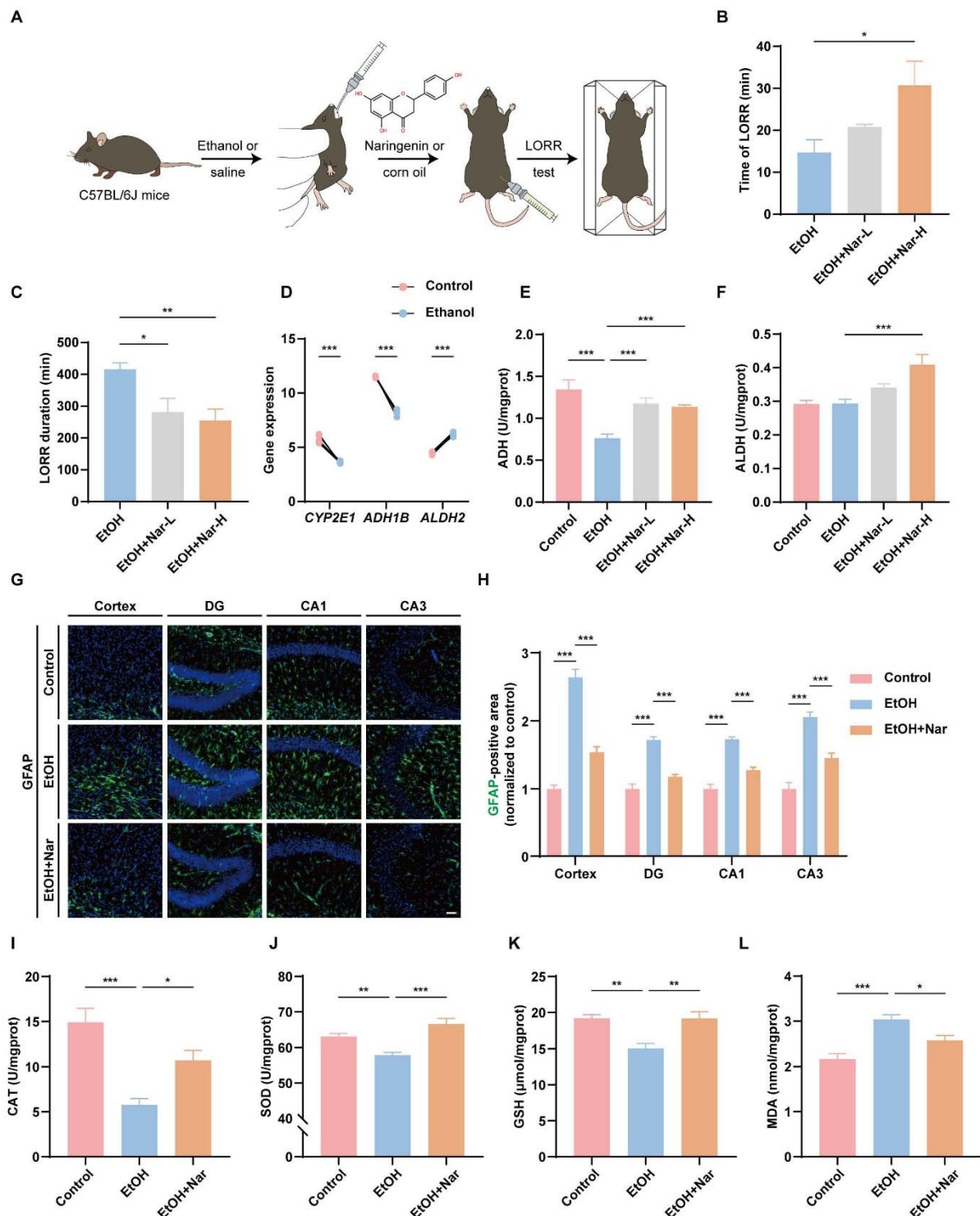


Figure 1. Naringenin exhibited neuroprotective effect in binge ethanol feeding mice. **A.** Schematic representation of the binge ethanol feeding, naringenin treatments, and the LORR test in mice. **B, C.** Effects of naringenin on mice inebriation, evaluated by time to LORR and LORR duration. **D.** The genes expression levels of key enzymes involved in alcohol metabolism following acute ethanol exposure in humans. **E, F.** Alcohol metabolizing enzymes assay of ADH and ALDH activities in the brain following naringenin treatment in ethanol exposure mice. **G.** Representative images of immunohistochemistry staining of GFAP in the cortex and hippocampus of mice after ethanol exposure and naringenin treatment. Scale bar = 50 μ m. **H.** Quantitative analyses of GFAP-positive area in the cortex and hippocampus of mice after ethanol exposure and naringenin treatment. **I, J, K, L.** Oxidative stress analysis of CAT, SOD, GSH, and MDA in the brain following naringenin treatment in ethanol exposure mice. All experiments were repeated at least three times. Data are represented as means $\pm$ SEM. * P < 0.05; ** P < 0.01; *** P < 0.001. EtOH, acute ethanol exposure group; EtOH+Nar-L, low-dose naringenin (50 mg/kg) treatment group following acute ethanol exposure; EtOH+Nar-H, high-dose naringenin (100 mg/kg) treatment group following acute ethanol exposure; EtOH+Nar, the treatment group with the optimal concentration of naringenin following acute ethanol exposure.

Metabolic enzyme mediated ethanol metabolism is crucial in ethanol detoxification [63]. Previous microarray data (GSE20489) [62] showed that the gene expression levels of *CYP2E1*, *ADH1B*, and *ALDH2*, which are key enzymes involved in alcohol metabolism, exhibited abnormal alterations following acute ethanol exposure (Figure 1D, Table S1). Given that ethanol metabolism in mammals primarily relies on ADH and ALDH enzymatic, we next examined whether these transcriptional changes were reflected at the functional level by measuring ADH and ALDH enzymatic activities in binge ethanol feeding mice. Our results showed that acute ethanol exposure significantly decreased ADH activity in the brain, while ALDH activity remained unchanged. In contrast, naringenin treatment induced a dose-dependent increase in ADH and ALDH activities compared with the EtOH group (Figure 1E-F). These findings suggest that naringenin promotes ethanol metabolism and attenuates ethanol-induced toxicity, with the high-dose naringenin group (100 mg/kg) exhibiting stronger protective effects.

Astrocytes are rapidly activated in a wide range of CNS pathologies. During neuronal injury, factors such as blood-brain barrier disruption, inflammation, metabolic disturbance, excitotoxicity, and oxidative stress collectively regulate the extent and characteristics of reactive gliosis [64]. Ethanol-induced neuronal injury is strongly linked to redox imbalance and elevated oxidative stress, which may further exacerbate astrocytic activation and neuronal damage [65]. To assess astrocyte reactivity, we performed GFAP immunostaining. Mice in the EtOH group displayed a significant increase in reactive astrocytes in both the cortex and hippocampus compared with control mice, whereas naringenin treatment markedly reduced GFAP-positive areas (Figure 1G-H). To further investigate the effects of naringenin on ethanol-induced oxidative stress, we quantified oxidative stress-related biomarkers in brain tissue. Compared with the control group, ethanol administration significantly decreased the activities of the antioxidant enzymes CAT and SOD as well as the level of the antioxidant GSH (Figure 1I-K). Conversely, levels of the oxidative damage marker MDA were significantly elevated in the EtOH group (Figure 1L). Naringenin treatment reversed these changes, significantly increasing CAT, SOD, and GSH levels while decreasing MDA accumulation (Figure 1I-L). These findings indicated that naringenin alleviated acute ethanol-induced neurotoxicity by reducing oxidative stress and astrocytes activation, thereby exerting a neuroprotective effect.

3.2 Naringenin Reduced Acute Ethanol Exposure-induced Nerve Injury

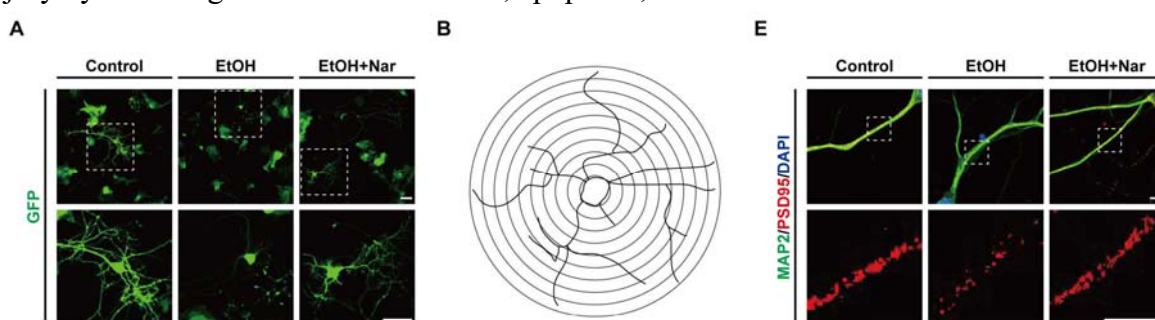
Previous proteomic studies have indicated that a single dose of ethanol intoxication leads to marked alterations in neuronal morphology and synaptic protein abundance [66]. To evaluate the protective effect of naringenin on neurons, we measured the viability of HT22 cells treated with a concentration gradient of ethanol and naringenin for 24 h. The results showed that ethanol reduced cell viability in a dose-dependent manner, with a pronounced reduction observed at 200 mM (Figure S1A). For consistency in subsequent experiments, the ethanol concentration was standardized to 400 mM ($P < 0.001$). Notably, treatment with 6.25 μ M naringenin significantly increased cell viability compared with ethanol alone (Figure S1B). We next evaluated neuronal morphology by adding 6.25 μ M naringenin to primary neurons exposed to 400 mM ethanol (Figure 2A). Sholl analysis, a widely used method to quantify dendritic complexity (Figure 2B) [67],

revealed that 24 h of ethanol exposure markedly disrupted neuronal dendritic arbors. Ethanol significantly reduced dendritic branch number at the radial distance and the intersection number within 300 μm of the soma, whereas naringenin treatment preserved dendritic arbors and prevented ethanol-induced morphological degeneration (Figure 2C-D).

Synapses are essential sites of neuronal communication and plasticity [68], and synaptic loss is a hallmark of neurodegeneration and nerve injury [69,70]. To determine whether naringenin prevents ethanol-induced synaptic impairment, we examined expression of the postsynaptic density protein PSD95 in primary neurons (Figure 2E). Ethanol exposure significantly decreased the number and size of PSD95-positive puncta. In contrast, naringenin treatment markedly increased synaptic density in ethanol-exposed neurons (Figure 2F-G), suggesting that naringenin effectively prevented ethanol-induced synaptic loss.

Acute ethanol intoxication is known to induce apoptotic neurodegeneration in mice, reportedly mediated by BAX-dependent pathways [71]. To evaluate neuronal apoptosis *in vivo*, we performed TUNEL staining. Ethanol exposure markedly increased TUNEL-positive neurons in the cortex and hippocampus, whereas naringenin treatment significantly inhibited apoptosis in both regions (Figure 2H-I). Consistent with these observations, western blot analysis showed that naringenin significantly reduced the expression of P53 and decreased the BAX/BCL-2 ratio in the hippocampus compared with the EtOH group (Figure 2J-L).

Our previous studies demonstrated that suppressing CHOP and p-JUN expression confers neuroprotection [31,72]. Since cellular stress is closely associated with the ER stress-mediated apoptotic pathway involving CHOP induction and JNK activation [73], we examined CHOP and p-JUN levels in the cortex and hippocampus using immunofluorescence staining (Figure 2M). Ethanol exposure markedly elevated CHOP and p-JUN levels in both regions, whereas naringenin treatment significantly attenuated their expression (Figure 2N-O). These findings indicated that naringenin attenuated ethanol exposure-induced nerve injury by reversing neuronal inactivation, apoptosis, and cellular stress.



