

Armillaria mellea polysaccharides alleviate chronic restraint stress-induced depression-like behaviors in mice by suppressing neuroinflammation via modulation of the JNK/p38 MAPK signaling pathway

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Abstract

For centuries, *Armillaria mellea* (A. mellea) has been used in East Asian traditional medicine, with its polysaccharides (AMP) known for anti-apoptotic and antioxidant properties, though their antidepressant effects remain unexplored. This study investigated AMP's effects in a chronic restraint stress (CRS)-induced mouse model of depression, assessing behavioral, cognitive, and molecular mechanisms. Male C57BL/6 mice (6–8 weeks old) were divided into control, CRS, CRS + low/high-dose AMP, and CRS+fluoxetine groups. Subsequent assessments included behavioral tests, Morris water maze (MWM) analysis, and Western blotting for inflammatory cytokines, apoptosis, and synaptic plasticity markers, alongside histopathological evaluation. Results showed AMP alleviated depression-like behaviors and improved cognitive function in CRS mice, while reducing neuroinflammation, hippocampal synaptic damage, and apoptosis, likely via JNK/p38 MAPK pathway modulation. These findings demonstrate AMP's antidepressant potential through anti-inflammatory, neuroprotective, and synaptic plasticity-enhancing mechanisms, bridging traditional use with modern pharmacological evidence.

Introduction

Depression is a debilitating mental disorder characterized by persistent depressed mood and anhedonia, severely compromising both physical and mental health^[1, 2]. Current treatments primarily involve antidepressant medications (e.g., SSRIs, SNRIs), psychotherapy, and lifestyle modifications. These drugs exert therapeutic effects by modulating monoamine neurotransmitters (5-HT, NE, DA) in the synaptic cleft^[3, 4]. While clinically effective, they demonstrate significant interindividual variability in efficacy and are associated with adverse effects (nausea, insomnia, sexual dysfunction), as well as potential dependence and organ toxicity with long-term use^[5]. Alarming, over 75% of patients in low-income countries lack access to treatment^[5]. The pathogenesis of depression involves multifaceted mechanisms including HPA axis dysregulation, monoaminergic dysfunction, neuroinflammation, and impaired neuroplasticity^[6]. No single existing theory can fully explain its complex pathophysiology^[7]. There is therefore an urgent need to develop novel, safer antidepressants with multi-target mechanisms of action. The present study aims to address this gap by systematically investigating the multi-target antidepressant mechanisms of *Armillaria mellea* polysaccharide (AMP), a natural compound with documented yet underexplored therapeutic potential.

Mitogen-activated protein kinase (MAPK) signaling cascades are evolutionarily conserved pathways that regulate critical cellular processes including proliferation, differentiation, apoptosis, and stress responses^[8]. The MAPK family comprises three major subfamilies: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPK^[9]. Particularly, JNK and p38 MAPK pathways serve as pivotal mediators of stress response, inflammation, and apoptosis. Upon activation by upstream kinases, these pathways modulate the expression of pro-inflammatory cytokines and apoptotic signals. Under stress conditions, JNK activation induces phosphorylation of Bcl-2 family proteins, triggering mitochondrial cytochrome c release and subsequent apoptotic cascade^[10]. In

inflammation, pathogen-stimulated JNK activates transcription factor AP-1, upregulating TNF- α and IL-6 production^[11]. Similarly, p38 MAPK mediates LPS-induced IL-1 and IL-6 synthesis, amplifying inflammatory responses^[12]. Notably, chronic stress dysregulates hippocampal MAPK signaling, evidenced by elevated phospho-JNK/p38 levels, which promotes neuronal apoptosis and contributes to depression-like behaviors^[13–14]. Pharmacological inhibition of MAPK/ERK signaling demonstrates neuroprotective effects, attenuating hippocampal damage and depressive symptoms in animal models^[14]. These findings highlight MAPK pathways as potential therapeutic targets for stress-related disorders.

Traditional Chinese medicine has demonstrated significant potential for treating depression with favorable safety profiles, particularly through formulations containing *Armillaria mellea*^[15]. This basidiomycete fungus of the Tricholomataceae family serves as both a nutritious food source and a medicinal agent. Notably, it is the symbiotic partner of the antidepressant herb *Gastrodia elata*, and the two share neuroactive properties^[16–17]. The fungus produces various bioactive compounds, with AMP representing the primary active components responsible for its broad pharmacological effects. AMP exhibits notable neuroprotective activity through multiple mechanisms, including potent antioxidant effects via superoxide radical scavenging^[18], anti-apoptotic action through preservation of mitochondrial membrane potential and inhibition of caspase-3 activation^[19], and significant anti-inflammatory properties that suppress neuroinflammation and platelet activation^[20–22]. These compounds also show promise for managing cognitive disorders, demonstrating potential benefits in Alzheimer's disease models^[19]. Although preliminary studies suggest that *Armillaria mellea* may exert antidepressant effects through multiple pathways, a comprehensive, integrative investigation of its mechanisms, particularly at the molecular and systems levels, remains insufficient. This study therefore aims to systematically elucidate the multi-target antidepressant mechanisms of AMP.

Materials and Methods

2.1 Animals

Fifty male C57BL/6 mice (4–6 weeks old) were obtained from the Experimental Animal Centre of North China University of Science and Technology. The animals were housed under controlled conditions ($22 \pm 2^\circ\text{C}$, $50 \pm 5\%$ humidity) with a 12-h light/dark cycle and ad libitum access to food and water. Following acclimatization, mice were randomly assigned to five experimental groups ($n = 10$ per group): (1) Control group (normal housing); (2) CRS model group (chronic restraint stress); (3) CRS + L-AMP group (50 mg/kg/day); (4) CRS + H-AMP group (100 mg/kg/day); and (5) CRS + FLX group (fluoxetine treatment).

The CRS procedure involved daily restraint in 50 mL conical tubes for 6 hours over 21 consecutive days, while control mice remained undisturbed in their home cages. The complete experimental timeline is illustrated in Fig. 2A. All treatments (AMP or fluoxetine) were administered concurrently with the CRS

protocol. This standardized approach ensured consistent stress exposure across treatment groups while allowing evaluation of potential therapeutic interventions.

2.2 Medication

Armillaria mellea polysaccharide (AMP) (TX20230812, purity $\geq 70\%$, Shanxi, China) was prepared as a 10 mg/mL aqueous solution and administered via oral gavage at doses of 50 mg/kg/day (CRS + L-AMP group) or 100 mg/kg/day (CRS + H-AMP group) for 3 weeks during the CRS procedure. The doses of AMP (50 and 100 mg/kg) were selected based on preliminary dose-ranging experiments in our laboratory. The polysaccharide used in all experiments was from a single, well-characterized batch to ensure consistency. The chemical profile (monosaccharide composition, molecular weight distribution) was confirmed to be consistent across key replicates. Fluoxetine hydrochloride (IF0390, C₁₇H₁₈F₃NO·HCl, purity $\geq 98\%$, Solarbio, China) was dissolved in DMSO to prepare a 20 mg/mL stock solution. For administration, this stock was diluted in sterile saline to a final concentration suitable for intraperitoneal injection, resulting in a vehicle containing 1% DMSO. Fluoxetine was administered intraperitoneally at a dose of 10 mg/kg/day for 3 weeks, concurrent with CRS exposure. All treatments were administered consistently throughout the 21-day experimental period.

