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Antidepressant effects of ershiwei roudoukou pills and its active ingredient Macelignan: Multiple mechanisms involving oxidative stress, neuroinflammation and synaptic plasticity

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Major depressive disorder (MDD) represents a significant global health burden, with current treatments showing limited efficacy and considerable side effects. While traditional medicines offer promising alternatives, their mechanisms often remain unclear. Here we demonstrate that Ershiwei Roudoukou Pills (ERP) and its active ingredient Macelignan exhibit potent antidepressant effects through multiple interconnected pathways in a chronic unpredictable mild stress (CUMS) mouse model. Both compounds significantly improved depression-like behaviors in forced swimming, tail suspension, and open field tests. Mechanistically, ERP and Macelignan restored oxidative balance by modulating multiple markers including SOD, CAT, and MDA across serum, hippocampus, and prefrontal cortex. They effectively suppressed neuroinflammation by reducing pro-inflammatory cytokines (IL-6, TNF-α) and microglial activation while increasing anti-inflammatory markers (IL-10). Furthermore, both compounds enhanced synaptic plasticity through upregulation of synaptic proteins (PSD-95, MAP2, SYP) and activation of the BDNF-TrkB signaling pathway. Notably, ERP demonstrated differential anti-inflammatory properties compared to Macelignan, with distinct effects on different inflammatory markers, suggesting potential synergistic effects from its multiple components. These findings reveal the multi-target therapeutic potential of ERP and Macelignan in treating depression, providing new insights for developing more effective antidepressant strategies, particularly for treatment-resistant cases.

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INTRODUCTION

Major depressive disorder (MDD) represents one of the most prevalent neuropsychiatric disorders, affecting approximately 280 million people worldwide and ranking as a leading cause of global disease burden [1]. The complex pathogenesis of MDD involves interactions between genetic vulnerability and environmental factors [2, 3], presenting significant challenges for both understanding and treatment. According to recent global epidemiological studies, the lifetime prevalence of MDD ranges from 10–20% across different populations [4], with particularly concerning trends in developing countries [5]. The socioeconomic burden of depression is substantial, with recent estimates suggesting depression-related disability accounts for 7.5% of all years lived with disability globally [6, 7].

Chronic unpredictable mild stress (CUMS) has been widely recognized as a crucial risk factor in the onset and development of MDD [8, 9]. Prolonged exposure to stressful life events triggers significant alterations in various brain regions, particularly in the prefrontal cortex and hippocampus [10, 11]. Neuroimaging studies have revealed that these stress-induced structural and functional changes are closely associated with disruptions in multiple biological processes, including neurotransmitter systems, neuroplasticity, and cellular resilience [12, 13]. The chronic unpredictable mild stress (CUMS) model has been extensively validated as a reliable approach for studying depression-like behaviors and evaluating potential therapeutic agents [14, 15]. This model effectively replicates many core features of human depression, including anhedonia, behavioral despair, and neurobiological alterations that parallel clinical observations [16, 17].

Currently available antidepressant treatments face significant limitations in both efficacy and tolerability. First-line antidepressants, primarily based on the monoamine hypothesis [18], have shown inadequate response rates, with approximately 30–50% of patients failing to achieve remission with initial treatment [19]. Moreover, conventional antidepressants typically require 2–4 weeks to exhibit therapeutic effects and are often accompanied by adverse effects that impact patient compliance [20]. While the recent FDA approval of ketamine and its derivatives has offered new hope for treatment-resistant depression [21], concerns about side effects and long-term safety remain significant challenges [22]. The limitations of current therapeutic approaches largely stem from our incomplete understanding of depression's complex pathophysiology, which involves multiple interconnected pathways including neuroinflammation, oxidative stress, neurotrophic factor dysfunction, and altered synaptic plasticity [23]. This multifaceted nature of depression pathology

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suggests that single-target therapeutic approaches may be inherently insufficient [24], highlighting the urgent need for novel treatment strategies that can address multiple pathological mechanisms simultaneously [25].

Over the past decade, there has been growing interest in natural products as therapeutic agents for neurological and psychiatric disorders [26]. This approach has gained particular attention due to the potentially favorable safety profiles and long-term tolerability of natural compounds [27]. Traditional medicine systems have historically utilized various natural products for treating mental disorders, with mounting scientific evidence now supporting their therapeutic potential [28]. Many natural compounds have demonstrated multiple mechanisms of action, including anti-inflammatory, antioxidant, and neurotrophic effects [29], which align well with the complex pathophysiology of depression [30]. Among these, traditional Chinese and Tibetan medicines have shown promising results in treating various neurological conditions, supported by both empirical evidence and modern scientific validation [31, 32].

Given the limitations of current antidepressant treatments and the promising potential of natural compounds, there is a compelling need to investigate novel therapeutic approaches for MDD [33]. While ERP has been traditionally used for various conditions, its potential antidepressant effects and underlying mechanisms remain largely unexplored [34]. Recent pharmacological studies have suggested that natural compounds with multiple active ingredients often exhibit superior therapeutic effects through synergistic actions [35, 36], particularly those possessing anti-inflammatory and antioxidant properties [37]. Therefore, this study aims to systematically evaluate the antidepressant-like effects of ERP and its active components in a well-validated chronic unpredictable mild stress mouse model. This comprehensive approach not only provides scientific validation for traditional medicine applications but also offers insights into potential new therapeutic strategies for depression treatment [23]. Understanding the molecular mechanisms underlying ERP's effects could facilitate the development of more effective, multi-target therapeutic approaches for MDD [25], particularly beneficial for patients who respond poorly to conventional treatments [38, 39].

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (six weeks old) were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The animals were housed under controlled environmental conditions with a 12-h light/dark cycle and provided free access to food and water. All experimental procedures were performed in compliance with the National Institutes of Health Laboratory Animal Care and Use Guidelines (NIH Publication No. 80-23) and received approval from the Animal Care and Use Committee of Minzu University of China.

CUMS model establishment and behavioral tests

Following a 5-day acclimation period, mice were subjected to CUMS to induce depressive-like behaviors according to our previously published protocol [40]. The CUMS model applied in our study includes 10 sources of stress: (1) 5 min tail suspension, (2) 5 min cold swim at 4–6 °C, (3) clipping the distal 1 cm of the tail tip with tongs for 5 min, (4) 5 min 45 °C hot water forced swimming, (5) 24 h food fasting, (6) 24 h water fasting, (7) 24 h of damp padding (200 mL of water per 100 g of padding), (8) 24 h cage tilting (cage inclined at 45° with respect to the horizontal), (9) 24 h of day and night reversal, and (10) 6 h of constraint. Mice were exposed to CUMS for 30 days, and two randomly selected pressure sources were applied to the animals daily. At the same time, animals in the control group maintained a normal feeding schedule.