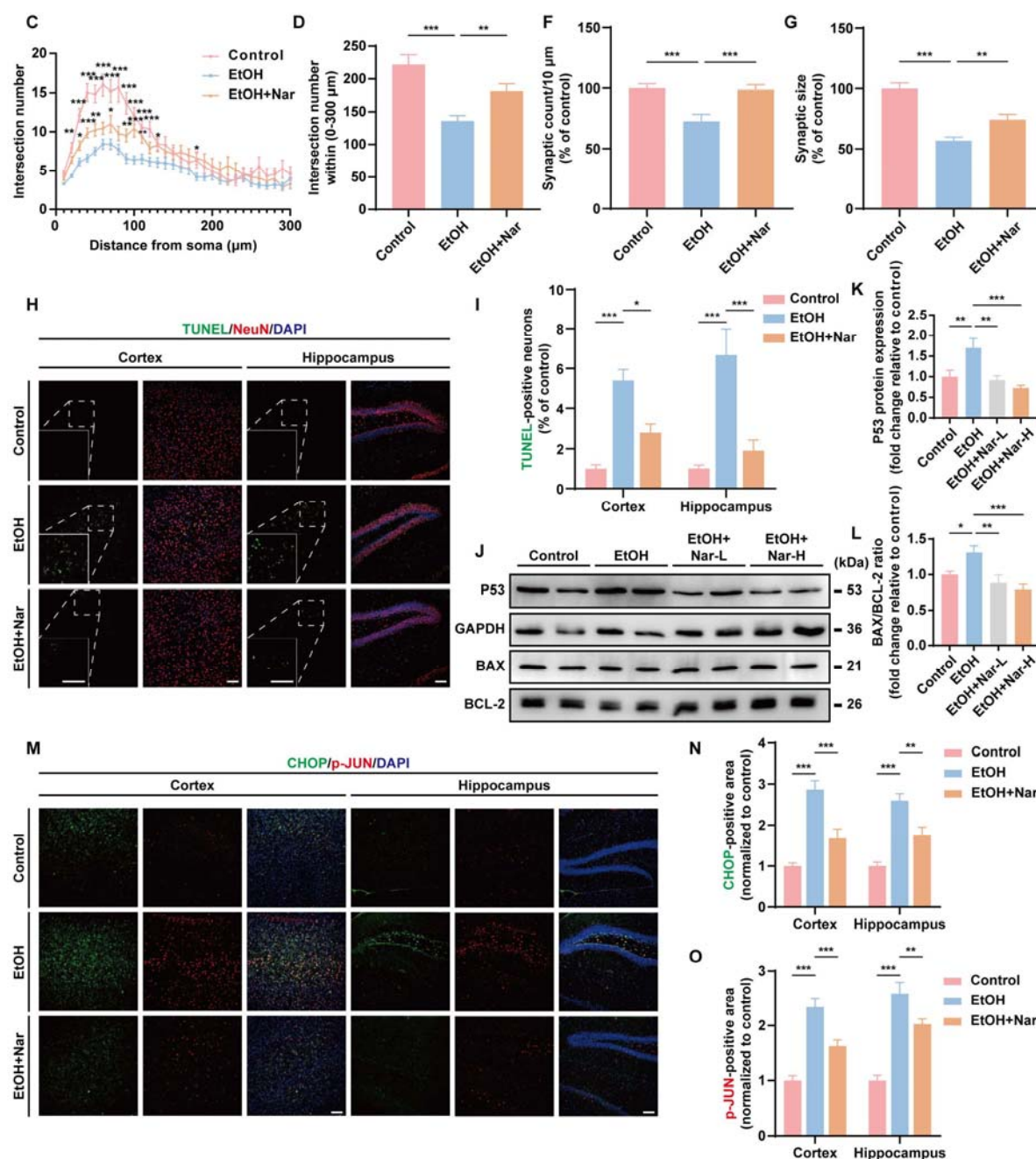
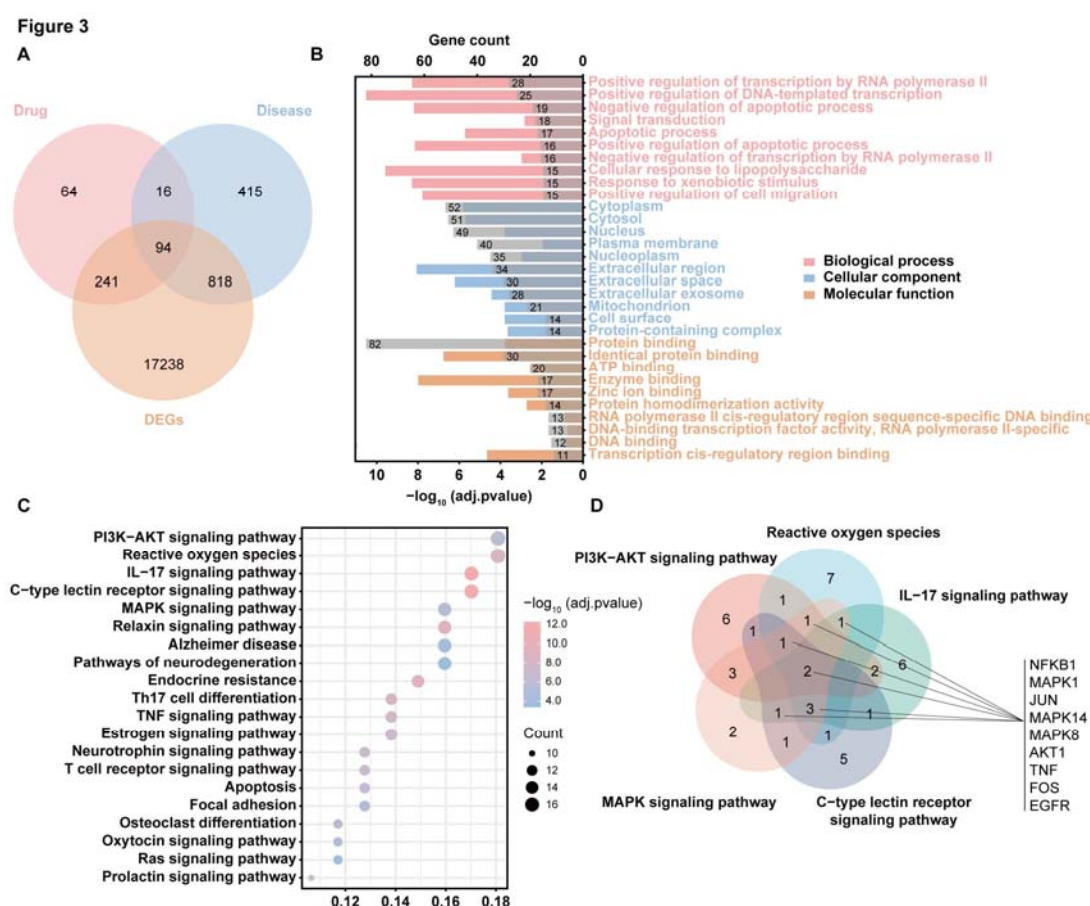


Figure 2. Naringenin attenuated ethanol exposure-induced nerve injury by reversing neuronal inactivation, apoptosis, and cellular stress. **A.** Representative images of GFP staining in primary neurons after ethanol exposure and naringenin treatment. Scale bar = 50 μm. **B.** Schematic representation of Sholl analysis. **C.** Line chart of intersection number in primary neurons after ethanol exposure and naringenin treatment. **D.** Statistical analysis of intersection number within 0-300 μm in primary neurons after ethanol exposure and naringenin treatment. **E.** Representative images of immunostaining staining of MAP2 (green) and PSD95 (red) in primary neurons after ethanol exposure and naringenin treatment. Scale bar = 5 μm. **F, G.** Statistical analysis of synaptic count per 10 μm and synaptic size in primary neurons after ethanol exposure and naringenin treatment. **H.** Representative images of TUNEL (green) and NeuN staining (red) in the cortex and hippocampus of mice after ethanol exposure and naringenin treatment. Scale bar = 100 μm. **I.** Quantitative analyses of TUNEL-positive neurons in the cortex and hippocampus of mice. **J.** Western blot results of P53, BAX, and BCL-2 protein expression in the hippocampus after ethanol exposure and naringenin treatment. **K, L.** Statistical analysis of P53 protein expression relative to GAPDH loading control and BAX/BCL-2 ratio after ethanol exposure and naringenin treatment. **M.** Representative immunohistochemistry images of CHOP (green) and p-JUN (red) staining in the cortex and hippocampus of mice after ethanol exposure and naringenin treatment. Scale bar = 100 μm. **N, O.** Quantitative analyses of CHOP and p-JUN-positive area in the cortex and hippocampus of mice. All experiments were repeated at least three times. Data are represented as means ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. EtOH, acute ethanol exposure group; EtOH+Nar-L, low-dose naringenin (50 mg/kg) treatment group following acute ethanol exposure; EtOH+Nar-H, high-dose naringenin (100 mg/kg) treatment group following acute ethanol exposure; EtOH+Nar, the treatment group with the optimal concentration of naringenin following acute ethanol exposure.

3.3 Naringenin Regulated Inflammation-Related Pathways and Critical Targets to Exert Neuroprotective Effects

To elucidate the mechanism by which naringenin exerts neuroprotective effects in acute ethanol-exposed mice, we identified genes associated with acute ethanol exposure using multiple online databases and obtained DEGs from microarray data (GSE20489) [62]. A total of 1783 overlapping genes were identified between DEGs and ethanol-related gene datasets (Figure S2A). GO and KEGG enrichment analyses were then performed to investigate biological functions and associated pathways. The results revealed that the inflammatory response and cytokine signaling in the immune system were the most significantly enriched biological processes and pathways (Figure S2B). GSEA further supported these findings, showing that acute inflammatory response-related gene sets were expressed at lower levels in healthy individuals and markedly upregulated following acute ethanol exposure (Figure S2C).

To further identify specific targets of naringenin involved in neuroinflammation, we retrieved naringenin-related and neuroinflammation-related targets from online databases. After merging and removing duplicates, 94 common genes shared among DEGs, disease-related genes, and potential naringenin targets were obtained via Venn analysis (Figure 3A). GO function enrichment analysis showed that these genes were primarily associated with the signal transduction, response to xenobiotic stimulus, and regulation of apoptotic process and cell migration, etc. (Figure 3B). Notably, these GO terms were consistent with the biological effects observed experimentally in this study, including restoration of the righting reflex, enhancement of neuronal activity, and reduced neuronal apoptosis.



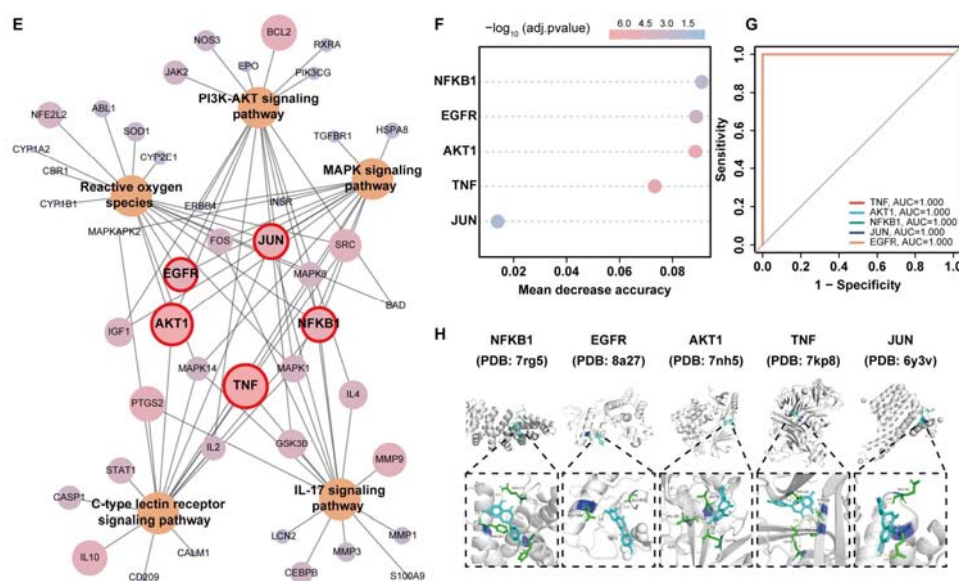


Figure 3. Naringenin regulated inflammation-related pathways and critical targets to exert neuroprotective effects. **A.** Venn diagram analysis of disease-drug-DEGs. **B.** GO analysis of cross-targets. The gene count (top X-axis and grey bar chart) indicates the number of genes contained in each GO term. **C.** KEGG pathway enrichment analysis of cross-targets. The count indicates the number of genes contained in each KEGG pathway. The gene ratio (X-axis) indicates the number of genes contained in a KEGG pathway as a proportion of all cross-targets. **D.** Venn diagram of genes involved in regulating multiple signaling pathways in top 5 KEGG pathways. **E.** PPI networks for KEGG-enriched genes. The nodes represent proteins corresponding to genes and edges indicate proteins interactions. The larger and red circles indicate higher centrality degree levels. The degree level refers to the number of direct connections a node has with other nodes, reflecting the ability of a protein to interact with other proteins. The color and size of nodes were determined based on the degree level of genes within the network, progressing from blue (0, low) to pink (68, high), with KEGG pathway nodes shown in orange. **F, G.** Random Forest analysis and ROC curve analysis of 5 critical targets. **H.** Molecular docking of 5 critical targets to naringenin: NFKB1, EGFR, AKT1, TNF, and JUN.