2.3 Analysis of the Physicochemical Properties of AMP

2.3.1 Molecular Weight Detection

The molecular weight distribution and homogeneity of AMP were characterized using high-performance gel permeation chromatography (HPGPC) on an Agilent chromatography system. For analysis, samples were precisely weighed and dissolved in mobile phase to achieve a final concentration of 1–3 mg/mL, followed by filtration through a 0.22 μm membrane. Molecular weight parameters including peak molecular weight (Mp), number-average molecular weight (Mn), weight-average molecular weight (Mw), and z-average molecular weight (Mz) were determined through viscosity analysis and retention time calibration using dedicated GPC software.

2.3.2 Monosaccharide Composition Analysis

The monosaccharide composition of AMP was analyzed using an Agilent 1200 HPLC system with ultraviolet detection. The analytical procedure consisted of two main steps: (1) Acid hydrolysis: Samples were treated with 2 mol/L trifluoroacetic acid (1 mL) under nitrogen atmosphere at 110°C for 5 h, followed by neutralization to pH 7.0; (2) 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization: The hydrolysate was reacted with 0.3 mol/L NaOH (0.2 mL) and 0.5 mol/L PMP methanol solution (0.4 mL) at 70°C for 60 min. After derivatization, the mixture was acidified with 0.3 mol/L HCl and subjected to triple chloroform extraction (1.5 mL $\times$ 3) to remove excess PMP reagent. The aqueous phase was filtered through a 0.45 μm membrane prior to HPLC analysis. Chromatographic separation was performed on a C18 column (4.6 mm $\times$ 250 mm, 5 μm) with UV detection at 250 nm.

2.3.3 Fourier transform-infrared (FT-IR) spectrometry

The infrared spectra were recorded using a Fourier transform infrared (FT-IR) spectrometer (Thermo Fisher Scientific, USA). The powdered sample was mixed with KBr at an appropriate ratio, finely ground, and pressed into a transparent pellet for analysis. Prior to measurement, the sample was thoroughly dried to eliminate moisture interference.

2.4 Chronic restraint stress model

Prior to chronic restraint stress (CRS) induction, all mice underwent a 7 day acclimatization period. Following adaptation, CRS group mice were subjected to daily restraint for 6 hours (09:00–15:00) over 21 consecutive days. Restraint was achieved using adjustable cylindrical restraints fabricated from 50 mL centrifuge tubes. Multiple ventilation holes were drilled along the tube walls to ensure adequate airflow while restricting movement.

2.5 Behavioural experiments

2.5.1 Sucrose preference test (SPT)

Following a 7 day acclimatization period, anhedonia was assessed using the sucrose preference test (SPT) at baseline (pre-CRS) and after 21 days of chronic restraint stress. During the 2 day habituation phase, mice were first exposed to two identical bottles of 1% (w/v) sucrose solution (Day 1), followed by one sucrose bottle and one water bottle (Day 2), with positions randomized to prevent side bias. After 24 hour food/water deprivation to enhance drinking motivation, mice were simultaneously presented with one sucrose and one water bottle for a 2 hour test session, with intake measured gravimetrically.

Sucrose preference was calculated as: $[\text{sucrose intake (g)} / (\text{sucrose} + \text{water intake (g)})] \times 100\%$.

2.5.2 Forced swimming test (FST)

Mice were individually placed in a transparent cylinder (20×25 cm) containing 20 cm of water ($24 \pm 2^\circ\text{C}$) for 6 min under normal lighting. The first 2 min served as acclimation; immobility (passive floating with minimal movement) was recorded during the final 4 min. Two parameters were measured: immobility duration and latency to first immobility. Water depth prevented tail/hindpaw contact with the bottom.

2.5.3 Tail suspension test (TST)

Mice were individually suspended by the tail tip using adhesive tape (50 cm above the surface) for 6 min under standardized conditions. To account for initial stress responses, the first 2 min served as acclimation, with only the final 4 min analyzed. Immobility time (defined as complete absence of limb/body movements except those required for respiration) was quantified. The apparatus included a white background to enhance contrast for automated scoring.

2.5.4 Open field experiments (OFT)

The OFT assessed anxiety-like behavior and locomotor activity in mice during a 10 min session. An automated tracking system (Activity Monitor, MED-Associates, USA) recorded total ambulatory distance (cm) and time spent mobile. Between trials, the apparatus was thoroughly cleaned with 95% ethanol to

eliminate olfactory cues. Testing occurred under standardized lighting conditions in a 40×40×30 cm arena, with center zone entries serving as an additional anxiety index.

2.5.5 Morris Water Maze (MWM)

The Morris water maze test was conducted to evaluate spatial learning and memory in mice. The apparatus consisted of a circular pool (120 cm diameter, 50 cm height) filled with opaque water (22–25°C) maintained at a consistent depth of 30 cm. Four distinct visual cues were positioned around the pool walls, corresponding to the four quadrants (NE, SE, SW, NW). A transparent escape platform (10×10 cm) was submerged 1 cm below the water surface in the target quadrant.

The experiment included four phases: (1) Acquisition phase (5 days, 4 trials/day) where mice learned platform location; (2) Probe test (day 6) assessing memory retention with the platform removed; (3) Reversal training (2 days) with the platform moved to the opposite quadrant; and (4) Final probe test (day 9). Each trial lasted 60 seconds or until the mouse found the platform. Mice were given 15 seconds on the platform after each successful trial. Between trials, a 30 minute inter-trial interval was maintained.

Key parameters recorded included escape latency, path length, swimming speed, time spent in target quadrant, and platform crossings. The pool was thoroughly cleaned and water changed daily to eliminate odor cues. All sessions were conducted under consistent lighting (50 lux) between 9:00–15:00. Behavioral data were automatically tracked and analyzed using the Smart 3.0 video tracking system (Panlab, Spain), with experimenters blinded to treatment groups.

2.6 Hematoxylin/eosin Staining (HE) and Nissl Staining

The brains of each mouse were then extracted and post-fixed by submersion in 4% paraformaldehyde at 4°C for at least 24 hours. The tissue was subsequently dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin. Sections of 4 µm thickness were cut using a microtome. Sections were stained with hematoxylin and eosin (HE) or toluidine blue (Servicebio, Wuhan, China) to evaluate histopathological alterations. Following staining, sections were dehydrated, cleared in xylene, and coverslipped with neutral mounting medium. Images were acquired using a Leica microscope (Wetzlar, Germany).