The animals were randomly assigned to four experimental groups: control group, CUMS model group, CUMS model plus ERP group, and CUMS model plus maceligan group. After 30 days of CUMS treatment, mice received intragastric administration of either ERP (1 g/kg, diluted in saline) or maceligan (5 mg/kg) for an additional 30 days (Fig. 1A). The ERP dosage was selected based on human prescription guidelines. Notably, During the 30-day drug administration period, mice were maintained under stress conditions with 4-h restraint daily to prevent the natural recovery of depression-like behaviors. The dosage of ERP (1 g/kg) was calculated based on the conventional human clinical dose (5 g/day for a 60 kg adult, equivalent to 0.083 g/kg) and converted to mouse equivalent dose using the human-to-mouse conversion factor (12.3) based on body surface area. Based on both previous literature reporting antioxidant effects at 10 mg/kg in mice [41] and our preliminary dose-finding experiments (Supplementary Figure), the dosage of macelignan (5 mg/kg) was selected.

Behavioral assessments were conducted to evaluate the antidepressant-like effects of ERP and Macelignan. Behavioral tests included open-field test (OFT), forced swimming test (FST), and tail suspension test (TST). For OFT, individual mice were carefully placed in the center of an open-field apparatus (50 × 50 × 45 cm, Chengdu Taimeng Software Co. Ltd., Chengdu, China), and their movement was recorded for 5 min via video camera and analyzed using open-field software (Chengdu Taimeng Software Co. Ltd.). For FST, mice were placed in a glass cylinder (Chengdu Taimeng Software Co. Ltd.) containing water (approximately 25 °C) for 6 min. Following a 1-min adaptation period, immobility time was recorded and analyzed during the final 5 min using FST software (Chengdu Taimeng Software Co. Ltd.). For TST, individual mice were suspended upside down in a tail suspension chamber (Chengdu Taimeng Software Co. Ltd.). After a 1-min adaptation period, immobility time was recorded and analyzed over the subsequent 5 min using TST software. The day following behavioral testing, all mice were sacrificed for collection of blood and brain tissues for biochemical analyses. The complete experimental timeline is illustrated in Fig. 1B.

Measurement of oxidative stress markers

Oxidative markers were analyzed using enzymatic colorimetric tests according to manufacturer's protocols. Peripheral blood samples (1.2 mL) were collected from the retroorbital vessels of mice and centrifuged at 3000 rpm for 15 min at room temperature to obtain serum layers (400 µL). To assess oxidative stress levels, serum, prefrontal cortex and hippocampal tissue samples were analyzed. Superoxide dismutase (SOD), malondialdehyde (MDA), nitric oxide (NO), total antioxidant capacity (T-AOC) and catalase (CAT) activities or levels were measured by commercial kits (Nanjing Jiancheng Bioengineering Institute).

Quantitative real-time PCR (qRT-PCR)

Quantitative assessment of cytokine (IL-6, IL-10, TNF-α and TGF-β) and apoptosis-related marker (Bax and Bcl-2) transcriptional expression levels was performed using qRT-PCR following our previously published protocol [42–44]. Hippocampal and prefrontal cortical tissues were isolated from mice across different treatment groups, and total RNA was extracted using trizol reagent (ThermoFisher Scientific, Waltham, MA, US). The extracted RNA was reverse-transcribed to cDNA using StarScript II RT Mix with gDNA Remover (GenStar, Beijing, China). PCR amplification was performed on a LightCycler® 96 system (Roche, Basel, Switzerland) using SYBR green I Master Mix (Solarbio, Beijing, China) with specific forward and reverse primers. The amplification protocol consisted of initial denaturation at 95 °C for 10 min, followed by 40 cycles of DNA synthesis (95 °C for 15 s and 60 °C for 1 min). Primer sequences are detailed in Table 1. Relative transcriptional expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Western blotting

Western blot analysis was conducted according to our previously established protocol [45]. Protein samples (30 µg) from hippocampal and prefrontal cortical tissue lysates were separated by 10% SDS-PAGE for 1.5 h and transferred to a 0.22 µm polyvinylidene fluoride membrane (ThermoFisher Scientific). Following membrane blocking, samples were incubated overnight with primary antibodies at 4 °C and washed three times. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc., PA, United States). Protein signals were visualized using a chemiluminescence detection system (Tanon 4200, Shanghai, China), and signal quantification was performed using ImageJ software. Primary antibodies included monoclonal mouse BDNF and p-Trkb were obtained from Abcam with dilution ratio 1:1000 (Cambridge, UK), SYP, Syn-1, PSD-95, MAP2 with dilution ratio 1:1000 and β-Actin with dilution ratio 1:10000 were obtained from Cell Signaling Technology (Boston, MA, US). Secondary antibodies (goat anti-mouse and goat anti-rabbit) were obtained from Abcam.