KEGG pathway enrichment analysis further revealed that these targets were involved in signaling pathways such as the PI3K-AKT signaling pathway, reactive oxygen species (ROS), IL-17 signaling pathway, C-type lectin receptor signaling pathway, and MAPK signaling pathway (Figure 3C). Among the top five enriched KEGG pathways, nine genes regulating multiple signaling pathways were identified through Venn analysis (Figure 3D). To explore the underlying molecular interactions, a PPI network was constructed using STRING, resulting in a network consisting of 93 nodes and 1062 edges (Figure S3A). To pinpoint hub genes within the network, we employed the cytoHubba plugin based on the degree level, identifying the top 10 nodes: TNF, AKT1, ACTB, JUN, NFKB1, PTGS2, EGFR, BCL2, IL10, and PPARG (Figure S3B). A subsequent Venn intersection identified NFKB1, EGFR, AKT1, TNF, and JUN as critical targets involved in multiple KEGG pathways (Figure S3C). A KEGG-gene interaction network was then generated to visualize pathway associations and rank genes by the degree level (Figure 3E).

To evaluate the importance and biological significance of these hub genes, we performed random forest analysis and ROC curve analysis. The results showed that NFKB1, EGFR, AKT1, TNF, and JUN contribute significantly to sample grouping, each with area under the curve (AUC) values greater than 0.9 (Figure 3F-G), indicating that these genes may serve as promising targets for the treatment of acute ethanol-induced neuroinflammation. Molecular docking, a computational method crucial in drug discovery, was employed to characterize the fundamental interactions between proteins and small molecule compounds at the atomic level [74]. All five protein-ligand docking scores were below -5 Kcal/mol (Table 2), indicating energetically

favorable binding. Lower binding energy corresponds to a more stable interaction between ligand and receptor, suggesting that naringenin is capable of forming stable binding conformations with these proteins (Figure 3H). These results indicated that NFKB1, EGFR, AKT1, TNF, and JUN represent potential binding targets of naringenin, and their regulation may underlie its neuroprotective effects.

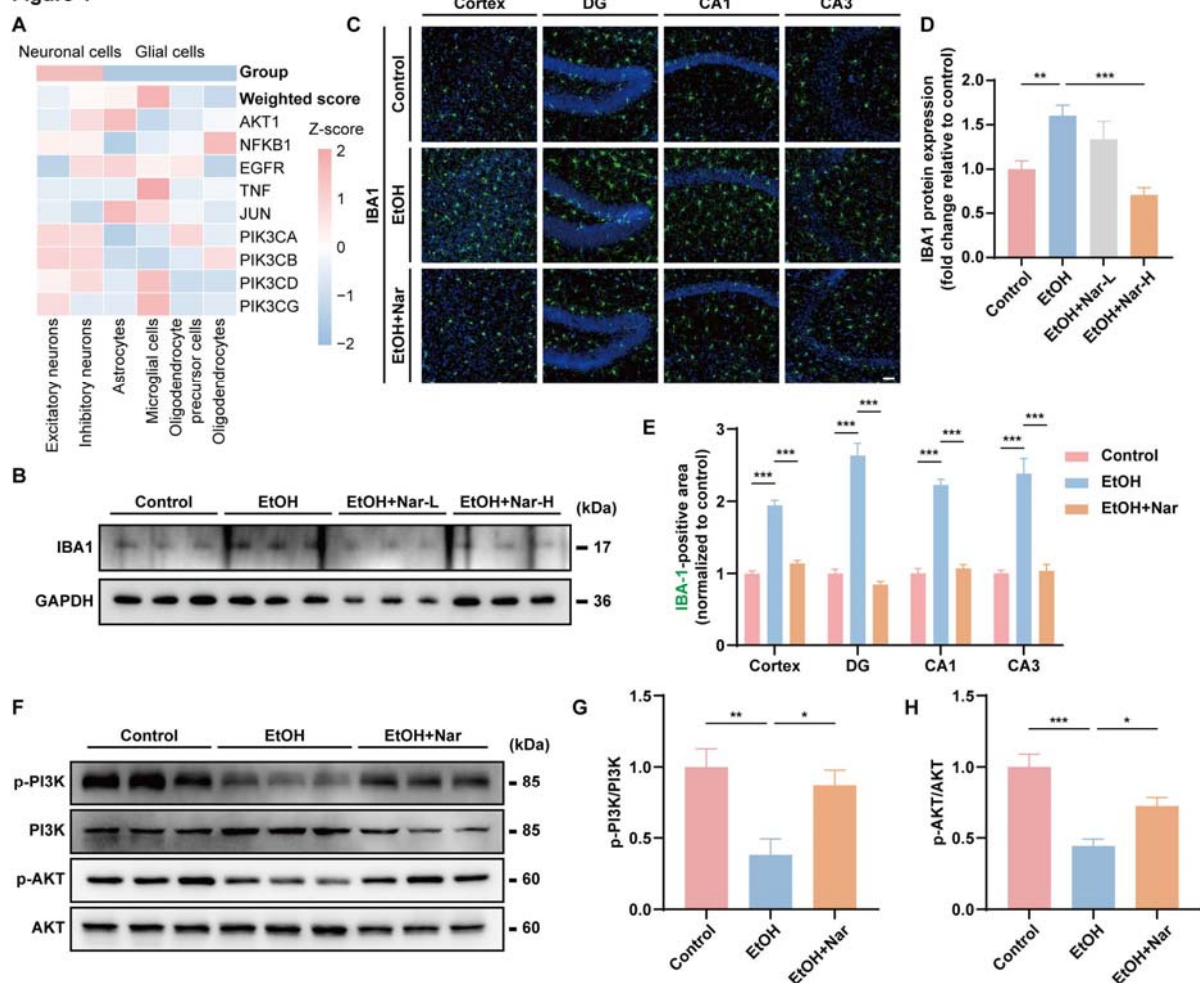
Table 2. Binding site and binding energy of molecular docking.

Gene Name	PDB ID	Hydrogen Bonds	Binding Energy (Kcal/mol)
NFKB1	7rg5	PHE207, HIS167, GLN171	-5.61
EGFR	8a27	GLU758	-5.45
AKT1	7nh5	GLN61, VAL185	-5.32
TNF	7kp8	PRO116	-7.00
JUN	6y3v	GLU31	-5.75

3.4 Naringenin Attenuated Microglial Activation by Activating the PI3K-AKT Pathway in Binge Ethanol Feeding Mice

To further investigate the cellular and molecular mechanisms underlying the neuroprotective effects of naringenin, we analyzed the protein expression levels of potential targets (NFKB1, EGFR, AKT1, TNF, JUN, and PI3K-AKT-related proteins) across different single-cell and single-nucleus clusters of brain tissue using the Human Protein Atlas database. The results indicated that these targets were predominantly enriched in microglial cells (Figure 4A and Figure S3D).

Figure 4



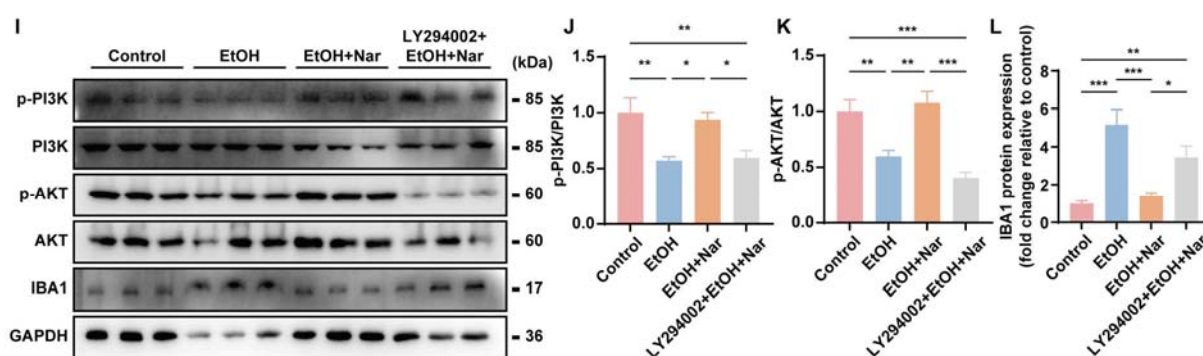


Figure 4. Naringenin attenuates microglial activation by activating the PI3K-AKT pathway in binge ethanol feeding mice. **A.** The analysis of protein expression levels of potential targets (NFKB1, EGFR, AKT1, TNF, JUN, and PI3K-AKT-related targets) in different single-cell type clusters of brain tissue. **B.** Western blot results of IBA1 protein expression in the hippocampus after ethanol exposure and naringenin treatment. **C.** Representative images of immunohistochemistry staining of IBA1 in the cortex and hippocampus of mice after ethanol exposure and naringenin treatment. Scale bar = 50 μ m. **D.** Statistical analysis of IBA1 protein expression relative to GAPDH loading control after ethanol exposure and naringenin treatment. **E.** Quantitative analyses of IBA1-positive area in the cortex and hippocampus of mice after ethanol exposure and naringenin treatment. **F.** Western blot results of p-PI3K, PI3K, p-AKT, and AKT protein expression in the hippocampus after ethanol exposure and naringenin treatment. **G, H.** Statistical analysis of p-PI3K/PI3K and p-AKT/AKT ratios after ethanol exposure and naringenin treatment. **I.** Western blot results of p-PI3K, PI3K, p-AKT, AKT, and IBA1 protein expression in the hippocampus after ethanol exposure, naringenin treatment, and LY294002 treatment. **J, K.** Statistical analysis of p-PI3K/PI3K and p-AKT/AKT ratios after ethanol exposure, naringenin treatment, and LY294002 treatment. **L.** Statistical analysis of IBA1 protein expression relative to GAPDH loading control after ethanol exposure, naringenin treatment, and LY294002 treatment. All experiments were repeated at least three times. Data are represented as means $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. EtOH, acute ethanol exposure group; EtOH+Nar-L, low-dose naringenin (50 mg/kg) treatment group following acute ethanol exposure; EtOH+Nar-H, high-dose naringenin (100 mg/kg) treatment group following acute ethanol exposure; EtOH+Nar, the treatment group with the optimal concentration of naringenin following acute ethanol exposure. LY294002+EtOH+Nar, LY294002 pre-treatment followed by ethanol exposure and high-dose naringenin (100 mg/kg) treatment group.

Although glial activation is essential for maintaining CNS homeostasis, ethanol exposure induces an aberrant neuroinflammatory response in which microglia become excessively activated and contribute to disease progression [75]. To determine whether naringenin modulates microglial activation following ethanol exposure, we examined microglial markers using western blot and IBA1 immunostaining. The IBA1 protein expression was markedly increased in the EtOH group, but naringenin treatment significantly reduced IBA1 levels in a dose-dependent manner (Figure 4B, D). Consistent with these findings, immunostaining revealed that naringenin significantly decreased the abundance of reactive microglia in the cortex and hippocampus, as evidenced by reduced IBA1-positive staining compared to the EtOH group (Figure 4C, E). These results suggested that naringenin effectively restored microglial activation to normal levels in both the cortex and hippocampus of ethanol-exposed mice (Figure 4B-E).