2.7 Western blotting

Hippocampal tissue samples were homogenized in RIPA lysis buffer (Beyotime, China) supplemented with protease inhibitors. Protein concentrations were determined using a BCA assay kit (Servicebio, China) according to the manufacturer's protocol. Equal amounts of protein (30 µg per lane) were separated by 10% SDS-PAGE and transferred onto 0.22 µm PVDF membranes (Millipore, USA). Membranes were blocked with 5% non-fat milk in TBST for 1 h at room temperature, then incubated overnight at 4°C with the following primary antibodies: TNF-α (1:2000), IL-6 (1:2000), IL-10 (1:1000), Caspase-3 (1:5000), Bax (1:5000), Bcl-2 (1:2000), NMDAR2A (1:500), BDNF (1:2000), PSD95 (1:2000) (all from HUABIO, China except β-actin, 1:100000, ABclonal, USA). After TBST washes, membranes were incubated with HRP-conjugated secondary antibodies (1:5000) for 90 min at room temperature. Protein

bands were visualized using ECL substrate and quantified using a chemiluminescence imaging system (CLINX-6300, China). β -actin served as the loading control for normalization.

3 Statistical analyses

Statistical analysis was performed using GraphPad Prism 8. Data were tested for normality and homoscedasticity with Shapiro-Wilk test and Levene's test. Data are expressed as mean $\pm$ S.E.M. A two-way repeated measures ANOVA was used with group and day as the factors to measure the performance of mice in MWM escape latencies. Other data, obtained from the sucrose preference test, the body weight measurement, the FST, the TST, the OFT, the SET/RET stage, and Western blotting, were assessed by a one-way ANOVA for multiple comparisons with stress or AMP. Group and daily differences were analysed post hoc using Bonferroni test. The significance level was considered as $p < 0.05$.

Results

4.1 Physicochemical Properties of AMP

Figure 1A shows the molecular weight distribution. HPSEC analysis revealed that AMP has a moderately broad molecular weight distribution ($M_w = 36,947$ g/mol; $M_p = 20,616$ g/mol; $M_n = 6,809$ g/mol), characterized by a major peak at 11–16 min, indicating a moderately heterogeneous population of medium-to-high molecular weight polysaccharides. The symmetrical peak shape and RI detector response confirm sample homogeneity and polysaccharide nature. Figure 1B presents monosaccharide composition. GC-MS analysis identified AMP as a heteropolysaccharide predominantly composed of glucose (Glc, $\sim 85\%$), with minor constituents including mannose (Man, $\sim 8\%$), arabinose (Ara, $\sim 4\%$), and xylose (Xyl, $\sim 3\%$). Trace monosaccharides eluting at 20–30 min accounted for $< 1\%$ of total composition. Figure 1C exhibits FT-IR spectral analysis. The infrared spectrum displayed characteristic polysaccharide signatures: 3425 cm^{-1} (O-H stretching), 1635 cm^{-1} (bound water $\delta\text{H-O-H}$), 1374 cm^{-1} (C-H bending), $928/851\text{ cm}^{-1}$ (pyranose ring vibrations). The absence of 1740 cm^{-1} (C = O) and 1250 cm^{-1} (S = O) peaks confirmed the neutral polysaccharide nature.

Comprehensive characterization confirms AMP as a neutral heteropolysaccharide with 1,4-linked glucan backbone and α -configuration (characteristic 850 cm^{-1} band), exhibiting moderate polydispersity and predominant glucose composition.

4.2 Effects of AMP on the sucrose test and body weight in stressed mice

The sucrose preference test revealed significant treatment effects (one-way ANOVA: $F_{(4, 45)} = 4.087$, $p < 0.01$, Fig. 2B-C). While no differences were observed in the first week, CRS exposure for 3 weeks significantly reduced sucrose preference compared to controls ($p < 0.05$), indicating successful

depression model establishment. Both low- and high-dose AMP (CRS + L-AMP and CRS + H-AMP) and fluoxetine (CRS + FLX) treatments reversed this effect ($p < 0.05$ and $p < 0.01$, respectively).

Body weight measurements showed similar treatment effects ($F_{(4, 45)} = 11.68, p < 0.001$). CRS significantly impaired weight gain ($p < 0.001$ vs control), demonstrating the physiological impact of chronic stress. The complete dataset is presented in Fig. 2B-C.

4.3 Effects of AMP on the force swim test (FST) and tail suspension test (TST) in stressed mice

The effects of CRS and AMP treatment on depressive-like behavior are presented in Fig. 2D-E. Statistical analysis revealed significant differences in immobility durations among the five groups in both the FST ($F_{(4, 45)} = 16.89, p < 0.001$) and TST ($F_{(4, 45)} = 10.65, p < 0.001$). Notably, AMP treatment effectively reversed the behavioral despair state in stressed mice, as demonstrated by significantly reduced immobility times in both tests. In the FST, all treatment groups showed marked improvements compared to the CRS group (CRS + L-AMP: $p < 0.001$; CRS + H-AMP: $p < 0.001$; CRS + FLX: $p < 0.001$). Similarly, in the TST, significant reductions were observed in the CRS + L-AMP ($p < 0.01$), CRS + H-AMP ($p < 0.001$), and CRS + FLX ($p < 0.001$) groups. These findings collectively demonstrate that AMP administration significantly alleviates stress-induced behavioral despair in mice.

4.4 Effects of AMP on the open field test (OFT) in stressed mice

To exclude the possibility that reduced immobility time reflected treatment-induced hyperactivity rather than true antidepressant effects, we assessed locomotor activity using OFT. As shown in Fig. 2F-G, one-way ANOVA revealed significant differences among groups in both ambulatory time ($F_{(4, 45)} = 3.977, p < 0.01$) and distance traveled ($F_{(4, 45)} = 8.678, p < 0.001$). CRS-exposed mice showed significantly reduced central area dwell time ($p < 0.01$ vs control) and decreased total ambulatory distance ($p < 0.001$ vs control), indicating stress-induced behavioral inhibition. Importantly, all treatment groups (CRS + L-AMP, CRS + H-AMP, and CRS + FLX) demonstrated significant recovery of central area exploration time ($p < 0.05, p < 0.05$, and $p < 0.01$, respectively) and ambulatory distance ($p < 0.01, p < 0.001$, and $p < 0.001$, respectively) compared to the CRS group. These results confirm that the antidepressant-like effects observed in behavioral despair tests were not attributable to general locomotor stimulation.

4.5 Effects of AMP on Morris water maze (MWM) in stressed mice

The Morris water maze test (Fig. 3A-F) revealed that chronic restraint stress (CRS) significantly impaired spatial learning and memory in male mice, as evidenced by prolonged escape latencies during training (Fig. 3A; $p < 0.01$ day 2; $p < 0.001$ days 3–5 vs controls). Both low and high-dose AMP treatment (L-AMP and H-AMP) effectively reversed these deficits (Fig. 3A), with H-AMP showing particularly robust effects comparable to fluoxetine (FLX). During spatial probe tests (Fig. 3C-D), CRS mice exhibited reduced

platform crossings ($F_{(4, 45)} = 3.53, p < 0.05$) and target quadrant time ($F_{(4, 45)} = 4.77, p < 0.001$), impairments that were significantly ameliorated by AMP treatment ($p < 0.05, p < 0.01$).