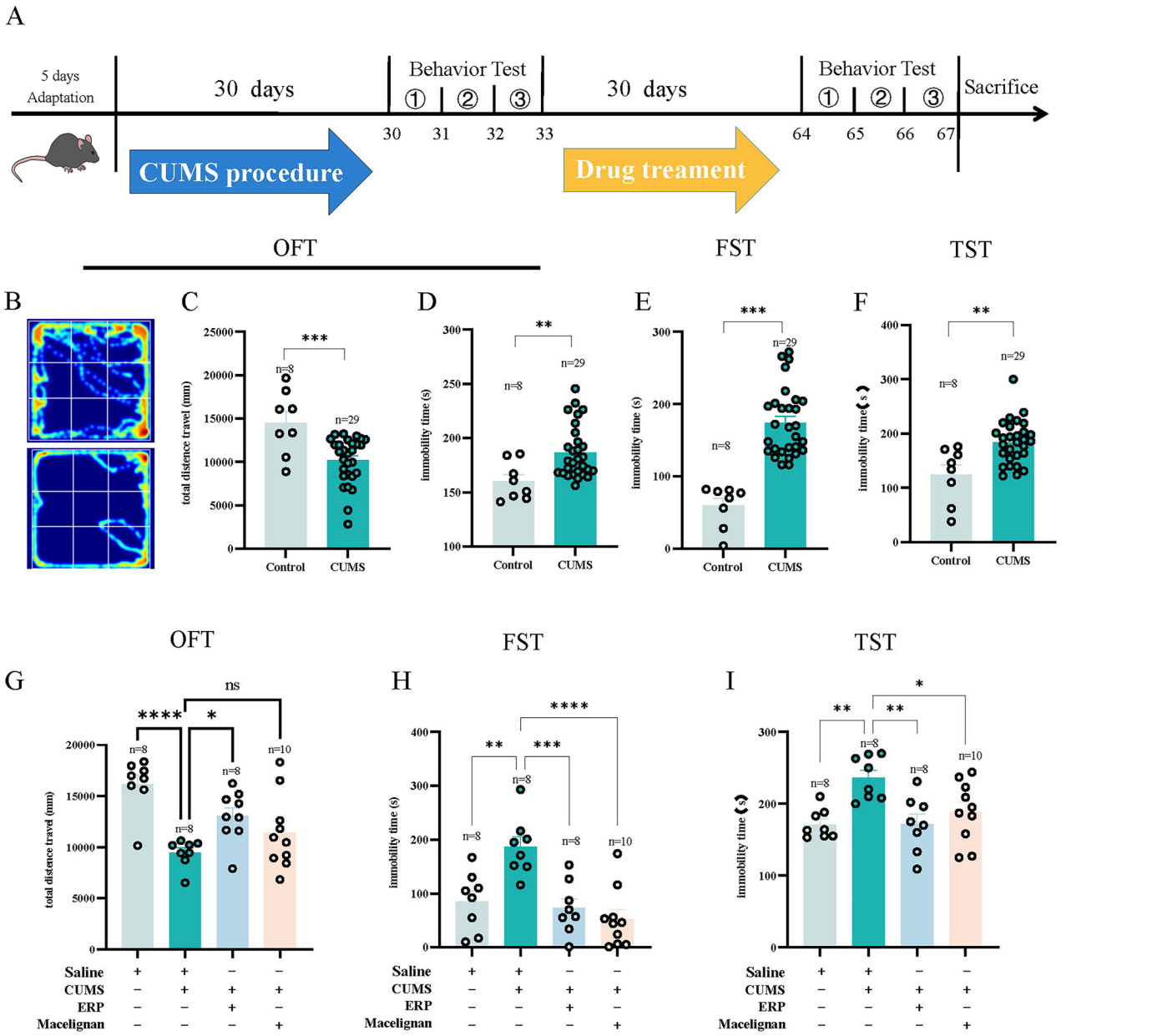

Fig. 1 Effects of ERP and maceligan treatment on depression-like behaviors in mice. Schematic representation of the experimental design. **A**. Representative heat maps showing locomotor trajectories in OFT from control, CUMS, CUMS & ERP and CUMS & maceligan groups (**B**). Total distance traveled in OFT showing reduced locomotor activity in CUMS-exposed mice (**C**). Immobility time in FST (**D**) and TST (**E**), and total duration in TST (**F**) demonstrating CUMS-induced depression-like behaviors. Effects of drug treatment on total distance in OFT (**G**), immobility time in FST (**H**) and TST (**I**) showing the antidepressant-like effects of ERP and maceligan. For ANOVA statistics: (G), $F(3, 30) = 8.948$, $p < 0.001$; (H), $F(3, 30) = 10.600$, $p < 0.001$; (I), $F(3, 30) = 6.293$, $p = 0.002$. Data are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: not significant). CUMS chronic unpredictable mild stress, OFT open field test, FST forced swimming test, TST tail suspension test, ERP ershiwei roudoukou pills.

Table 1. Primer sequences for qRT-PCR.

Primers	Forward primer	Reverse primer
m-IL-6	TGGCTAAGGACCAAGACCATCCAA	AACGCACTAGGTTTGCCGAGTAGA
m-IL-10	CCAAGGTGTCTACAAGGCCA	GCTCTGTCTAGGTCCTGGAGT
m-TNF-α	GCTCCTCCACTTGGTGGTTTGT	ACTCCAGGCGGTGCCTATGTC
m-TGF-β	AATTCCTCGAGACAGGCCATT	CCAGCTGACTGCTTTTCTGTAG
m-Bax	TGCTAGCAAACTGGTGCTCA	CTTGGATCCAGACAAGCAGC
m-Bcl-2	GGCCTTCTTTGAGTTCGGTG	GCATGCTGGGGCCATATAGTT
m-Actin	AGACCTCTATGCCAACACAGT	TCCTGCTTGCTGATCCACAT

qRT-PCR quantitative real-time polymerase chain reaction, *IL-6* interleukin 6, *IL-10* interleukin 10, *TNF-α* tumour necrosis factor alpha-like, *Bax* Bcl-2-associated X protein, *Bcl-2* Bcl-2 apoptosis regulator.

Immunohistochemisty

Based on previous findings indicating BDNF's role in preventing neuronal death [46], we performed immunohistochemical analysis following our established protocol [47] to evaluate mature neurons and glial cells in the hippocampus across different treatment groups. This analysis involved immunostaining for Microtubule-Associated Protein 2 (MAP2) and Ionized calcium binding adaptor molecule 1 (IBA1). Primary antibodies included monoclonal mouse anti-GFAP (1:1000, Cell Signaling Technology), and visualization was achieved using Alexa Fluor 488 goat anti-mouse secondary antibody (1:1000, ThermoFisher Scientific).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0 software. Data are presented as mean ± SEM (standard error of the mean). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by multiple comparison tests. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Antidepressant-like effects of ERP and Macelignan in CUMS-induced depression model mice

To investigate the therapeutic potential of ERP and Macelignan in treating depression, we conducted behavioral assessments using the forced swimming test (FST), tail suspension test (TST), and open field test (OFT) following intragastric administration of these compounds to CUMS-treated mice. Initial evaluations revealed that CUMS treatment successfully induced a depression-like phenotype, evidenced by significantly decreased total distance traveled in the OFT and prolonged immobility times in both FST and TST (Fig. 1B–F). Following treatment interventions, we observed differential effects between ERP and Macelignan. While ERP administration significantly altered locomotor activity in CUMS-treated mice compared to the saline control group, Macelignan showed no such effect (Fig. 1G). To further validate the antidepressant efficacy of both compounds, we conducted additional FST and TST analyses. These assessments demonstrated that both ERP and Macelignan significantly reduced immobility time in CUMS-treated mice (Fig. 1H, I), suggesting an improvement in depression-like behaviors. Collectively, these findings demonstrate that ERP and its active component maceligan effectively ameliorated depression-like behaviors in the CUMS-treated mouse model.

ERP and Macelignan reduced oxidative stress in CUMS-treated mice

Given the critical role of oxidative stress in depression pathophysiology, we evaluated multiple oxidative stress markers across different treatment groups. In serum analysis, CUMS-treated mice exhibited significant alterations in oxidative stress markers compared to saline-treated controls, including decreased T-AOC activities (Fig. 2A), elevated MDA (Fig. 2B) and NO levels (Fig. 2C). Both ERP and Macelignan treatments effectively reversed these changes, significantly increasing T-AOC activities while reducing MDA and NO levels (Fig. 2A–C). Notably, while CAT levels were significantly reduced in CUMS-treated mice, neither ERP nor Macelignan treatment significantly altered these levels (Fig. 2D). SOD levels remained consistent across all groups (Fig. 2E).