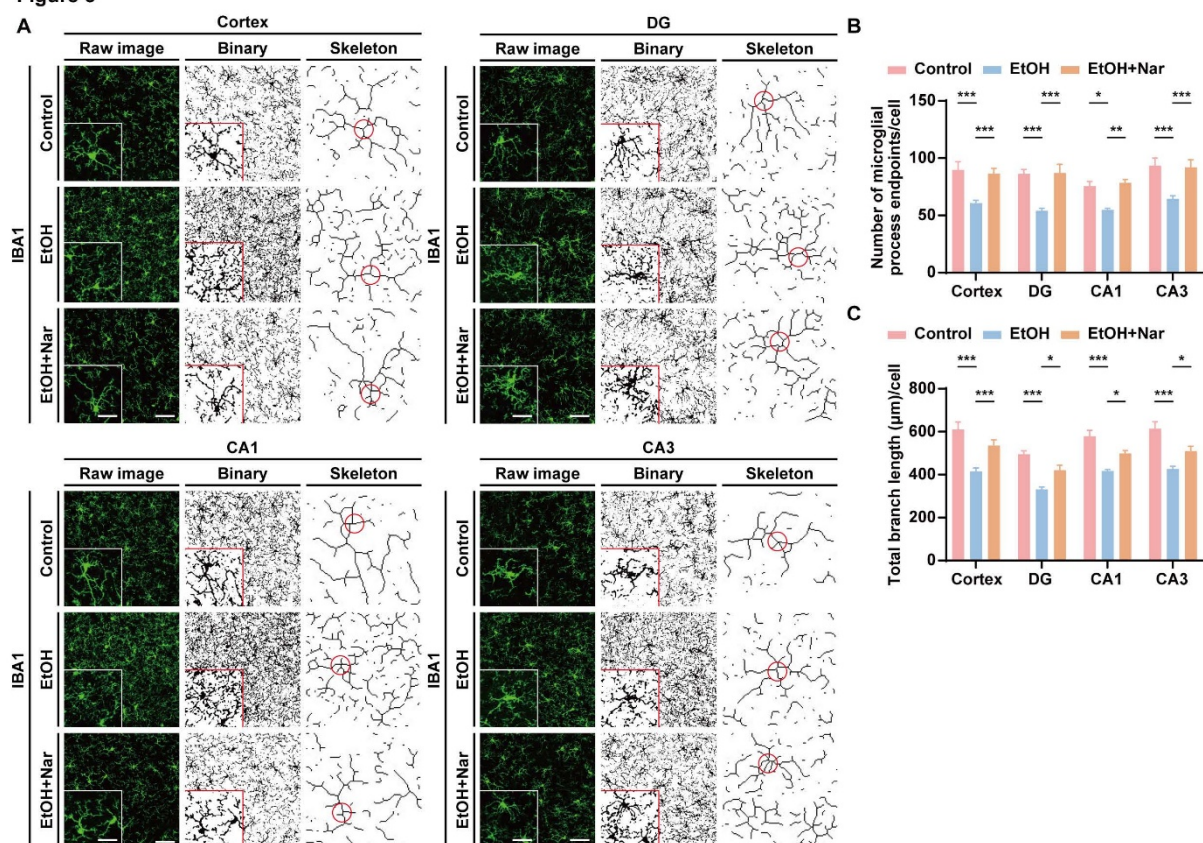
The PI3K-AKT pathway plays a critical role in regulating cell survival, proliferation, and stress responses, and is well known for its anti-inflammatory, anti-oxidative, and anti-apoptotic effects in neurons [45,76]. To determine whether this pathway contributes to the protective effects of naringenin, we examined the protein expression of PI3K-AKT-related signaling molecules in hippocampal using western blot analysis. The results showed that ethanol exposure significantly decreased the phosphorylation of PI3K (p-PI3K) and AKT (p-AKT) compared with the control group (Figure 4F-H). Naringenin treatment reversed these ethanol-induced reductions, restoring p-PI3K/PI3K and p-AKT/AKT ratios to near-normal levels. To determine whether PI3K-AKT signaling mediated the effects of naringenin, we administered the PI3K

inhibitor LY294002 prior to naringenin treatment to block this signaling cascade. LY294002 abolished the naringenin-induced increases in PI3K and AKT phosphorylation (Figure 4I-K). Furthermore, inhibition of the PI3K-AKT pathway elevated IBA1 protein expression (Figure 4I, L), indicating enhanced microglial activation. To further validate pathway specificity, we additionally used wortmannin, a second inhibitor of the PI3K-AKT pathway. Consistently, both LY294002 and wortmannin markedly attenuated the naringenin-induced reduction in IBA1- and GFAP-positive areas in the cortex and hippocampus (Figure S4), indicating a reversal of the naringenin-mediated suppression of reactive microglia and astrocytes. These finds suggested that naringenin inhibited microglia activation through activation of the PI3K-AKT signaling pathway.

3.5 Naringenin Restored Microglial Morphological Alterations in Binge Ethanol Feeding Mice

Morphological transformation of microglia is widely recognized as a hallmark of microglial activation and serves as a reliable indicator of CNS pathology [77]. To further investigate the effects of naringenin on microglial morphology, we analyzed microglial structural characteristics using confocal microscopy and the ImageJ skeleton analysis plugin (Figure 5A). Compared with the control group, ethanol exposure significantly reduced both the number of microglial process endpoints per cell and total branch length per cell in the cortex and hippocampus. In contrast, naringenin treatment significantly increased the number of microglial process endpoints per cell and the total branch length per cell in the cortex as well as in the DG, CA1, and CA3 regions of the hippocampus (Figure 5B-C), indicating that naringenin enhanced microglial branching complexity after ethanol exposure.

Figure 5



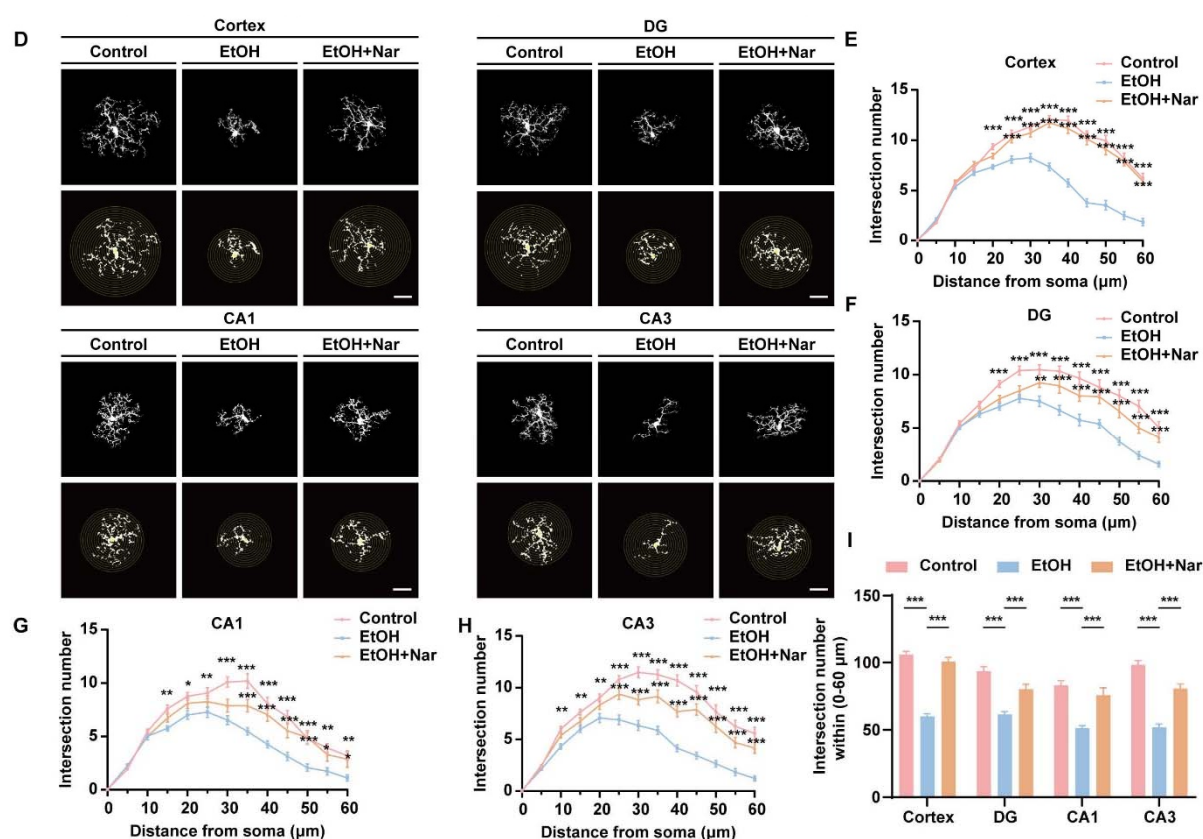


Figure 5. Naringenin protected microglial morphology in binge ethanol feeding mice. **A.** Representative images of IBA1-positive cells (raw image) used for binary and skeletonization analysis, along with skeletonized cells with a single origin soma. Scale bar = 12.5 μm (left) and 50 μm (right). **B, C.** Quantitative analyses of microglial morphology, including the number of microglial process endpoints per cell and total branch length per cell in the cortex and hippocampus of mice after ethanol exposure and naringenin treatment. **D.** Schematic representation of microglia Sholl analysis. **E, F, G, H.** Line chart of intersection number in microglia from the cortex, DG, CA1, and CA3 regions after ethanol exposure and naringenin treatment. **I.** Statistical analysis of intersection number within 0-60 μm in microglia from the cortex, DG, CA1, and CA3 regions after ethanol exposure and naringenin treatment. Scale bar = 25 μm. All experiments were repeated at least three times. Data are represented as means ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. EtOH, acute ethanol exposure group; EtOH+Nar, the treatment group with the optimal concentration of naringenin following acute ethanol exposure.

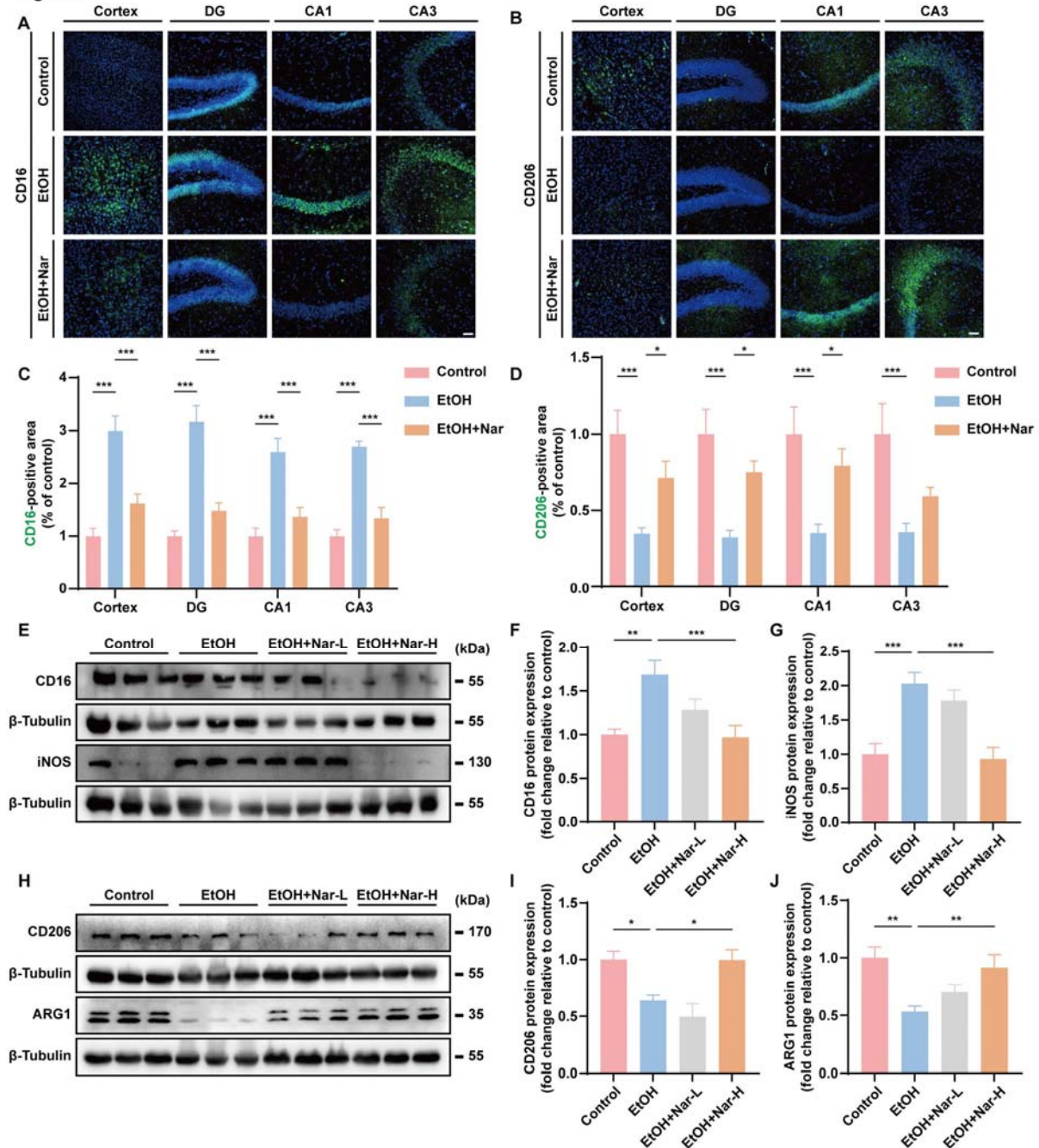
Furthermore, microglial Sholl analysis was performed to quantitatively assess morphological complexity (Figure 5D). Ethanol-exposed mice exhibited a marked reduction in the number of intersections with concentric circles, reflecting decreased branching complexity. Notably, naringenin treatment attenuated this reduction in the cortex, DG, CA1, and CA3 regions (Figure 5E-I). These results indicated that naringenin treatment not only reduced the abundance of reactive microglia but also restored their ramified morphology in both cortical and hippocampal regions, thereby mitigating microglia-mediated neuroinflammation induced by ethanol exposure.