Reversal training (Fig. 3E-F) demonstrated AMP's ability to enhance cognitive flexibility. While CRS mice struggled to learn the new platform location (Fig. 3E), all treated groups showed improved performance by day 2 (L-AMP: $p < 0.05$; H-AMP: $p < 0.01$; FLX: $p < 0.001$ vs CRS). Retention testing (Fig. 3G-H) further confirmed AMP's protective effects, with H-AMP significantly improving both platform crossings and target quadrant time ($p < 0.05$). Importantly, no group differences in swimming speed were observed (Fig. 3B, F; $p > 0.05$), confirming that cognitive improvements were not due to motor effects.

These findings demonstrate that AMP treatment, particularly at higher doses, can effectively mitigate CRS-induced spatial learning and memory deficits across multiple cognitive domains.

4.6 Effects of AMP on HE and NISSL staining in stressed mice.

Our histological examination of hippocampal tissue revealed that chronic restraint stress (CRS) induced significant neuronal damage in both the dentate gyrus (DG) and CA3 regions, while AMP treatment demonstrated notable neuroprotective effects (Figs. 4–5). In control mice, the DG region exhibited normal hippocampal architecture with densely packed, well-organized neurons and thick pyramidal cell layers (Fig. 4). However, CRS exposure led to marked neurodegeneration characterized by pyknotic nuclei and neuronal injury, despite preserved cell bodies.

Nissl staining of the CA3 region (Fig. 5) further confirmed these pathological changes, showing dispersed pyknotic neurons with light staining and disorganized Nissl body distribution. Importantly, both AMP and fluoxetine treatments effectively attenuated these stress-induced alterations, restoring neuronal density and organization in DG while improving Nissl body distribution and staining intensity in CA3. These morphological improvements in key hippocampal subregions correlate with our behavioral findings and suggest that AMP treatment preserved neuronal structural integrity and maintained the abundance of Nissl bodies, which are indicative of active protein synthesis capacity.

4.7 Effects of AMP on inflammatory factors in the hippocampus in mice with CRS induced depression

In addition to causing notable variations in the levels of protein expression, CRS stimulation dramatically raised the levels of proinflammatory cytokines such as TNF- α and IL-6 and lowered those of the anti-inflammatory cytokine IL-10 (TNF- α : $F_{(4, 10)} = 6.543, p < 0.01$; IL-6: $F_{(4, 10)} = 5.880, p < 0.05$; IL-10: $F_{(4, 10)} = 4.552, p < 0.05$). As shown in Fig. 6A-D, The expression levels of TNF- α and IL-6 were significantly increase in CRS group mice ($p < 0.01, p < 0.01$). However, the expression levels of IL-10 were significantly reduced ($p < 0.05$). Compared with the CRS group, CRS + H-AMP group ($p < 0.05, p < 0.05, p < 0.05$), and CRS + FLX group ($p < 0.01, p < 0.05, p < 0.05$) significantly reduced the levels of the IL-6 and TNF- α inflammatory factors and the expression levels of IL-10 was significantly increased. Whereas, the CRS +

L-AMP group ($p < 0.05$) only reduced IL-6 levels, there were no significant differences for the remaining factors. The findings imply that AMP can increase the synthesis of anti-inflammatory factors while suppressing the creation of pro-inflammatory factors in the brains of mice given CRS.

4.8 Effects of AMP on apoptosis-related proteins in stressed mice

To further elucidate the molecular mechanisms underlying AMP's neuroprotective effects, we investigated its impact on apoptosis-related pathways in the hippocampus (Fig. 6E-H). Chronic restraint stress (CRS) significantly altered the expression of key apoptotic regulators, with marked increases in pro-apoptotic proteins caspase-3 ($F_{(4, 10)} = 3.669, p < 0.05$) and Bax ($F_{(4, 10)} = 10.14, p < 0.01$), coupled with decreased expression of anti-apoptotic Bcl-2 ($F_{(4, 10)} = 7.226, p < 0.01$). Specifically, CRS-exposed mice exhibited significantly elevated caspase-3 ($p < 0.05$) and Bax levels ($p < 0.001$), along with reduced Bcl-2 expression ($p < 0.01$) compared to controls.

Notably, AMP treatment dose-dependently reversed these apoptotic changes. H-AMP significantly decreased both caspase-3 ($p < 0.05$) and Bax expression ($p < 0.01$), while L-AMP specifically reduced Bax levels ($p < 0.05$). The positive control FLX group showed comparable effects to H-AMP (caspase-3: $p < 0.05$; Bax: $p < 0.05$). Importantly, all treatment groups (L-AMP: $p < 0.05$; H-AMP: $p < 0.05$; FLX: $p < 0.01$) effectively restored Bcl-2 expression, rebalancing the Bcl-2/Bax ratio toward anti-apoptotic conditions. These findings demonstrate that AMP exerts antidepressant effects at least partially through modulating hippocampal apoptosis pathways, with high-dose AMP showing efficacy similar to fluoxetine in normalizing the expression of critical apoptotic regulators.

4.9 Effects of AMP on the expressions of synaptic plasticity-related proteins in stressed mice

To elucidate the neuroprotective mechanisms of AMP, we evaluated the expression levels of key synaptic-associated proteins. CRS significantly altered the expression of synaptic plasticity-related proteins, with statistically significant differences observed for NR2A ($F_{(4, 10)} = 7.578, p < 0.01$), PSD95 ($F_{(4, 10)} = 8.234, p < 0.01$), and BDNF ($F_{(4, 10)} = 4.173, p < 0.01$). As illustrated in Fig. 7A-D, CRS exposure markedly reduced protein expression levels compared to control conditions (NR2A: $p < 0.01$; PSD95: $p < 0.01$; BDNF: $p < 0.05$).

Notably, treatment with both high-dose AMP (CRS + H-AMP) and fluoxetine (CRS + FLX) significantly reversed these CRS-induced deficits, restoring expression levels of NR2A ($p < 0.05$ for both), PSD95 ($p < 0.01$ for both), and BDNF ($p < 0.05$ for both). The low-dose AMP group (CRS + L-AMP) showed selective improvement, with significant elevation in PSD95 expression ($p < 0.01$) but no significant effects on other proteins. These data indicate that AMP exerts neuroprotective effects, at least in part, through modulation of synaptic plasticity markers, with dose-dependent effects observed for specific synaptic proteins.

4.10 Effects of AMP on the expressions of JNK and p38 MAPK pathways in stressed mice.

Western blot analysis was performed to assess the activation of JNK and p38 MAPK signaling pathways in the hippocampal tissues of each experimental group. The phosphorylation levels of JNK (p-JNK) and p38 (p-p38) were normalized to their respective total protein levels (JNK and p38). CRS exposure significantly upregulated the phosphorylation of both JNK and p38, indicating pathway activation (JNK: $F_{(4, 10)} = 12.04, p < 0.001$; p38: $F_{(4, 10)} = 5.918, p < 0.05$). As illustrated in Fig. 7E-F, CRS-treated mice exhibited elevated p-JNK ($p < 0.001$) and p-p38 ($p < 0.01$) levels compared to controls, confirming stress-induced activation of the JNK/p38 MAPK cascade.