We extended our investigation to examine oxidative stress markers in both hippocampal and prefrontal cortex regions. In the hippocampus, CUMS treatment led to significantly increased T-AOC activities, which were further elevated by both ERP and Macelignan treatments (Fig. 2F). While MDA levels showed no significant inter-group differences (Fig. 2G), NO levels were significantly elevated (Fig. 2H), and both CAT (Fig. 2I) and SOD (Fig. 2J) levels were significantly reduced compared to saline controls. ERP and Macelignan administration effectively normalized these alterations, decreasing NO levels while increasing CAT and SOD levels (Fig. 2H–J).

In the prefrontal cortex, we observed similar patterns of oxidative stress marker alterations. CUMS treatment significantly increased T-AOC activities, which were further enhanced by both ERP and Macelignan treatments (Fig. 2K). Both MDA (Fig. 2L) and NO (Fig. 2M) levels were significantly elevated in CUMS-treated mice compared to saline controls, and these elevations were effectively reduced by both ERP and Macelignan treatments (Fig. 2L, M). Additionally, CUMS treatment significantly reduced CAT (Fig. 2N) and SOD (Fig. 2O) levels, which were successfully restored by both ERP and Macelignan administration (Fig. 2N, O).

Collectively, these findings demonstrate that both ERP and Macelignan exhibit potent antioxidant effects in stressed mice, effectively modulating various oxidative stress markers across multiple tissues.

Regulation of neuroinflammation and apoptotic pathways by ERP and Macelignan

Next, we investigated the expression patterns of inflammatory cytokines and apoptosis-related markers across different treatment groups. In the prefrontal cortex, CUMS treatment significantly elevated the transcriptional expression of pro-inflammatory genes IL-6 and TNF-α compared to saline controls. Macelignan administration effectively reduced both TNF-α and IL-6 transcriptional levels, while ERP selectively reduced IL-6 but not TNF-α expression (Fig. 3A, B). Regarding anti-inflammatory markers, both ERP and Macelignan successfully restored the CUMS-induced reduction in IL-10 mRNA levels (Fig. 3C). Interestingly, while TGF-β mRNA expression remained consistent across saline, CUMS, and ERP groups, it showed a significant decrease in the Macelignan group compared to CUMS-treated mice (Fig. 4D).

Analysis of hippocampal tissue revealed distinct patterns of inflammatory marker expression. IL-6 mRNA levels were significantly elevated in the CUMS group compared to saline controls, with both ERP and Macelignan treatments effectively reducing these elevated levels (Fig. 4E). In contrast, TNF-α and IL-10 mRNA expression showed no significant variations across treatment groups (Fig. 4F, G). TGF-β expression demonstrated a unique pattern: while no significant difference was observed between saline and CUMS groups, ERP treatment significantly increased its expression compared to the CUMS group. However, no significant difference was detected between ERP and Macelignan treatments (Fig. 4H).

We further examined apoptosis-related markers, focusing on Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) mRNA expression. CUMS treatment significantly increased Bax mRNA levels in both the prefrontal cortex and hippocampus compared to saline controls, while both ERP and Macelignan administration effectively reduced these elevated levels (Fig. 3I, J). Similarly, the CUMS-induced reduction in Bcl-2 mRNA levels in both brain regions was successfully restored by ERP and Macelignan treatment (Fig. 3K, L).

Comprehensively, these findings demonstrate that both ERP and Macelignan exhibit significant anti-inflammatory and anti-apoptotic properties in CUMS-treated mice, though with some region-specific and marker-specific variations in their effects.

ERP and Macelignan suppress microglial activation in hippocampal subregions

Based on previous reports indicating that activated microglia are key contributors to neuroinflammatory conditions, we investigated microglial activation by examining the expression of the microglial marker Iba-1 through immunofluorescence. Our analysis revealed increased numbers of Iba1-positive cells in the DG, CA1, and CA2 regions of the hippocampus in CUMS-treated mice. Notably, both ERP and Macelignan administration effectively reversed these microglial morphological alterations across all examined hippocampal regions (Fig. 4A). Quantitative analysis further confirmed that both compounds significantly reduced the number of activated microglia throughout all hippocampal subregions (Fig. 4B–D), demonstrating their potent anti-neuroinflammatory effects.