3.6 Naringenin Regulated the Balance of Microglial M1/M2 Polarization by Activating the PI3K-AKT Pathway in Binge Ethanol Feeding Mice

Microglia-mediated inflammation and the accompanying M1/M2 polarization are key cellular events following brain injury [78]. Excessive alcohol consumption-induced neuroinflammation has been shown to be alleviated by correcting the imbalance in microglial polarization [79]. Our findings suggested that ethanol exposure-induced microglial activation and morphological disruption were modulated by naringenin

treatment. To further determine whether the neuroprotective effects of naringenin were mediated through regulating the balance of microglial M1/M2 phenotypes, we analyzed the expression of the M1 marker CD16 and the M2 marker CD206 in the cortex and hippocampus using immunofluorescence staining (Figure 6A-B). Ethanol exposure significantly increased CD16-positive areas and reduced CD206-positive areas, whereas naringenin treatment markedly decreased CD16 expression in both cortical and hippocampal regions and significantly increased CD206 expression in the cortex, DG, and CA1 subregions (Figure 6C-D).

Figure 6



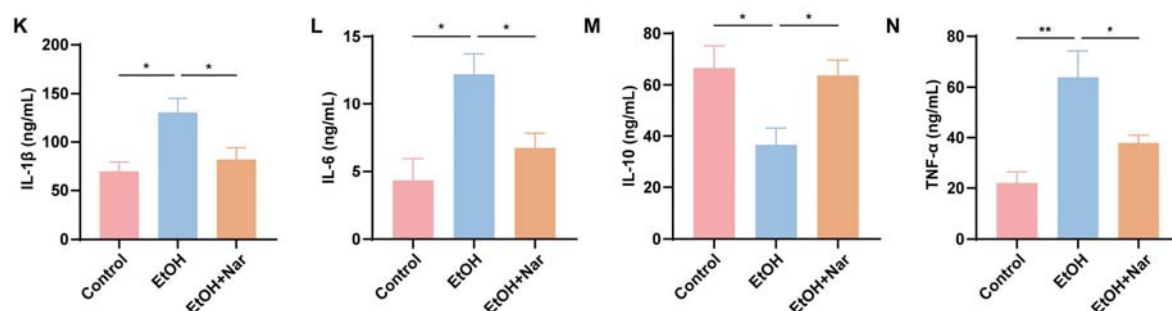


Figure 6. Naringenin promoted microglia polarization from pro-inflammatory M1 phenotype to anti-inflammatory M2 state. **A, B.** Representative images of immunohistochemistry staining of CD16 and CD206 in the cortex and hippocampus of mice after ethanol exposure and naringenin treatment. Scale bar = 50 μ m. **C, D.** Quantitative analyses of CD16 and CD206-positive areas in the cortex and hippocampus of mice after ethanol exposure and naringenin treatment. **E.** Western blot results of CD16 and iNOS protein expression in the hippocampus after ethanol exposure and naringenin treatment. **F, G.** Statistical analysis of CD16 and iNOS protein expression relative to β -tubulin loading control after ethanol exposure and naringenin treatment. **H.** Western blot results of CD206 and ARG1 protein expression in the hippocampus after ethanol exposure and naringenin treatment. **I, J.** Statistical analysis of CD206 and ARG1 protein expression relative to β -tubulin loading control after ethanol exposure and naringenin treatment. **K, L, M, N.** ELISA analysis of IL-1 β , IL-6, IL-10, and TNF- α levels in the brain following naringenin treatment in ethanol-exposed mice. All experiments were repeated at least three times. Data are represented as means $\pm$ SEM. * P < 0.05; ** P < 0.01; *** P < 0.001. EtOH, acute ethanol exposure group; EtOH+Nar-L, low-dose naringenin (50 mg/kg) treatment group following acute ethanol exposure; EtOH+Nar-H, high-dose naringenin (100 mg/kg) treatment group following acute ethanol exposure; EtOH+Nar, the treatment group with the optimal concentration of naringenin following acute ethanol exposure.

Western blot analysis further demonstrated that ethanol exposure elevated hippocampal protein expression of M1 markers (CD16 and iNOS) (Figure 6E-G) while decreasing the expression of M2 markers (CD206 and ARG1) (Figure 6H-J). Naringenin treatment significantly reversed these alterations, reducing CD16 and iNOS levels while increasing CD206 and ARG1 levels compared with the EtOH group (Figure 6E-J). Acute ethanol exposure is known to trigger the release of pro-inflammatory cytokines and chemokines, resulting in neuroinflammation and neuronal injury [80]. Therefore, we performed ELISA to examine whether naringenin modulated cytokine production by shifting microglial polarization from a pro-inflammatory M1 state toward an anti-inflammatory M2 phenotype. Ethanol exposure significantly elevated IL-1 β , IL-6, and TNF- α levels, all of which were reduced by naringenin treatment (Figure 6K, L, N). Conversely, the anti-inflammatory cytokine IL-10 decreased following ethanol exposure but increased after naringenin treatment (Figure 6M). These results indicated that naringenin alleviated brain inflammation induced by acute alcohol exposure.

However, prophylactic administration of LY294002 or wortmannin abolished the naringenin-mediated restoration of M1/M2 balance. Compared to the control and EtOH+Nar groups, the LY294002 group exhibited a significantly increased expression of CD16 and iNOS proteins (Figure 7A-C), accompanied by a marked reduction in CD206 and ARG1 expression (Figure 7D-F). Similarly, both LY294002 and wortmannin significantly reversed the naringenin-induced decrease in CD16-positive area and increase in CD206-positive area in the cortical and hippocampal (Figure S5A-B). This disruption of M1/M2 polarization was accompanied by elevated levels of pro-inflammatory cytokines (IL-1 β , IL-6, and TNF- α) and a reduced level of the anti-inflammatory cytokine IL-10 in both the LY294002 and wortmannin groups (Figure 7G-J). Altogether, these results demonstrated that naringenin treatment inhibited M1 microglial polarization and

promoted M2 microglial polarization in the cortex and hippocampus by activating the PI3K-AKT pathway, thereby mitigating neuroinflammation induced by acute ethanol exposure.