Notably, treatment with AMP as well as FLX effectively suppressed this activation. Both AMP doses significantly reduced p-JNK ($p < 0.05$) and p-p38 ($p < 0.05$), while FLX exerted a more pronounced inhibition on p38 phosphorylation ($p < 0.001$). These results suggest that AMP exerts its antidepressant-like effects, at least in part, by attenuating CRS-induced hyperactivity of the JNK/p38 MAPK signaling pathways in the hippocampus.

Discussion

This study employed the chronic restraint stress (CRS) model to evaluate the therapeutic potential of AMP^[23]. Treatment with AMP effectively reversed CRS-induced behavioral deficits. Specifically, AMP significantly reduced immobility time in the FST and TST, indicating attenuated behavioral despair. Furthermore, in the OFT, AMP treatment reversed anxiety-like behaviors by increasing exploration of the central zone.

To evaluate AMP's neuroprotective potential on cognitive function, we employed the Morris water maze (MWM) test, a gold-standard assessment of hippocampal-dependent spatial learning and memory^[24]. CRS mice displayed significant deficits in escape latency and platform quadrant preference, consistent with previous reports of stress-induced cognitive impairment. Remarkably, AMP treatment restored spatial memory performance to near-normal levels, corroborating earlier findings that AMP enhances neurogenesis in the hippocampal dentate gyrus and improves cognitive function in D-galactose-induced memory impairment models^[25]. These results collectively demonstrate AMP's multifaceted therapeutic potential in addressing the behavioral, emotional, and cognitive dimensions of depression.

Growing evidence suggests that neuroinflammation plays a pivotal role in depression pathogenesis, characterized by elevated pro-inflammatory cytokines (TNF- α , IL-6) and reduced anti-inflammatory IL-10^[26–28]. Our study demonstrated that CRS induced hippocampal neuroinflammation, consistent with clinical findings in depressed patients^[27]. Importantly, AMP treatment significantly attenuated these inflammatory changes, mirroring previous reports of *Armillaria mellea*'s anti-depressant effects via anti-inflammatory mechanisms^[20]. These cytokine alterations may disrupt synaptic function and

neuroplasticity^[29], suggesting AMP's therapeutic potential may involve modulating neuroinflammatory pathways.

The neuroinflammatory hypothesis of depression suggests that chronic inflammation disrupts neuronal function through multiple pathways. Our study demonstrates that CRS significantly decreased expression of synaptic proteins (PSD95, NR2A, BDNF) while increasing pro-apoptotic markers (Bax, caspase-3) and reducing anti-apoptotic Bcl-2. These molecular alterations correlated with structural neuronal damage in hippocampal DG and CA3 regions, as shown by HE and Nissl staining. AMP treatment effectively counteracted these pathological changes by restoring synaptic protein levels, normalizing apoptotic markers, and preserving neuronal morphology. These neuroprotective effects were comparable to fluoxetine but likely mediated through distinct anti-inflammatory mechanisms^[20]. AMP's ability to target multiple pathological pathways suggests its potential as a novel treatment for inflammation-associated depression, particularly for cases resistant to current therapies. Further research should explore AMP's precise molecular mechanisms and clinical potential.

The MAPK signaling pathway plays a central role in depression pathogenesis, with chronic stress activating JNK and p38 MAPK subpathways to induce hippocampal dysfunction, neuroinflammation, and apoptosis^[11, 30, 31]. Substantial evidence shows these pathways are hyperactivated in depressed patients, correlating with elevated inflammatory markers and neuronal damage^[32]. Our study found that CRS significantly increased hippocampal levels of phosphorylated JNK and p38 MAPK in mice, confirming pathway activation during depressive states. Importantly, both AMP and fluoxetine treatments effectively normalized these phosphorylation levels, suggesting their antidepressant effects may involve MAPK pathway modulation. These findings align with clinical evidence demonstrating that p38 MAPK inhibitors can alleviate depressive symptoms by reducing neuroinflammation^[33], highlighting the pathway's therapeutic potential.

The JNK pathway appears particularly responsive to stress-induced apoptosis, while p38 MAPK primarily mediates inflammatory responses^[10, 32]. Our results corroborate previous reports that antidepressant treatments can reverse stress-induced MAPK activation^[30, 31], possibly by restoring neuroplasticity. The differential effects of AMP and fluoxetine on these pathways warrant further investigation, particularly regarding their anti-inflammatory versus neuroprotective properties. These findings strengthen the evidence linking MAPK signaling to depression pathophysiology and treatment response, suggesting pathway components may serve as biomarkers or novel therapeutic targets.

Mechanistically, AMP's efficacy converges on the suppression of neuroinflammation. By normalizing CRS-elevated cytokines (TNF- α , IL-6) and inhibiting the activation of JNK/p38 MAPK signaling, AMP interrupts a critical pathogenic cascade. This prevents the ensuing synaptic deficits (e.g., reduced PSD95, BDNF) and neuronal apoptosis, ultimately restoring hippocampal integrity and explaining its combined antidepressant and neuroprotective effects.

The AMP preparation used in this study is a heterogeneous polysaccharide mixture with a broad molecular weight distribution. Given that large polysaccharides are unlikely to cross the blood-brain barrier in substantial quantities, we propose that its observed central effects are indirectly mediated. This structural heterogeneity may, in fact, be bioactively advantageous, as the diverse molecular sizes could enable multi-target engagement within the intestinal lumen, potentially modulating gut-immune signaling to exert systemic and neuroprotective anti-inflammatory effects^[25, 34].

Conclusion

Our results demonstrate that AMP alleviates CRS-induced depression by reducing hippocampal inflammation, inhibiting apoptosis, and enhancing synaptic plasticity, potentially via JNK/p38 MAPK pathways. These findings highlight AMP's neuroprotective effects and provide a molecular basis for its antidepressant potential, offering new insights for depression treatment strategies.

Declarations

Ethical statement

All experiments were conducted in accordance with the Regulations of the Ministry of Health of the People's Republic of China on Animal Management and approved by the Animal Research Ethics Committee of North China University of Science and Technology (20230062).

Competing interests

All authors disclosed no relevant relationships.

Author Contribution

X.H. contributed to methodology, software, investigation, formal analysis, and writing of the original draft. S.F. contributed to methodology, software, investigation, and formal analysis. Q.L., J.X., and S.Z. contributed to formal analysis and data curation. H.W. contributed to conceptualization, resources, and supervision. X.S. contributed to conceptualization, resources, funding acquisition, supervision, and review and editing of the manuscript. All authors reviewed and approved the final manuscript.

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Data availability

All data generated or analysed during this study are included in this published article. Not applicable.