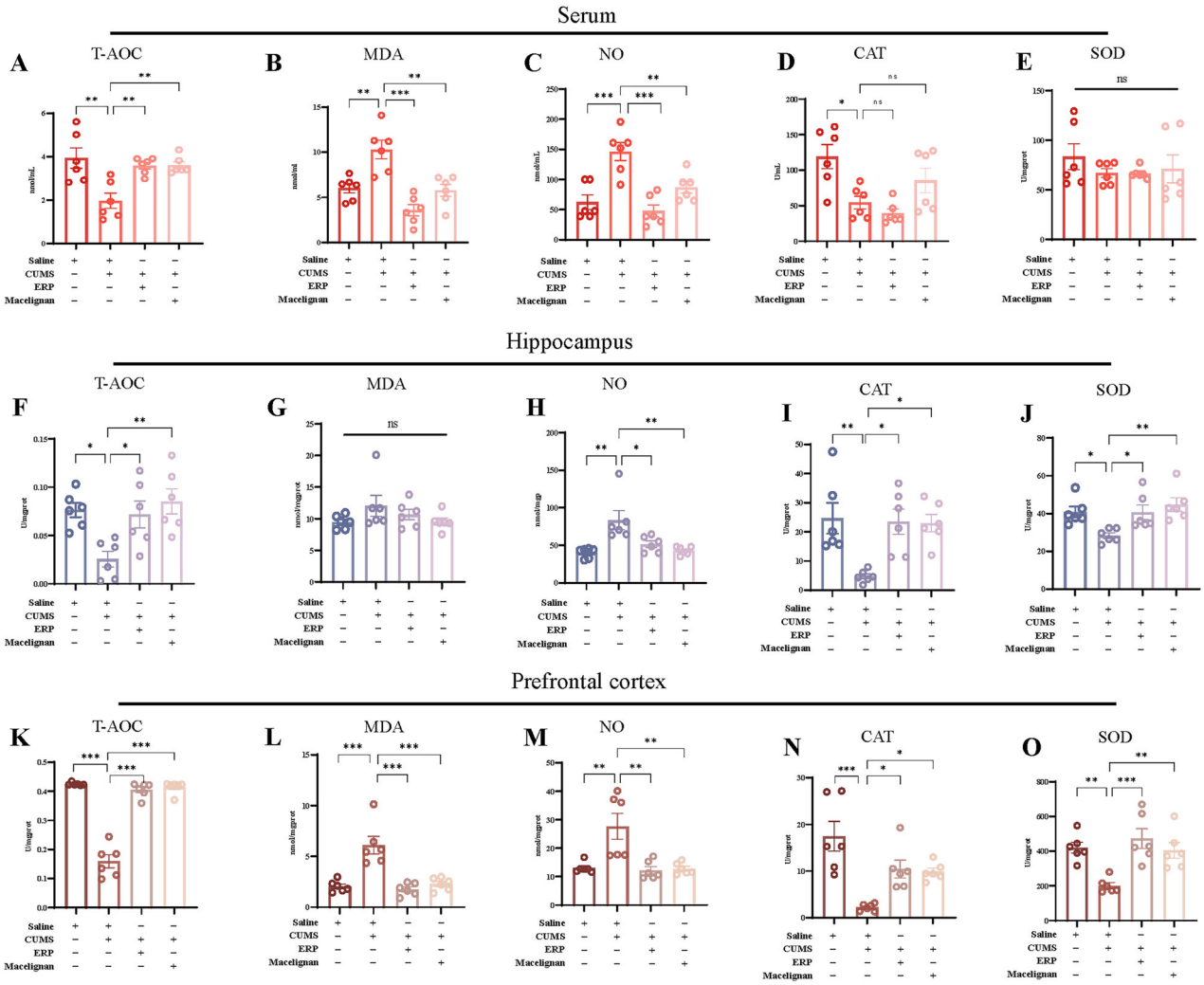

Fig. 2 Effects of ERP and maceligan treatment on oxidative stress markers in multiple tissues. Levels of oxidative stress markers in serum including T-AOC (**A**), MDA (**B**), NO (**C**), CAT activity (**D**), and SOD activity (**E**) from different treatment groups. Levels of oxidative stress markers in hippocampus including T-AOC (**F**), MDA (**G**), NO (**H**), CAT activity (**I**), and SOD activity (**J**). Levels of oxidative stress markers in prefrontal cortex including T-AOC (**K**), MDA (**L**), NO (**M**), CAT activity (**N**), and SOD activity (**O**). For ANOVA statistics: (**A**), $F(3, 20) = 7.953$, $p = 0.001$; (**B**), $F(3, 20) = 14.440$, $p < 0.001$; (**C**), $F(3, 20) = 13.490$, $p < 0.001$; (**D**), $F(3, 20) = 7.091$, $p = 0.002$; (**E**), $F(3, 20) = 0.6116$, $p = 0.615$; (**F**), $F(3, 20) = 5.906$, $p = 0.047$; (**G**), $F(3, 20) = 1.467$, $p = 0.254$; (**H**), $F(3, 20) = 8.208$, $p < 0.001$; (**I**), $F(3, 20) = 6.425$, $p = 0.003$; (**J**), $F(3, 20) = 5.354$, $p = 0.007$; (**K**), $F(3, 20) = 95.44$, $p < 0.001$; (**L**), $F(3, 20) = 18.320$, $p < 0.001$; (**M**), $F(3, 20) = 9.559$, $p < 0.001$; (**N**), $F(3, 20) = 10.750$, $p < 0.001$; (**O**), $F(3, 20) = 8.706$, $p < 0.001$. Data are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). T-AOC total antioxidant capacity, MDA malondialdehyde, NO nitric oxide, CAT catalase, SOD superoxide dismutase.

Restoration of synaptic proteins by ERP and Macelignan treatment

We proceeded to assess the expression of synapse-associated proteins in mice subjected to various treatments. Western blot analysis demonstrated that CUMS exposure significantly suppressed the expression of essential synaptic proteins in the hippocampus, including PSD-95, MAP2, and SYP (Fig. 5A). Importantly, both ERP and Macelignan treatments effectively restored the expression levels of these synaptic markers (Fig. 5A), suggesting their capacity to preserve synaptic integrity and function. Subsequent quantitative analysis validated these findings, showing that both ERP and Macelignan significantly enhanced the expression of PSD-95, MAP2, and SYP in hippocampal tissue (Fig. 5B–D), highlighting their effectiveness in maintaining synaptic homeostasis.

ERP and Macelignan enhance BDNF signaling pathway

Analysis of hippocampal tissue revealed a marked decrease in MAP2-positive cells in CUMS-treated mice compared to the saline-treated group. Remarkably, both ERP and Macelignan treatments significantly increased the number of MAP2-positive cells relative to the CUMS-treated group (Fig. 6A, B). We further investigated the expression patterns of BDNF and its receptor p-TrkB in hippocampal tissue across treatment groups (Fig. 6C). Our findings showed that CUMS-induced reductions in both BDNF and p-TrkB protein levels in the hippocampus were effectively normalized by ERP and Macelignan treatments (Fig. 6D, E). Similarly, both compounds successfully restored BDNF and p-TrkB protein levels in the prefrontal cortex (Fig. 6F–H). These results indicate that ERP and Macelignan exhibit dual therapeutic effects: neuroprotection and activation of the BDNF/TrkB signaling pathway.

DISCUSSION

Depression represents a major global health burden, affecting more than 280 million people worldwide and ranking as a leading cause of disability [1]. Despite decades of research and various available treatments, current antidepressants show limited