Figure 7

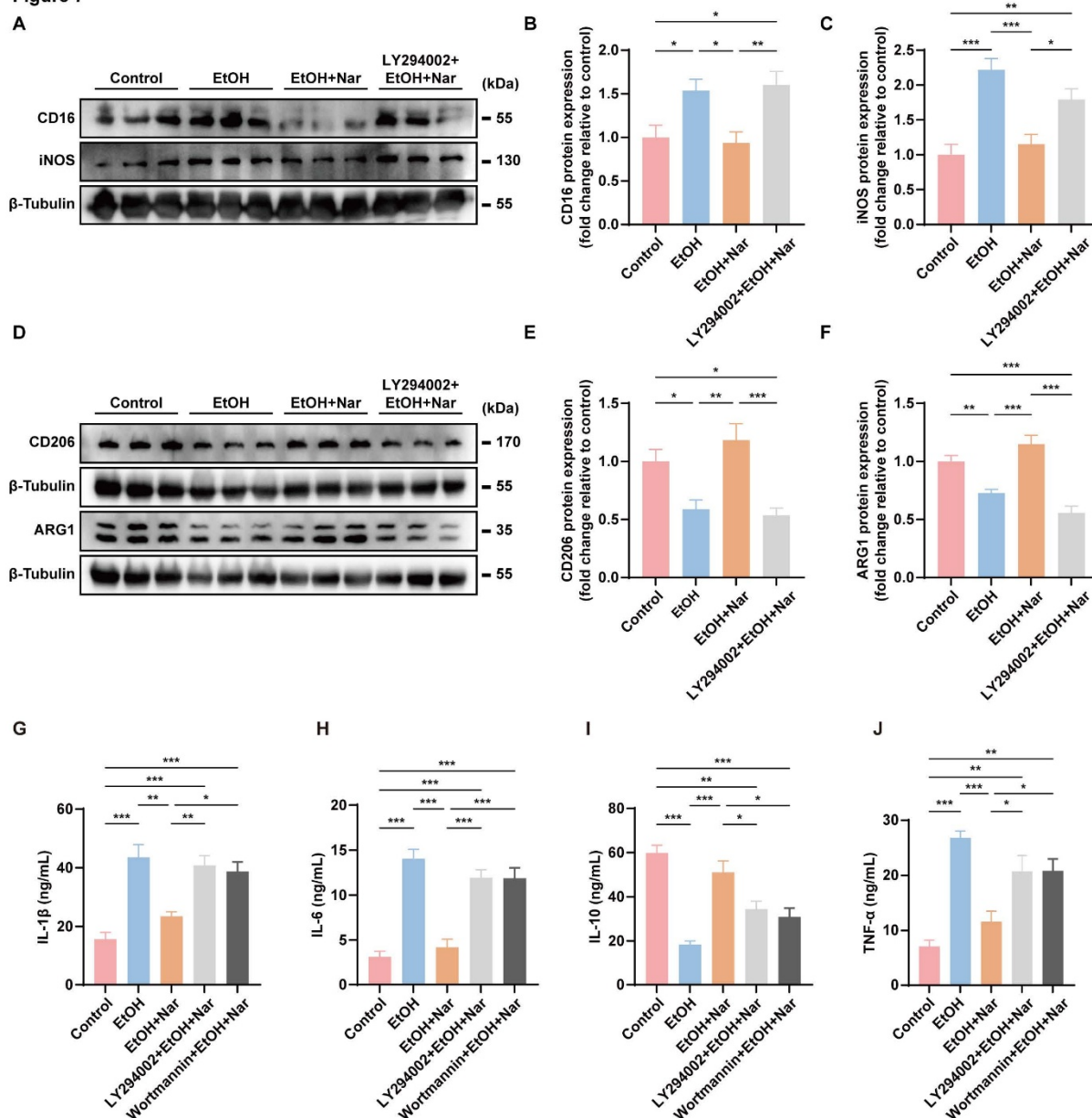


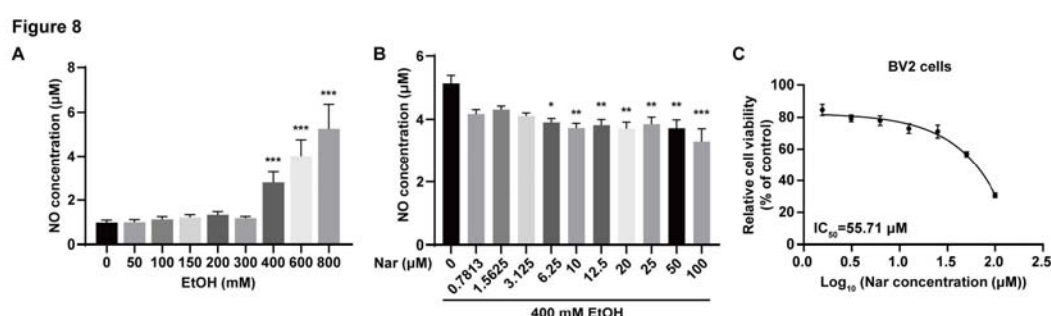
Figure 7. Naringenin regulated the balance of microglial M1/M2 polarization through the PI3K-AKT pathway. **A.** Western blot results of CD16 and iNOS protein expression in the hippocampus after ethanol exposure, naringenin treatment, and LY294002 treatment. **B, C.** Statistical analysis of CD16 and iNOS protein expression relative to β-tubulin loading control after ethanol exposure, naringenin treatment, and LY294002 treatment. **D.** Western blot results of CD206 and ARG1 protein expression in the hippocampus after ethanol exposure, naringenin treatment, and LY294002 treatment. **E, F.** Statistical analysis of CD206 and ARG1 protein expression relative to β-tubulin loading control after ethanol exposure, naringenin treatment, and LY294002 treatment. **G, H, I, J.** ELISA analysis of IL-1β, IL-6, IL-10, and TNF-α levels in the brain following naringenin, LY294002, and wortmannin treatment in ethanol-exposed mice. All experiments were repeated at least three times. Data are represented as means ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. EtOH, acute ethanol exposure group; EtOH+Nar, the treatment group with the optimal concentration of naringenin following acute ethanol exposure; LY294002+EtOH+Nar, LY294002 pre-treatment followed by ethanol exposure and high-dose naringenin (100 mg/kg) treatment group. Wortmannin+EtOH+Nar, wortmannin pre-treatment followed by ethanol exposure and high-dose naringenin (100 mg/kg) treatment group.

Since naringenin exerts neuroprotective effects by modulating PI3K-AKT signaling and suppressing neuroinflammation, we next examined whether the molecular inhibition observed was functionally reflected

in behavioral phenotypes. Gait analysis was performed to evaluate stride length and swing speed in mice [53]. Naringenin-treated mice exhibited significantly increased stride length and swing speed in both the forelimbs and hindlimbs compared with ethanol-exposed mice and those treated with PI3K inhibitors (Figure S5C-D). The rotarod test, a standard assessment of motor coordination, further demonstrated that ethanol exposure markedly reduced both the time and rotational speed that mice were able to remain on the rotating rod compared with the control group. In contrast, naringenin-treated mice showed prolonged latency to fall and sustained higher rotational speeds compared with the EtOH, LY294002, and wortmannin groups (Figure S5E-F). The LORR test revealed that pharmacological inhibition of the PI3K-AKT pathway abolished the naringenin-induced prolongation of LORR onset and the restoration of the righting reflex (Figure S5G-H). These findings indicated that activation of the PI3K-AKT pathway was essential for the behavioral and functional improvements mediated by naringenin following acute ethanol exposure.

3.7 Naringenin Restored Neuronal Activity by Reducing Microglia-mediated Pro-inflammatory Cytokine Release

In addition to their critical function as immune sentinels in the brain, microglia are essential for preserving neuronal homeostasis and maintaining overall brain health [81]. To further verify whether naringenin attenuated nerve injury through regulating microglia, we collected CM and E-CM from the supernatants of normally cultured and ethanol-exposed BV2 cells, respectively, and co-cultured them with HT22 cells for 24 h. We first quantified NO, an inflammatory mediator, in BV2 supernatants after 24 h of ethanol and naringenin treatment across a concentration gradient to establish optimal incubation conditions. The results showed that NO concentration was markedly elevated after exposure to 400 mM ethanol but declined with naringenin treatment starting at 6.25 μ M (Figure 8A-B). For consistency in subsequent experiments, ethanol and naringenin concentrations were standardized to 400 mM and 6.25 μ M, respectively. Importantly, cell viability assays in both BV2 and HT22 cells confirmed that this concentration of naringenin was non-toxic (Figure 8C and Figure S6A). The mRNA expression of key inflammatory genes (such as *Il-6*, *p50*, *Tnf- α* , and *Relb*, etc.) was upregulated by ethanol exposure but decreased after naringenin treatment (Figure 8D). iNOS and COX2, two pivotal enzymes involved in NO production and inflammatory signaling [82], were also markedly elevated in ethanol-treated BV2 cells. Naringenin reduced iNOS and COX2 protein expression by approximately 100% and 50%, respectively (Figure 8E-F). These findings indicated that the neuroprotective effects of naringenin were mediated by the inhibition of pro-inflammatory cytokine and chemokine expression in microglia.



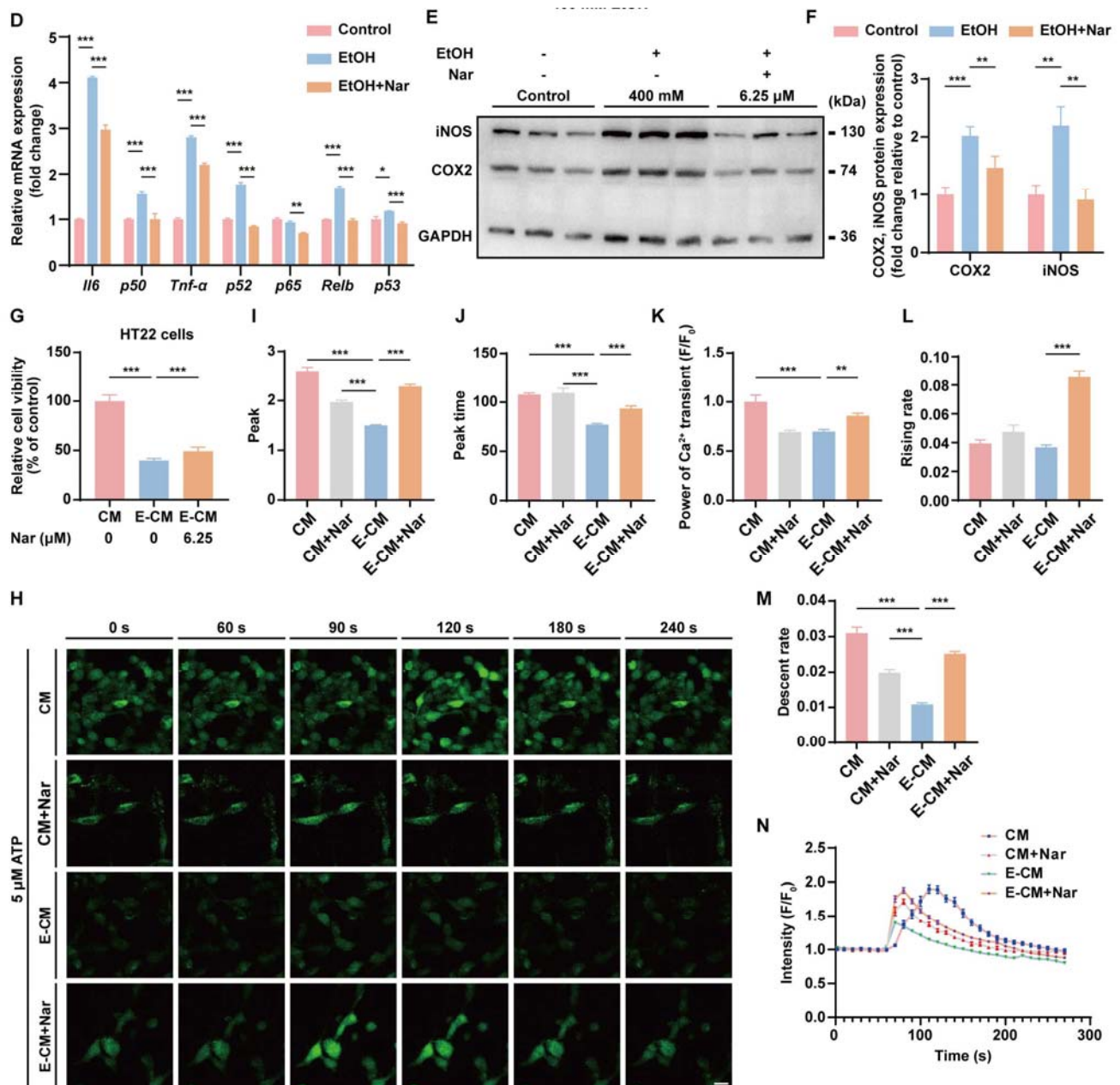


Figure 8. Naringenin restored neuronal activity by reducing microglia-mediated pro-inflammatory cytokine release. **A.** NO concentration assay in BV2 cells following treatment with different ethanol concentrations. **B.** NO concentration at different naringenin concentrations following 400 mM ethanol treatment in BV2 cells. **C.** Relative cell viability analysis of BV2 cells treated with a gradient of naringenin concentrations. **D.** Fold changes in mRNA expression of pro-inflammatory genes reversed by 6.25 μM naringenin treatment after 400 mM ethanol exposure, normalized to β-actin levels. **E.** Western blot results of the iNOS and COX2 protein expression in BV2 cells following 400 mM ethanol exposure and 6.25 μM naringenin treatment. **F.** Statistical analysis of iNOS and COX2 protein expression relative to GAPDH loading control after ethanol exposure and naringenin treatment. **G.** Relative cell viability analysis of HT22 cells co-cultured with CM and E-CM from BV2 cells, following 6.25 μM naringenin treatment. CM represents the supernatant of normal cultured BV2 cells, and E-CM represents the supernatant of ethanol-exposed BV2 cells. **H.** Representative images of calcium imaging in HT22 cells after CM, E-CM, and naringenin treatment, with 5 μM ATP added at 60 s. Scale bar = 20 μm. CM+Nar represents HT22 cells co-cultured with CM and treated with 6.25 μM naringenin. E-CM+Nar represents HT22 cells co-cultured with E-CM and treated with 6.25 μM naringenin. **I, J, K, L, M.** Statistical analysis of peak value, peak time, Ca^{2+} transient power, and calcium transients' rising and descent rates in HT22 cells following CM, E-CM, and naringenin treatment. **N.** Line chart of intensity values during calcium imaging in HT22 cells following CM, E-CM, and naringenin treatment. All experiments were repeated at least three times. Data are represented as means ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. EtOH, acute ethanol exposure group; EtOH+Nar, the treatment group with the optimal concentration of naringenin following acute ethanol exposure.

Co-culture experiments further demonstrated that HT22 cell viability was significantly reduced by E-CM but effectively rescued by naringenin treatment (Figure 8G). Calcium imaging is a widely used approach for monitoring intracellular Ca^{2+} dynamics and serves as a functional indicator of neuronal activity [83]. ATP is known to induce transient increases in intracellular calcium levels during Ca^{2+} imaging [84]. We therefore evaluated Ca^{2+} signaling in HT22 cell using Fluo-4 as a calcium indicator after co-culturing with E-CM for 24 h. To optimize E-CM preparation, we first investigated the impact of BV2 seeding density on HT22 (1.0×10^5 cells/mL) Ca^{2+} responses following ethanol exposure and naringenin treatment (Figure S7). BV2 density significantly affected the amplitude and kinetics of Ca^{2+} transients in HT22 cells exposed to E-CM (Figure S8A). Increasing BV2 density progressively reduced Ca^{2+} transient peak, power, and both rising and descent rates (Figure S8B-E), suggesting that excessive BV2 density reduces the bioactivity of the conditioned medium. This attenuation is likely due to increased release of pro-inflammatory cytokines and a concomitant reduction of neuroprotective factors at high BV2 densities.