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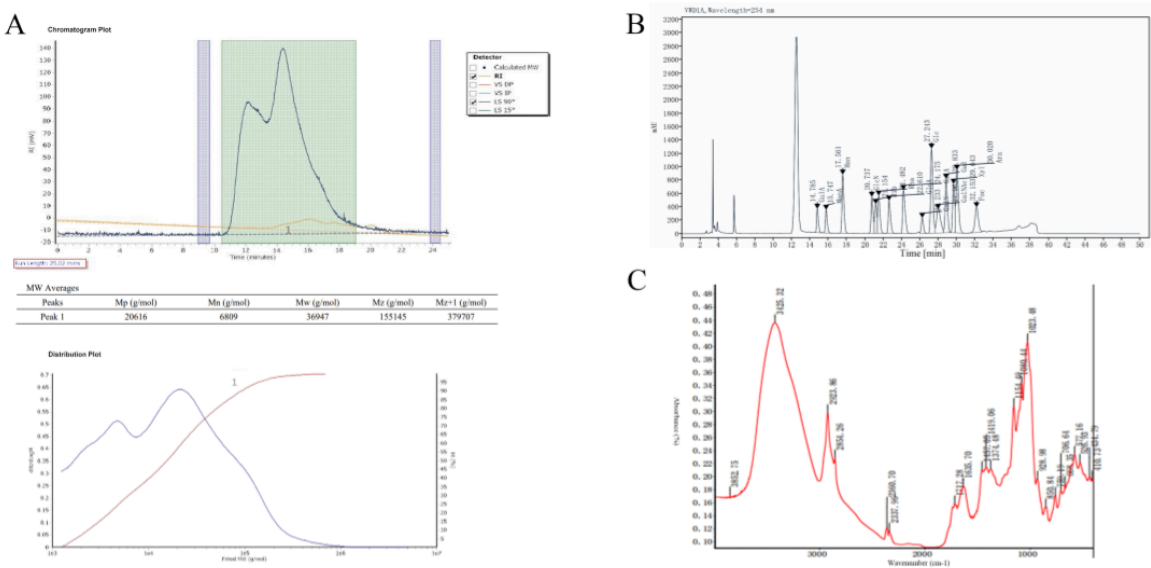
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Figures



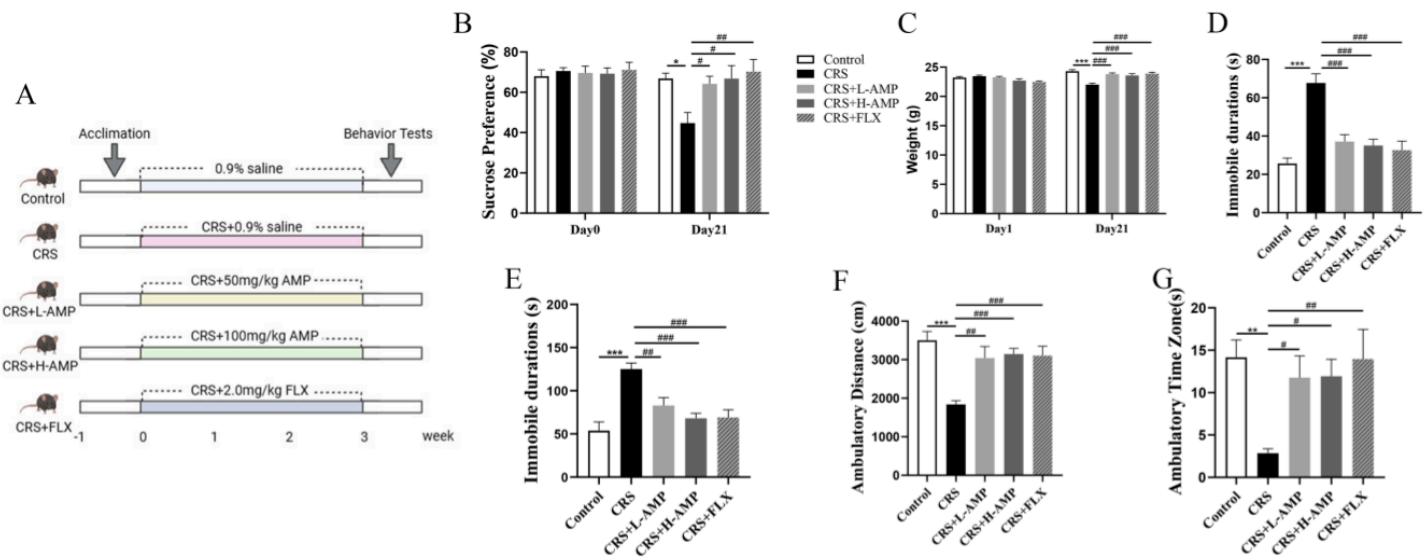


Figure 2

The experimental schedule *in vivo* (A) and effects of AMP administration on depressive-like behavior, anxiety-like behavior in stressed mice. (B) The percentage of sucrose consumption in the SPT. (C) Body weight on mice. (D-E) Immobility time was determined in the FST and TST. (F) Ambulatory distance in the OFT. (G) Ambulatory time zone in the OFT. Data are presented as mean $\pm$ SEM (n = 10). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, v.s. the Control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, v.s. the CRS group; n.s., not significant.

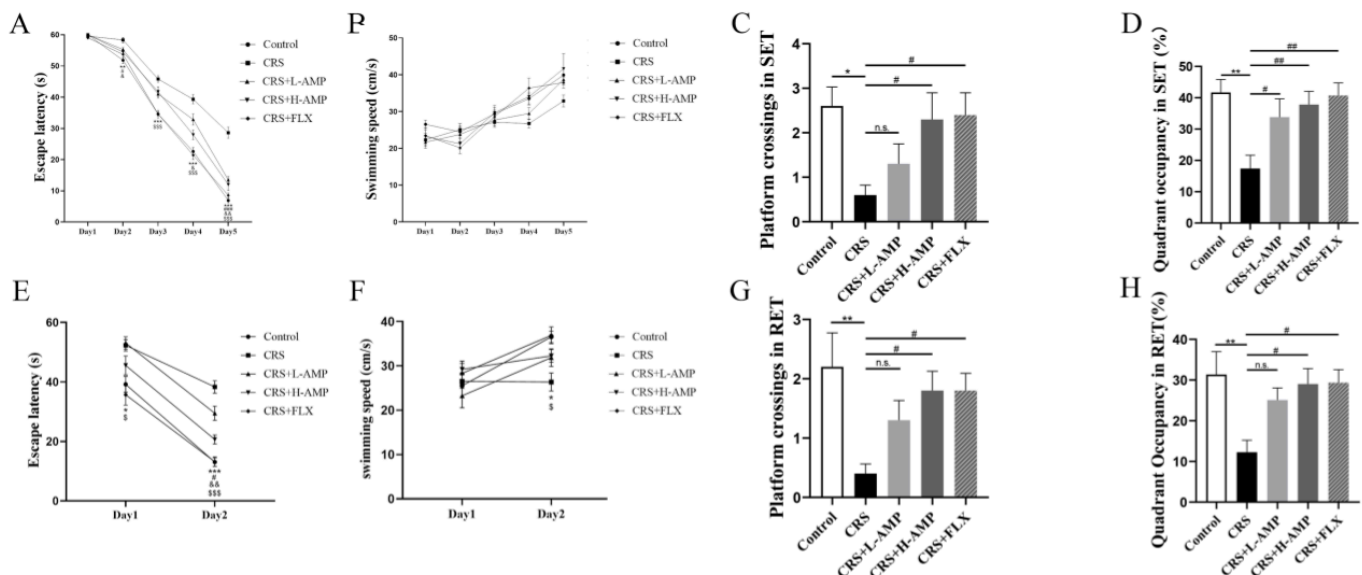


Figure 3

Effects of AMP administration on cognitive impairment in stressed mice. (A, E) Mean escape latency was determined for each day in the IT and RT stages among five groups. (B, F) Mean swimming speed in the IT and RT stages. (C) Number of platform area crossings in the SET stage. (D) Quadrant occupancy