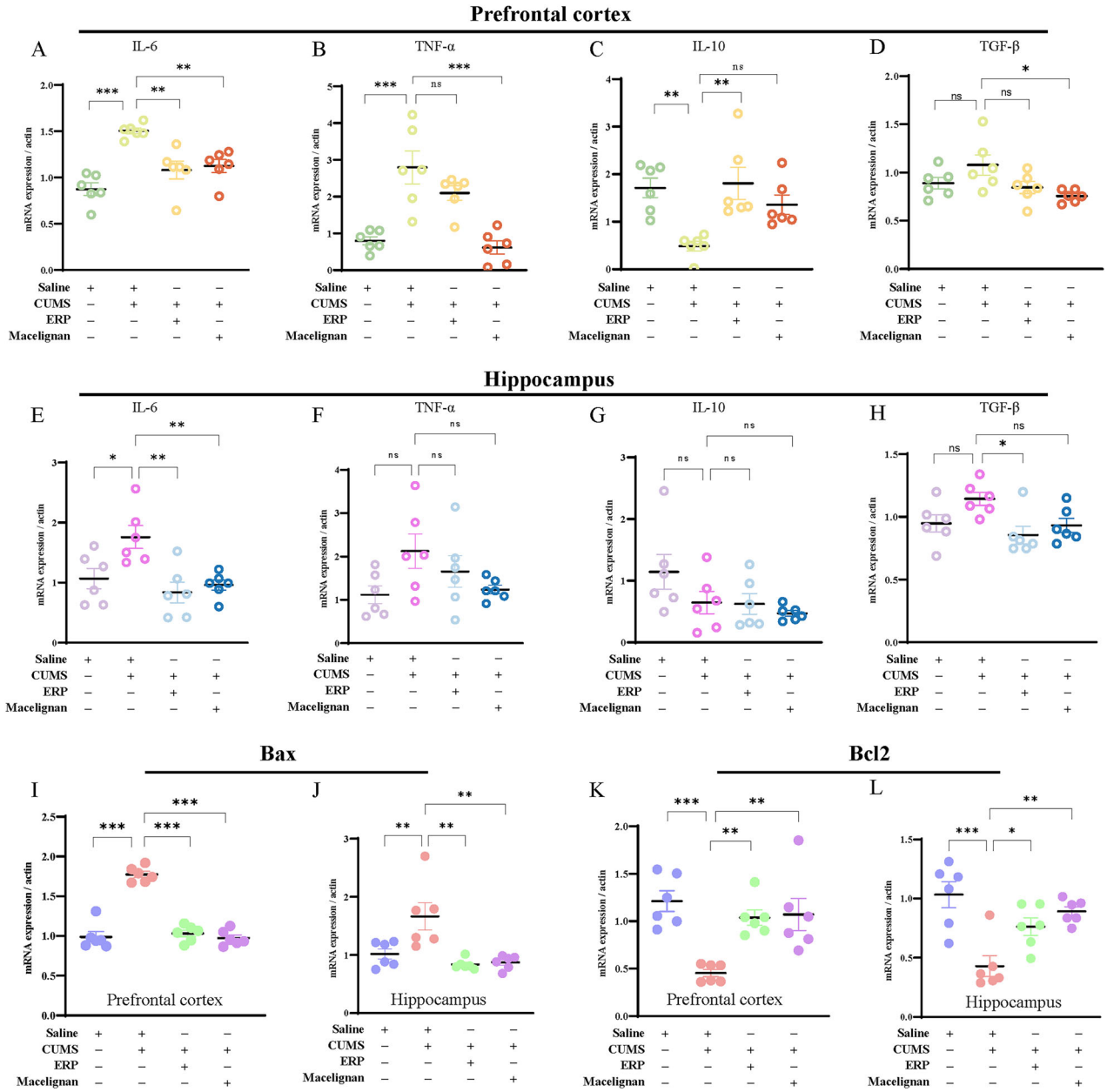

Fig. 3 Effects of ERP and maceligan treatment on neuroinflammatory markers and apoptosis-related proteins in brain tissues. Expression levels of inflammatory cytokines in prefrontal cortex, showing IL-6 (**A**), TNF-α (**B**), IL-1β (**C**), and TGF-β (**D**) from four treated groups. Expression levels of inflammatory cytokines in hippocampus, showing IL-6 (**E**), TNF-α (**F**), IL-1β (**G**), and TGF-β (**H**). Expression levels of apoptotic proteins showing Bax in prefrontal cortex (**I**) and hippocampus (**J**), and Bcl2 in prefrontal cortex (**K**) and hippocampus (**L**). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. n = 6–8 mice per group. For ANOVA statistics: (**A**), $F(3, 20) = 13.910$, $p < 0.001$; (**B**), $F(3, 20) = 15.450$, $p < 0.001$; (**C**), $F(3, 20) = 7.000$, $p = 0.002$; (**D**), $F(3, 20) = 3.844$, $p = 0.025$; (**E**), $F(3, 20) = 6.735$, $p = 0.002$; (**F**), $F(3, 20) = 2.427$, $p = 0.095$; (**G**), $F(3, 20) = 2.348$, $p = 0.103$; (**H**), $F(3, 20) = 3.844$, $p = 0.024$; (**I**), $F(3, 20) = 65.58$, $p < 0.001$; (**J**), $F(3, 20) = 8.980$, $p < 0.001$; (**K**), $F(3, 20) = 9.187$, $p < 0.001$; (**L**), $F(3, 20) = 9.866$, $p < 0.001$. Data are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). IL-6 interleukin-6, TNF-α tumor necrosis factor-alpha, IL-1β interleukin-1beta, TGF-β transforming growth factor-beta.

efficacy and considerable side effects, with approximately one-third of patients failing to achieve remission [4, 48]. This therapeutic challenge stems largely from our incomplete understanding of depression's complex pathophysiology and the limitations of single-target approaches [14]. In this study, we demonstrated that Ershiwei Roudoukou Pills (ERP) and its active component Macelignan significantly improved depression-like behaviors in the CUMS mouse model. We identified three key mechanisms underlying their antidepressant effects: (1) restoration of oxidative balance through modulation of antioxidant enzymes and reduction of oxidative stress markers, (2) suppression of neuroinflammation via regulation of pro- and anti-inflammatory cytokines and microglial activation, and (3) enhancement of synaptic plasticity through upregulation of synaptic proteins and BDNF-TrkB signaling. Notably, ERP showed differential anti-inflammatory properties compared to Macelignan, suggesting potential synergistic effects from its multiple components.

The CUMS model has been extensively validated as a reliable approach for studying depression-like behaviors and evaluating potential therapeutic agents [14, 16]. This model effectively replicates many core features of human depression, including