Interestingly, the restorative effect of naringenin on Ca^{2+} signaling was also density dependent. At lower BV2 densities (approximately $1.0\text{--}1.25 \times 10^5$ cells/mL), naringenin treatment markedly enhanced the Ca^{2+} transient amplitude and recovery kinetics of HT22 cells, suggesting that E-CM derived under these conditions retains optimal responsiveness to naringenin. In contrast, at higher BV2 densities, the protective effect of naringenin was diminished, likely due to the overwhelming inflammatory milieu (Figure S8). Based on these findings, a BV2 density of 1.0×10^5 cells was selected for subsequent experiments to ensure physiological relevance and sensitivity to naringenin-mediated modulation. Accordingly, a BV2:HT22 ratio of 1:1 was used in subsequent calcium imaging experiments.

For calcium imaging, the first 60 s were recorded as baseline, followed by the addition of 5 μM ATP at 60 s and continued imaging for 4 min (Figure 8H). Ethanol-exposed cells exhibited a shorter time to peak but showed markedly reduced peak value, power, rising rate, and descent rate compared with the CM group (Figure 8I-N). To further assess the ability of cells to cope with calcium overload, we evaluated Ca^{2+} signaling in response to a 20 μM ATP challenge (Figure S6B). The results showed that ethanol exposure cells failed to mount a prompt response to the high-ATP stimulus, and their capacity to recover from abnormal elevations in intracellular calcium was significantly impaired compared with the CM, CM+Nar, and E-CM+Nar groups (Figure S6C-H). Calcium dynamics in the CM+Nar group were slightly lower than those in the CM group, indicating that naringenin did not adversely affect basal neuronal activity. In contrast, naringenin markedly restored Ca^{2+} dynamics in HT22 cells exposed to E-CM (Figure 8H-N, Figure S6B-H). These findings suggested that naringenin restores neuronal activity by suppressing microglia-derived pro-inflammatory cytokine and chemokine release.

3.8 Naringenin Pharmacokinetics Analysis and Its Therapeutic Relevance in Binge Ethanol Feeding Mice

To confirm that naringenin reached pharmacologically relevant concentrations in the CNS during the therapeutic window, we performed a pharmacokinetic analysis in serum and brain following intraperitoneal administration in binge ethanol feeding mice (Figure S9A). Naringenin (100 mg/kg) was rapidly absorbed,

with the aglycone achieving peak levels in both serum and brain (C_{max} ~653 vs. 222 ng/mL) and displaying similar elimination half-lives ($t_{1/2}$ ~2.2 h) (Table S2). These values are consistent with previous reports showing a ~2 h half-life in rats, dogs, and humans [59]. Interestingly, glucuronide and sulfate conjugates of naringenin displayed substantially higher exposure than the parent compound. Serum C_{max} values for glucuronides and sulfates were ~14081 and ~3825 ng/mL, respectively, while their brain C_{max} reached ~684 and ~476 ng/mL. Although serum AUC values were higher overall, the detectable brain concentrations confirm naringenin and its conjugates cross the blood-brain barrier. These findings are consistent with previous studies reporting that naringenin and its conjugated metabolites are capable of distributing into the rodent brain after administration [58,85].

Time-course analysis showed that dynamic changes in pro-inflammatory cytokines (IL-1 β , IL-6, and TNF- α) and the anti-inflammatory cytokine IL-10 in both serum and brain closely paralleled the exposure profiles of naringenin and its metabolites (Figure S9B-E). Spearman correlation analysis further revealed significant associations between compound levels and inflammatory markers in both serum and brain (Figure S9F), suggested that naringenin together with its sulfate and glucuronide metabolites may collectively mediate the observed systemic and central anti-inflammatory effects. These findings demonstrated that naringenin and its conjugated metabolites could enter systemic circulation and reach the brain at concentrations sufficient to exert anti-inflammatory and neuroprotective effects.

3.9 Naringenin Exhibited a More Pronounced Protective Effect Against Acute Ethanol-Induced Neuronal Damage Than Its Representative Conjugated Metabolite

To further investigate whether the major metabolites of naringenin retain neuroprotective activity, we performed additional *in vitro* experiments using its representative conjugated metabolites. Based on our pharmacokinetic analysis, glucuronide conjugates were identified as predominant circulating and brain-exposed metabolites of naringenin. Among them, naringenin-7-O-glucuronide, a representative glucuronidated metabolite, was selected for subsequent cell experiments. BV2 cells were treated with naringenin-7-O-glucuronide under the same experimental conditions used for naringenin to evaluate and compare their neuroprotective effects against acute ethanol-induced neuronal injury.

The results showed that treatment with 12.5 μ M naringenin-7-O-glucuronide significantly reduced NO production in BV2 cells (Figure S10A). NO analysis and cell viability assays confirmed that 12.5 μ M naringenin-7-O-glucuronide did not induce obvious inflammatory activation or cytotoxicity in BV2 cells (Figure S10B-C). Consistently, treatment of HT22 cells with a concentration gradient of naringenin-7-O-glucuronide showed low neurotoxicity, further supporting its cellular safety under the tested conditions (Figure S10I). mRNA expression analysis further showed that naringenin-7-O-glucuronide reduced the expression of key inflammatory genes after ethanol treatment, although the magnitude of this effect was weaker than that of naringenin (Figure S10D). Similarly, naringenin more effectively suppressed the ethanol-induced upregulation of iNOS and COX2 than naringenin-7-O-glucuronide (Figure S10E-G). In the co-culture system, the reduction in HT22 cell viability induced by E-CM was significantly attenuated by

both naringenin-7-O-glucuronide and free naringenin treatment (Figure S10H). However, analysis of neuronal function-, apoptosis-, and oxidative stress-related genes in HT22 cells showed that naringenin exerted a more pronounced neuroprotective effect than naringenin-7-O-glucuronide following E-CM treatment (Figure S10J). These findings suggested that glucuronidated metabolite of naringenin retains detectable anti-inflammatory and neuroprotective activity, but its protective effect is weaker than that of naringenin at the same optimal dose.

4. Discussion

Excessive alcohol consumption exerts detrimental effects on the brain, contributing to neurodegeneration, neuronal dysfunction, and a wide range of neuropathological outcomes [86]. Although the underlying mechanisms are not fully elucidated, a previous study had linked ethanol consumption to both systemic and organ-specific inflammatory responses [14]. Ethanol-induced neuroinflammation is now recognized as a key driver of alcohol-related brain injury and an important antecedent of neurodegeneration [10]. In the present study, we demonstrated that naringenin provided neuroprotection and alleviated nerve injury triggered by acute ethanol exposure. These protective effects were mediated through the PI3K-AKT pathway, which suppressed microglial activation and morphological alterations, maintained the balance of M1/M2 polarization, reduced the release of pro-inflammatory cytokines, and ultimately restored neuronal activity.

Ethanol exposure has been shown to produce injury in the nervous system across various experimental paradigms. Recent studies indicated that chronic alcohol consumption induced hippocampal neuronal damage associated with neuronal loss, oxidative stress, and inflammatory response [87,88]. Binge ethanol intoxication also shortened the axon initial segment and caused persistent alterations in proteins essential for neuronal morphology and synaptic function [66]. However, most existing research on ethanol-induced injury has focused on gastrointestinal effects or the long-term consequences of chronic alcohol exposure. Metadoxine, an ion pair of pyridoxine and pyrrolidone-carboxylate, is currently the primary pharmacological agent used clinically for acute alcohol intoxication, functioning mainly to accelerate the clearance of ethanol and acetaldehyde. However, there is limited evidence that metadoxine directly protects the brain from acute binge-induced neuroinflammation or microglial activation. In the present study, we found that even a single binge dose of ethanol acutely induced ataxia in mice, as demonstrated by the LORR test, and resulted in substantial nerve injury. Naringenin significantly promoted ethanol metabolism and provided protection against ethanol-induced intoxication and oxidative stress (Figure 1). The brain is particularly susceptible to oxidative damage due to its high metabolic rate and oxygen demand. Oxidative stress contributes to synaptic loss and neuronal degeneration and is a key pathological mechanism in numerous neurological disorders [89,90]. Therefore, maintaining antioxidant homeostasis in the brain is essential not only for mitigating ethanol-induced neurotoxicity but also for supporting a shift toward an anti-inflammatory response [91].

Furthermore, acute ethanol intoxication has been reported to induce widespread apoptotic neurodegeneration in the mouse brain [71]. These detrimental effects are associated with the endoplasmic

reticulum stress-mediated apoptotic pathway, including the upregulation of CHOP and activation of JNK [73]. Recent studies have indicated that ethanol exposure, either directly or indirectly through oxidative stress, activates JNK signaling [92]. Our previous studies have also indicated that inhibiting the expression of CHOP and p-JUN have neuroprotective effects [31,72]. In the present study, we further demonstrated that naringenin mitigated the loss of neuronal morphology and synaptic function induced by single-dose ethanol intoxication. Naringenin also reduced apoptosis and decreased the expression of cellular stress markers (CHOP and p-JUN) in the brains of binge ethanol feeding mice (Figure 2). These results demonstrated that naringenin exerts neuroprotective effects to alleviate acute ethanol exposure-induced nerve injury.

Network pharmacology is a powerful approach for identifying potential therapeutic targets and elucidating pharmacological mechanisms [93]. To investigate how naringenin alleviates ethanol-induced nerve injury, we performed network pharmacology analysis. Our findings revealed that genes associated with acute ethanol exposure were enriched in neuroinflammatory pathways, whereas the neuroprotective effects of naringenin were closely associated with ROS regulation and the PI3K-AKT signaling pathway. Acute binge ethanol exposure has been shown to elevate the expression of neuroinflammation-associated proteins in the brain [86]. Among the potential targets identified, NFKB1, EGFR, AKT1, TNF, and JUN play critical roles in the regulation of neuroinflammation (Figure 3). A defining feature of neuroinflammation is the activation of resident immune cells, particularly microglia, which respond robustly to excessive alcohol intake and serve as primary mediators of the brain's immune response to ethanol [45,94]. Pharmacological blockade or genetic silencing of NFKB1, EGFR, TNF, and JUN has been shown to ameliorate microglia-driven neuroinflammation and substantially reduce associated neurological impairment [95-98]. AKT is an important signaling molecule that regulates numerous cellular processes and plays an important role in inflammatory development [99]. PI3K activates AKT through phosphorylation, thereby modulating cell survival, growth, and angiogenesis in response to extracellular signals [100]. Previous studies have demonstrated that the PI3K-AKT pathway exerts anti-neuroinflammatory, anti-oxidative, and anti-apoptotic functions in neurons [45,76]. Consistently, Guo et al. reported that flavonoids alleviated ethanol-induced apoptosis via the PI3K-AKT pathway [101]. In the present study, the predicted targets were predominantly enriched in microglia, and naringenin treatment effectively reduced ethanol-induced microglial activation. Naringenin counteracted the ethanol-mediated inhibition of PI3K-AKT signaling. However, when the PI3K-AKT pathway was pharmacologically blocked using LY294002 or wortmannin, the regulatory effects of naringenin on microglial activation were significantly reversed (Figure 4 and S4). This finding is consistent with a previous study, which reported that naringenin ameliorated amyloid- β pathology and neuroinflammation in Alzheimer's disease by significantly reducing microglial activation in mice [102].

A previous mouse experiment showed that ethanol not only sensitized the production of pro-inflammatory cytokines but also altered morphological features [103]. Activated microglia typically adopts an amoeboid-like morphology, characterized by reduced process length and increased soma size [104]. In the present study, naringenin treatment effectively restored ethanol-induced microglial morphological

changes in the mouse brain (Figure 5). In addition to morphological remodeling, activated microglia are commonly categorized into two functional phenotypes depending on the molecular signals they receive: the pro-inflammatory M1 phenotype and the immunosuppressive M2 phenotype [91]. The M1 phenotype is associated with the expression of markers such as CD16 and the release of proinflammatory mediators, including iNOS and TNF- α [105,106]. In contrast, the M2 phenotype is characterized by the production of anti-inflammatory cytokines and neuroprotective chemokines, with IL-10, CD206, and ARG1 serving as representative biomarkers [107,108]. Correcting the imbalance in microglial polarization has been shown to ameliorate alcohol-induced neuroinflammation [79]. Consistent with these findings, we observed that naringenin restored the M1/M2 balance in the brains of mice subjected to acute binge ethanol feeding. Specifically, naringenin significantly reduced the expression of M1 phenotype markers (CD16 and iNOS) and increased the expression of M2 phenotype markers (CD206 and ARG1) (Figure 6). These results are supported by recent studies showing that naringenin attenuates microglia-mediated neuroinflammation by promoting M2 polarization in rodent models of Alzheimer's disease and chronic pain [105,107]. These results indicated that naringenin alleviated ethanol-induced neuroinflammation by restoring microglial branching complexity and rebalancing M1/M2 polarization.