in the SET stage. (G) Number of platform area crossings in the RET stage. (H) Quadrant occupancy in the RET stage. Data are presented as mean $\pm$ SEM (n = 10). * p < 0.05, ** p < 0.01, *** p < 0.001, v.s. the Control group; # p < 0.05, ## p < 0.01, ### p < 0.001, v.s. the CRS group; & p < 0.05, && p < 0.01, &&& p < 0.001, v.s. the CRS group; \$ p < 0.05, \$\$ p < 0.01, \$\$\$ p < 0.001, v.s. the CRS group; n.s., not significant.

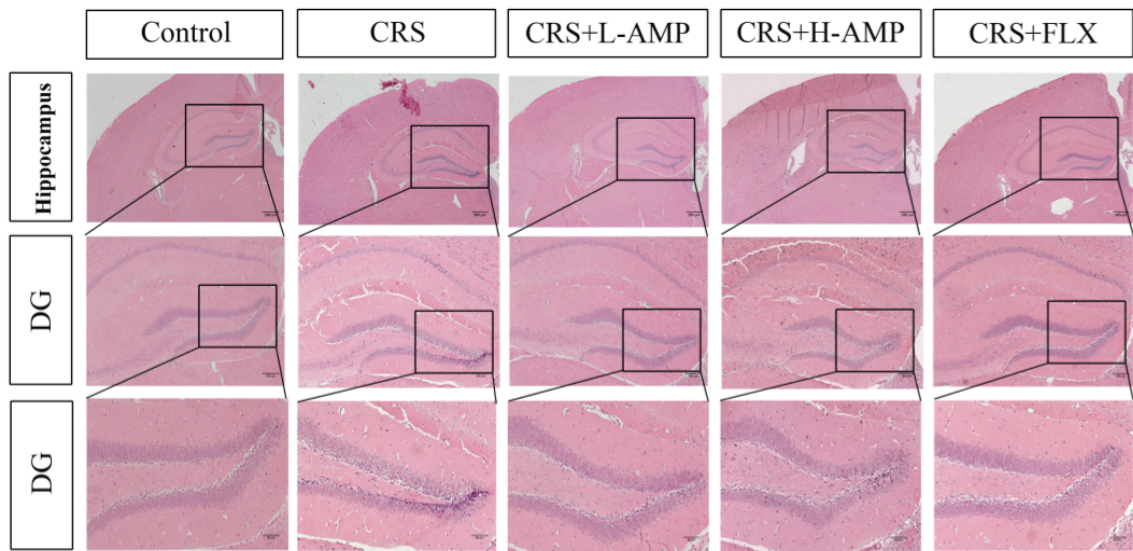


Figure 4

Effects of AMP administration on neuronal damage in the hippocampal of CRS mice. The representative photographs of HE stained hippocampus DG region in the five groups. Scale bar = 200 μ m in the five pictures above, Scale = 100 μ m in the five middle pictures, Scale bar = 50 μ m in the five pictures below.

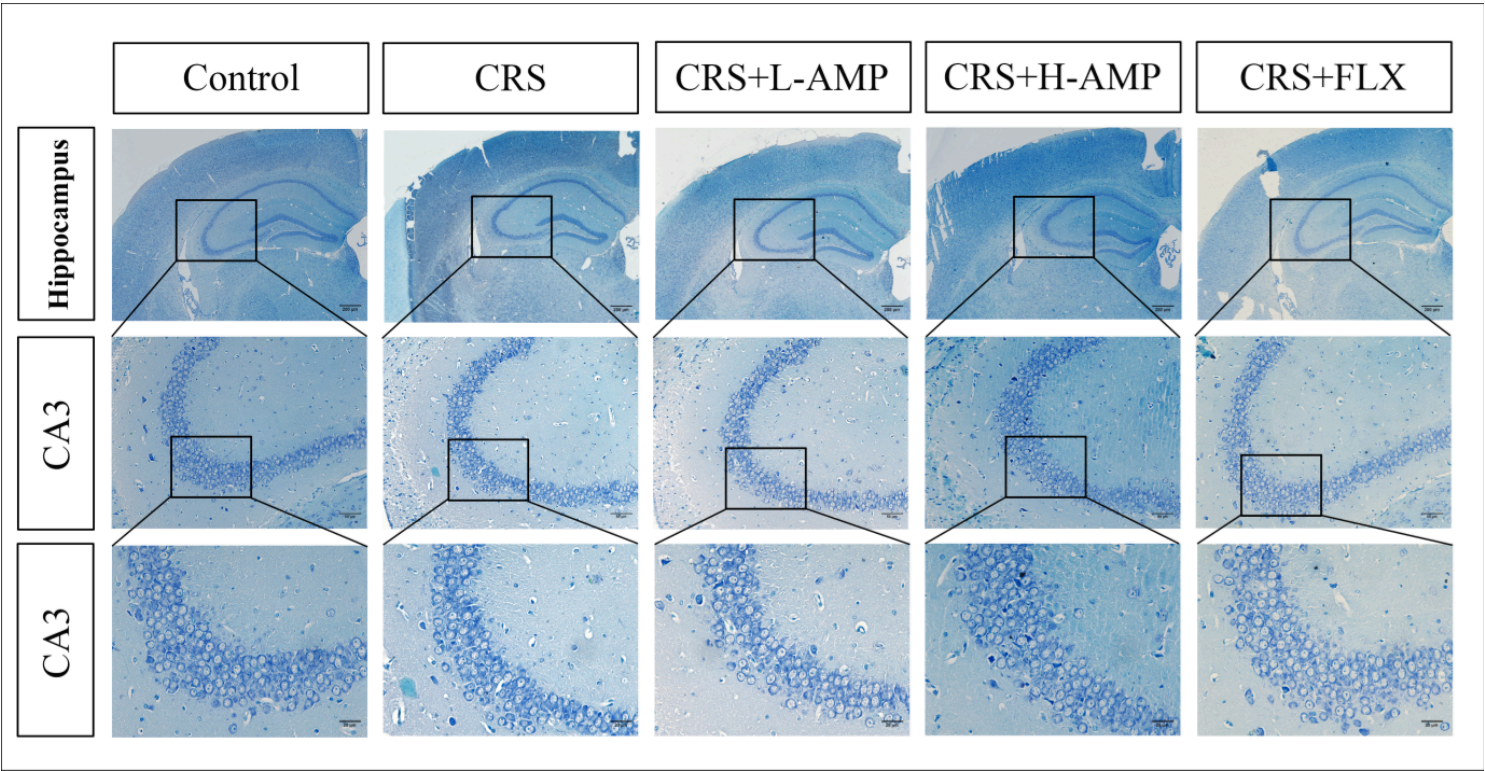


Figure 5

Effects of AMP administration on neuronal damage in the hippocampal of CRS mice. The representative photographs of NISSL stained hippocampal CA3 area region in the five groups. Scale bar = 200 μ m in the five pictures above, Scale = 50 μ m in the five middle pictures, Scale bar = 20 μ m in the five pictures below.