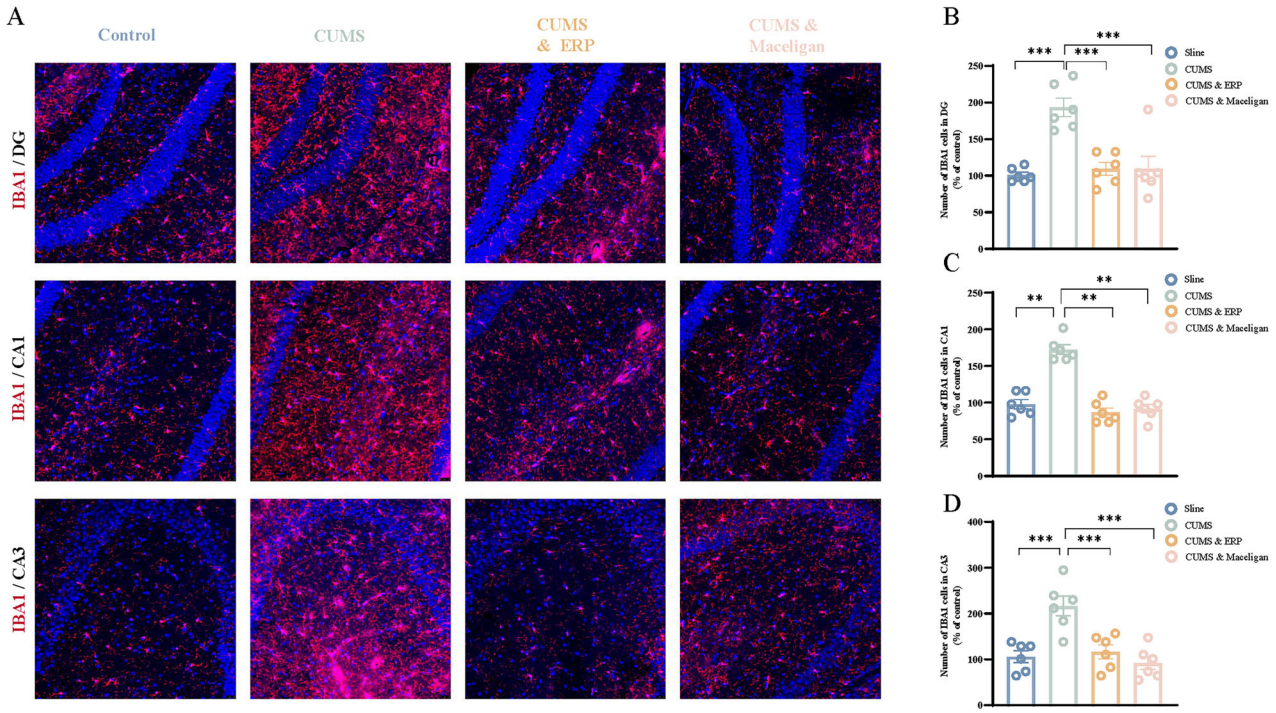

Fig. 4 Effects of RP and maceligan treatment on IBA1 expression in hippocampus. Representative immunofluorescence images showing IBA1 co-stained with DG, CA1, and CA3 regions in hippocampus from control, CUMS, CUMS & ERP and CUMS & Moxifloxacin groups (**A**). Number of IBA1-positive cells in DG (**B**), CA1 (**C**), and CA3 (**D**) regions. For ANOVA statistics: (**B**), $F(3, 20) = 14.040$, $p < 0.001$; (**C**), $F(3, 20) = 42.250$, $p < 0.001$; (**D**), $F(3, 20) = 12.140$, $p < 0.001$. Data are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). IBA1 ionized calcium binding adaptor molecule 1, DG dentate gyrus, CA1/3 cornu ammonis 1/3.

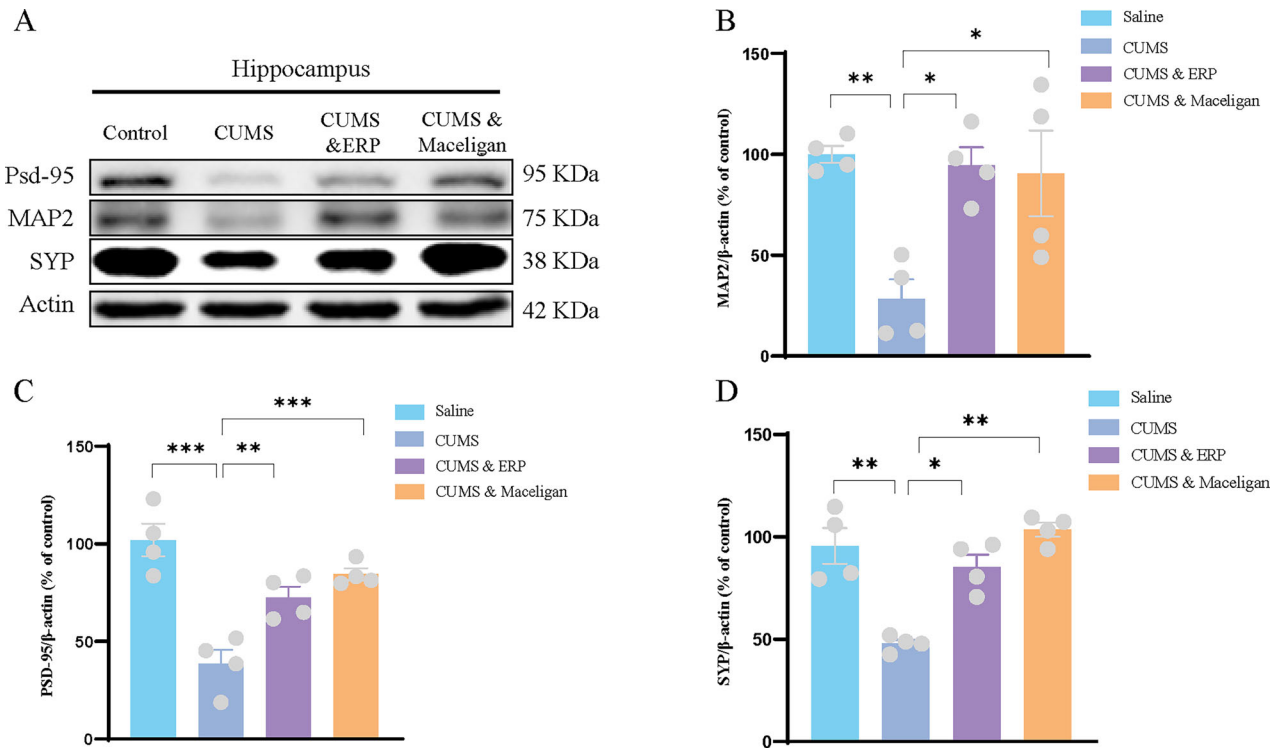

Fig. 5 Effects of RP and maceligan treatment on synaptic proteins in hippocampus. Representative Western blot bands showing protein levels of Psd-95, MAP2, SYP and Actin in hippocampus from different treatment groups (**A**). Relative MAP2 protein levels normalized to Actin (**B**). Relative Psd-95 protein levels normalized to Actin (**C**). Relative SYP protein levels normalized to Actin (**D**). For ANOVA statistics: (**B**), $F(3, 12) = 7.040$, $p = 0.006$; (**C**), $F(3, 12) = 18.030$, $p < 0.001$; (**D**), $F(3, 12) = 19.160$, $p < 0.001$. Data are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). MAP2 microtubule-associated protein 2, Psd-95 postsynaptic density protein 95, SYP synaptophysin.