Previous study has shown a negative correlation between the PI3K-AKT pathway and microglial activation and dysfunction [109]. Reduced PI3K-AKT signaling promotes a shift toward the pro-inflammatory M1 phenotype, whereas its activation is associated with enhanced the anti-inflammatory microglial M2 phenotype [110]. Upon activation, PI3K catalyzes the formation of PIP3, which recruits AKT to the plasma membrane, enabling its phosphorylation at Thr308 and Ser473. Activated AKT in turn regulates multiple downstream targets that determine microglial phenotype and inflammatory response [111]. A central downstream effector is GSK3 β , whose inhibition via AKT-mediated Ser9 phosphorylation suppresses NF- κ B-dependent transcription of IL-1 β , IL-6, iNOS, and TNF- α , while concurrently promoting M2-associated markers such as ARG1 and IL-10 [112]. AKT also engages in bidirectional crosstalk with mTOR complexes, which promote anti-inflammatory and tissue-repair programs through regulation of CREB, PPAR γ , and metabolic reprogramming toward oxidative phosphorylation [113,114]. Additionally, AKT phosphorylates FOXO transcription factors, preventing their nuclear translocation and inhibiting the transcription of pro-inflammatory and pro-apoptotic genes while supporting microglial survival and functional remodeling [115,116]. Consistent with these mechanistic insights, our findings demonstrated that pharmacological inhibition of the PI3K-AKT pathway using LY294002 or wortmannin reversed the naringenin-induced suppression of the pro-inflammatory M1 phenotype and the enhancement of the anti-inflammatory M2 phenotype. These effects were accompanied by corresponding changes in cytokine expression (Figure 7 and S5). These findings suggested that naringenin exerted its anti-neuroinflammatory and neuroprotective effects by activating the PI3K-AKT pathway to regulate microglia function.

Activated microglia-mediated excessive neuroinflammation is widely recognized as a major contributor to neuronal dysfunction and behavioral impairments across numerous neurodegenerative and toxin-induced

models [117,118]. Thus, the anti-inflammatory and microglia-modulating effects of naringenin observed in the present study likely underlie its capacity to improve behavioral outcomes. To determine whether these molecular and cellular effects translate into functional benefits, we assessed behavioral phenotypes in ethanol-exposed mice. Gait analysis, rotarod test, and LORR test revealed that naringenin significantly ameliorated ethanol-induced behavioral deficits, whereas pharmacological inhibition of the PI3K-AKT pathway using LY294002 or wortmannin abolished these improvements (Figure 1 and S5). These findings suggested that the activation of the PI3K-AKT signaling cascade and subsequent suppression of microglia-mediated neuroinflammation are critical for the protective effects of naringenin.

Additionally, the present study showed that naringenin effectively regulated the expression of pro-inflammatory mediators induced by ethanol exposure in microglia, including NO, iNOS, and COX-2 (Figure 8). The upregulation of pro-inflammatory cytokines and enzymes such as iNOS and COX-2 is a key driver of microglia-mediated nerve injury [119,120]. Consistent with this, Wu et al. demonstrated that naringenin inhibited neuroinflammation by reducing NO production and downregulating iNOS, COX-2, and other pro-inflammatory cytokines in microglia [121]. Beyond their role as immune sentinels, microglia are essential for maintaining neuronal homeostasis [81]. Numerous studies emphasize the bidirectional communication between microglia and neurons: microglia sense neuronal activity via neurotransmitter receptors [122], while microglia-derived mediators, including IL-10, IL-1 β , and TNF- α , modulate neuronal excitability and synaptic function [123]. Indeed, this bidirectional communication is altered in brain injury. An imbalance in microglial M1/M2 polarization impair these communication pathways and compromise neuronal regulatory mechanisms [124-126]. In the present study, naringenin significantly reduced neuronal death and restored neuronal activity following ethanol exposure in a co-culture of HT22 neuronal cells and BV2 microglia cells (Figure 8). These findings aligned with previous reports showing that ethanol exposure increased glutamate-induced excitotoxicity and oxidative stress, ultimately leading to irreversible neuronal damage [127]. The co-culture of neurons and microglia sensitizes neurons to ethanol-induced cell death, while alleviating GSK3 β -mediated neuroinflammation has been shown to reduce ethanol neurotoxicity [128]. Conversely, ethanol-induced neuronal injury can further activate microglia and amplify tissue damage, thereby impairing brain repair processes [129]. These findings suggested that naringenin alleviated ethanol-induced nerve injury by inhibiting pro-inflammatory cytokine expression in microglia.

Notably, our pharmacokinetic analysis showed that naringenin, along with its glucuronide and sulfate conjugates, reached measurable levels in both serum and brain. These findings suggested CNS exposure of both naringenin and its metabolites at pharmacologically relevant concentrations and further support their ability to cross the blood-brain barrier (Figure S9). The temporal patterns of cytokine expression closely paralleled drug exposure, supporting the conclusion that the observed neuroprotective effects occur at pharmacologically relevant concentrations. This is consistent with previous studies showing that naringenin undergoes extensive phase II metabolism *in vivo*, and that glucuronide and sulfate conjugates are the predominant circulating and tissue-exposed forms after naringenin administration [130]. To address the

biological relevance of naringenin metabolites, we further added *in vitro* validation experiments using naringenin-7-O-glucuronide as a representative major metabolite. Our results showed that this metabolite retained detectable anti-inflammatory and neuroprotective activity, although its effect was weaker than that of naringenin under the present experimental conditions (Figure S10). These findings suggested that the conjugated metabolites of naringenin retain biological activity. This result is also consistent with previous study reporting therapeutic activities of both naringenin and naringenin-7-O-glucuronide in Alzheimer's disease-related models [131]. At the same time, accumulating evidence indicates that flavonoid conjugates may not always represent the principal intracellular bioactive species, particularly under inflammatory conditions. Instead, glucuronidated metabolites are often regarded as relatively stable transport or reservoir forms that can be locally deconjugated by β -glucuronidase released from inflammatory cells, thereby regenerating the more cell-permeable aglycone at sites of injury or inflammation [132–134]. Therefore, these findings suggested that naringenin is the primary form underlying the neuroprotective effects in the present study, while its conjugated metabolites may contribute to the overall efficacy *in vivo*. These findings further strengthened the translational relevance of naringenin in mitigating acute ethanol-induced nerve injury.

To improve the translational relevance of our findings to food-derived intake, we further compared intragastric gavage and intraperitoneal injection administration *in vivo*. Our results showed that intraperitoneal injection administration produced a stronger neuroprotective effect than intragastric gavage administration, as reflected by the LORR test, the activities of alcohol-metabolizing enzymes, oxidative stress indices, and glial activation, although intragastric gavage administration still conferred detectable protection (Figure S11). This difference is pharmacokinetically explainable because naringenin has poor aqueous solubility, low oral bioavailability, and undergoes extensive intestinal and hepatic first-pass metabolism after oral administration. Previous studies have shown that the water solubility of naringenin is only about 46 ± 6 $\mu\text{g/mL}$ and its oral bioavailability is approximately 5.81%, which substantially limits systemic exposure to the free aglycone after oral delivery [135,136]. In addition, oral exposure is rapidly shifted toward glucuronide and sulfate conjugates, further reducing circulating levels of free naringenin [59]. The present study used intraperitoneal injection administration for mechanistic validation, because it reduced the confounding influence of gastrointestinal absorption and presystemic metabolism and enabled more reliable systemic exposure to evaluate the direct neuroprotective effect of naringenin. It is consistent with pharmacology literature indicating that intraperitoneal injection administration in rodent experimental studies is a justifiable route for proof-of-concept and pharmacodynamic investigations when the goal is to evaluate target engagement rather than the formulation properties or clinical pharmacokinetics of a compound [137]. Experimental studies have also shown that intraperitoneal injection administration of small-molecule agents generally results in faster and more complete absorption, as well as greater bioavailability, than oral administration, and systemic exposure following intraperitoneal injection delivery is often considered to be closer to that achieved by intravenous administration [138,139]. Importantly, the detectable protection observed after intragastric gavage administration still supports the relevance of naringenin to food science and human wellness under a

more physiologically relevant route of administration. In the present study, intraperitoneal injection administration should be interpreted primarily as a proof-of-concept strategy for evaluating efficacy and underlying mechanisms, whereas the additional intragastric gavage data extend the translational significance of our findings to oral intake. Given the limitations of oral absorption, future studies should further optimize oral delivery systems or food-compatible formulations to better bridge neuroprotective effects and practical dietary application.

Metadoxine is one of the few clinically approved drugs for the treatment of acute alcohol intoxication. A double-blind randomized trial in acutely intoxicated patients demonstrated that a single 900 mg intravenous dose significantly reduced the half-life of blood ethanol (from ~6.70 h to ~5.41 h) and accelerated the recovery of psychomotor and behavioral impairments [140]. Mechanistically, metadoxine primarily enhances ethanol and acetaldehyde clearance by increasing ALDH activity and stabilizing hepatic and cerebral energy metabolism through the preservation of ATP and glutathione levels [141,142]. However, despite its metabolic benefits, metadoxine has limited direct effects on microglial activation or CNS-specific pathways related to neuroinflammation and nerve injury. In contrast, naringenin has been reported to attenuate neuroinflammation and apoptosis in various models of neurotoxicity and Alzheimer's disease [143,144]. The present study extends this framework to an acute binge ethanol model, demonstrating that naringenin not only promotes ethanol metabolism (via ADH/ALDH) and reduces oxidative stress, but also modulates the PI3K-AKT-microglia axis to rebalance M1/M2 polarization and preserve neuronal integrity. By integrating the established metabolic benefits of metadoxine with the CNS-directed neuroprotective actions of naringenin, our findings suggested that naringenin represented a promising therapeutic candidate or potentially a dietary supplement for the prevention and treatment of acute alcohol-induced nerve injury.

Nonetheless, several limitations should be acknowledged. Several evidence indicate that microglial responses to alcohol are sexually dimorphic. A recent study in a stress-induced alcohol drinking model reported dynamic sex differences in microglial phenotypes at baseline and in response to both stress and chronic alcohol exposure [145]. Specifically, microglia in the hippocampus exhibited more pronounced sex differences and heightened reactivity to stress and alcohol compared with those in the amygdala, while chronic alcohol exposure suppressed microglial density in the amygdala and reduced expression of the lysosomal marker CD68 (a key indicator of microglial phagocytic activity) across multiple brain regions. Similar findings have been reported in other ethanol exposure paradigms, where female rodents display increased microglial priming and cytokine release relative to males [146]. In addition, estrogens regulate neuroinflammation by suppressing NF- κ B and NLRP3 activity while activating PI3K-AKT signaling in microglia and other CNS cells [147,148]. Because the present study utilized only male mice, the influence of sex hormones on the PI3K-AKT-microglia axis remains unresolved. Future investigations incorporating female cohorts and controlling for estrous-cycle stage will be essential to determine whether the neuroprotective effects of naringenin are conserved across sexes or exhibit sex-dependent variability.

5. Conclusion

In conclusion, our study demonstrated that naringenin is an effective neuroprotective agent capable of mitigating nerve injury caused by acute ethanol exposure. Naringenin promoted ethanol metabolism and alleviated ethanol-induced oxidative stress, neural damage, and apoptosis, with these protective effects primarily mediated through microglial regulation. Specifically, naringenin inhibited microglial activation, restored microglial morphological alterations, and maintained the M1/M2 polarization balance through activation of the PI3K-AKT pathway. These regulatory effects ultimately suppressed pro-inflammatory cytokine expression in microglia and preserved neuronal activity. Therefore, naringenin represents a promising drug candidate or potentially a dietary supplement for the prevention and treatment of acute alcohol-induced nerve injury.

Data availability

Data will be made available on request.

Competing interests

The authors declare that they have no competing interests.

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