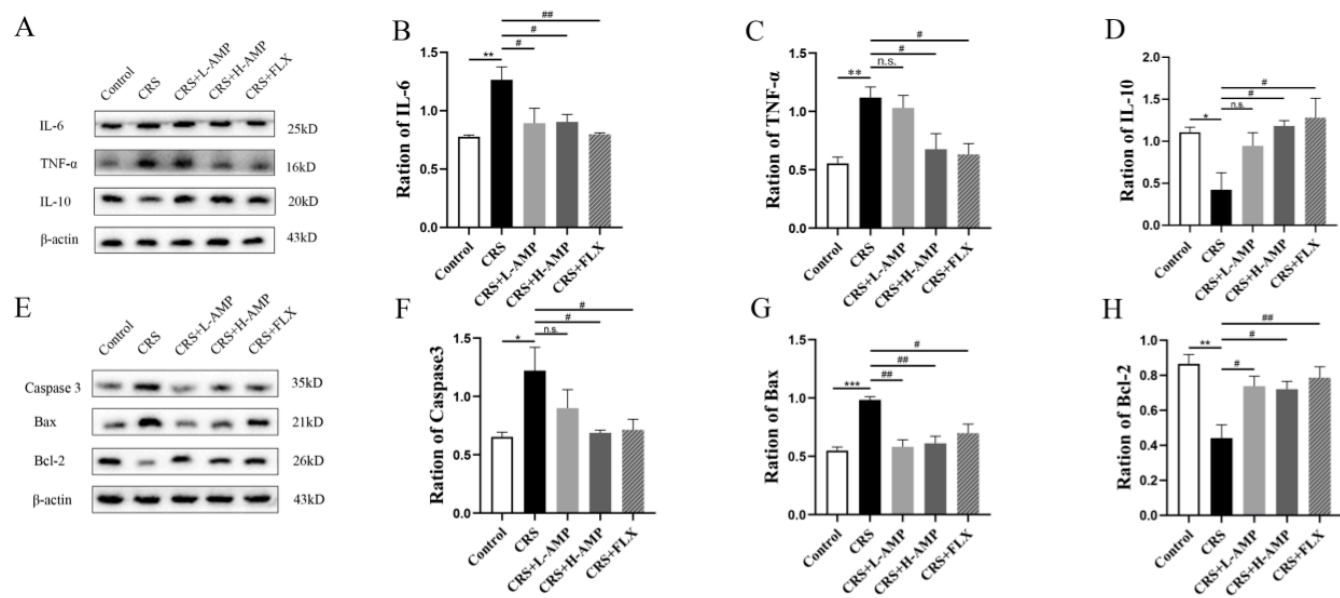


Figure 6

Effects of AMP administration on inflammatory factors and apoptosis. (A, E) Results are immunoblots from single representative experiments. The expression values of IL-6 (B), TNF- α (C), IL-10 (D), Caspase 3 (F), Bax (G), Bcl-2 (H) were normalized with β -actin value. Data are presented as mean $\pm$ SEM ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, denote significant difference from the control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, denote a significant difference from the CRS group. n.s. not significant.

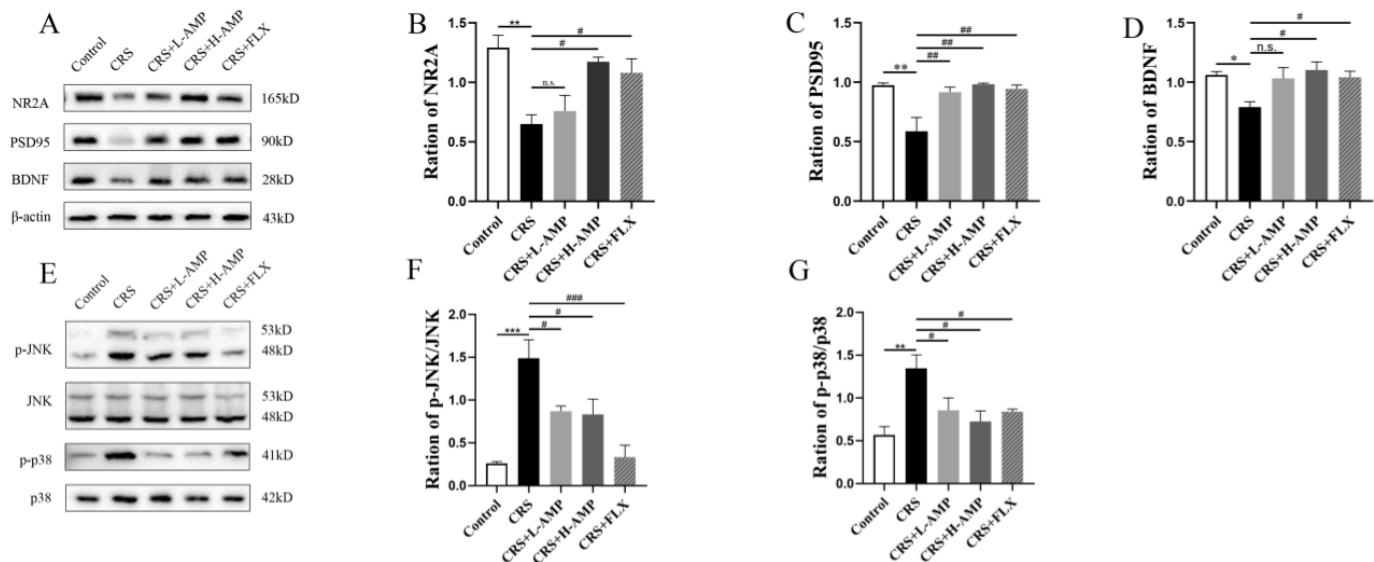


Figure 7

Effects of AMP administration on synaptic plasticity and JNK/p38MAPK signaling pathway. (A, E) Results are immunoblots from single representative experiments. The expression values of NR2A (B), PSD95 (C), BDNF (D) were normalized with β-actin value. The expression values of p-JNK (F), p-p38 (G) were normalized with JNK, p38 respectively. Data are presented as mean ± SEM (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, denote significant difference from the control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, denote a significant difference from the CRS group. n.s. not significant.