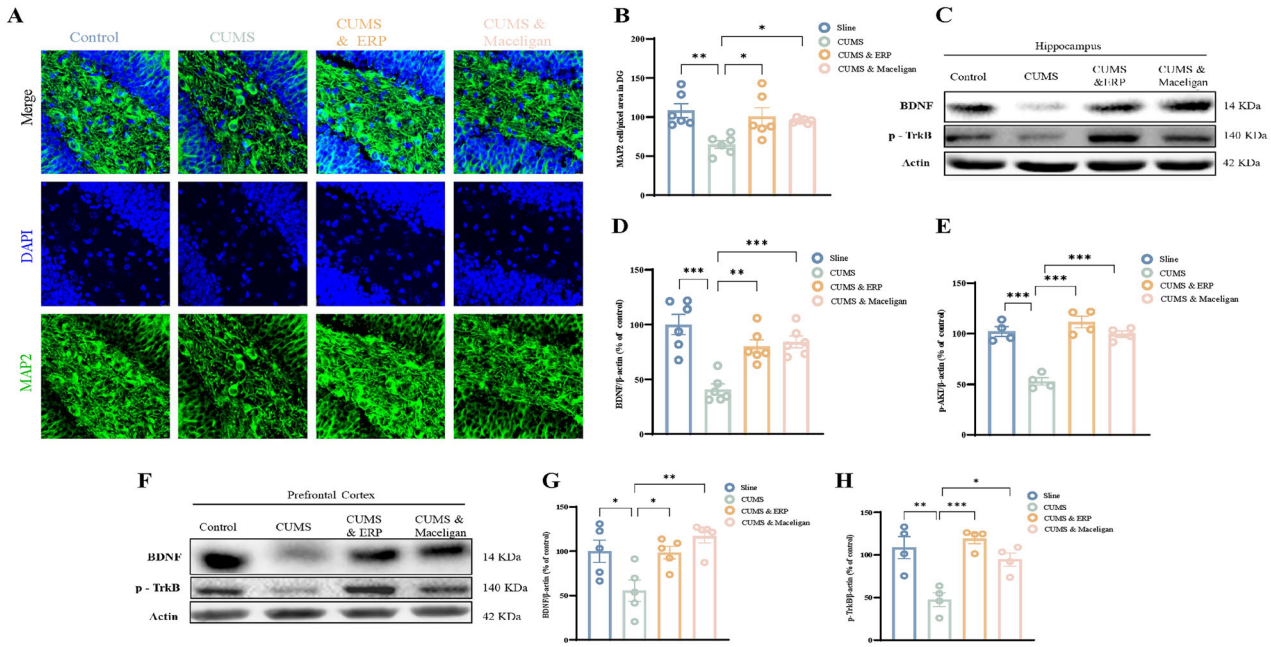

Fig. 6 Effects of RP and maceligan treatment on BDNF signaling pathway in hippocampus and prefrontal cortex. Representative immunofluorescence images showing Merge, DAPI and MAP2 staining in hippocampus from different treatment groups (**A**). Quantification of MAP2-positive area (**B**). Representative Western blot bands showing BDNF, p-TrkB and Actin in hippocampus (**C**) and their relative protein levels normalized to Actin (**D, E**). Representative Western blot bands showing BDNF, p - TrkB and Actin in prefrontal cortex (**F**) and their relative protein levels normalized to Actin (**G, H**). For ANOVA statistics: (**B**), $F(3, 20) = 6.196$, $p = 0.004$; (**D**), $F(3, 16) = 14.620$, $p < 0.001$; (**E**), $F(3, 12) = 35.060$, $p < 0.001$; (**G**), $F(3, 16) = 6.715$, $p = 0.004$; (**H**), $F(3, 12) = 12.160$, $p < 0.001$. Data are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). BDNF brain-derived neurotrophic factor, MAP2 microtubule-associated protein 2, p-TrkB phosphorylated tropomyosin receptor kinase B.

anhedonia, behavioral despair, and neurobiological alterations that parallel clinical observations [24]. Our behavioral assessments confirmed the successful establishment of the depression model, evidenced by increased immobility time in forced swimming and tail suspension tests, reduced sucrose preference, and decreased social interaction. Importantly, the behavioral improvements observed with ERP and Macelignan treatment were comparable to those induced by conventional antidepressants, without affecting baseline locomotor activity, suggesting specific antidepressant efficacy rather than general motor stimulation [19, 49].

Our molecular analyses revealed multiple interacting mechanisms underlying the antidepressant effects of ERP and Macelignan. Recent studies have emphasized that effective antidepressant treatments often involve concurrent modulation of multiple pathways [50, 51], aligning with our findings of comprehensive pathway regulation. This multi-target therapeutic potential particularly addresses the complex nature of depression pathophysiology, as highlighted in recent reviews [23].

A primary mechanism involves the modulation of oxidative stress, a critical factor in depression pathophysiology [52]. Both compounds effectively normalized multiple oxidative stress markers, with ERP showing particularly robust effects. Specifically, they restored the activities of key antioxidant enzymes including SOD and CAT, while reducing MDA and NO levels. This antioxidant effect was notably pronounced in the hippocampus, where oxidative damage has been strongly linked to depressive behaviors [37]. Recent evidence has demonstrated that oxidative stress can trigger mitochondrial dysfunction and cellular damage in critical brain regions, leading to depressive symptoms [53]. The ability of our compounds to enhance antioxidant defense systems therefore represents a fundamental therapeutic mechanism.

Previous studies have shown that Macelignan can modulate multiple cellular signaling pathways that may contribute to its antidepressant effects. Macelignan has been reported to regulate MAPK and PI3K/Akt pathways, leading to reduced JNK and ERK activation and subsequent downregulation of pro-inflammatory mediators such as MMP-9 and COX-2 [54, 55]. Literature evidence also suggests that Macelignan can suppress IL-1β/NF-κB signaling, a pathway crucial in neuroinflammation and implicated in both neurodegenerative diseases and depression [56]. Additionally, studies have demonstrated that Macelignan enhances cholinergic neurotransmission through acetylcholinesterase (AChE) inhibition [57]. These previously reported molecular mechanisms could potentially explain our experimental observations of reduced inflammatory markers and enhanced synaptic plasticity, though direct evidence for these pathways in our depression model requires further investigation. Together with our findings, these studies support Macelignan's potential role as a multi-target therapeutic agent capable of simultaneously modulating oxidative stress, inflammation, and neuronal function.

The anti-neuroinflammatory properties of both compounds emerged as another crucial mechanism. Chronic stress-induced neuroinflammation has been increasingly recognized as a key pathogenic factor in depression, with elevated pro-inflammatory cytokines directly linked to symptom severity [37, 58]. Our findings showed that ERP and Macelignan effectively suppressed microglial activation across multiple hippocampal subregions, particularly in the DG and CA1 areas [59, 60]. This was accompanied by significant reductions in pro-inflammatory cytokines (IL-6, TNF-α) and increased anti-inflammatory markers (IL-10), consistent with recent findings on neuroinflammation in depression [58, 60]. Notably, the compounds showed differential effects on specific inflammatory markers - while ERP demonstrated broader anti-inflammatory properties, Macelignan exhibited more selective cytokine modulation, suggesting distinct mechanistic pathways.The regulation of apoptotic pathways represents another significant mechanism. Our analysis revealed that both compounds effectively modulated the Bcl-2/Bax ratio, a critical

marker of cellular survival. Recent studies have highlighted the importance of cell survival pathways in depression treatment [61, 62]. The restoration of this balance was observed in both the hippocampus and prefrontal cortex, suggesting widespread neuroprotective effects. This is particularly significant given recent evidence linking neuronal loss in specific brain regions to depression severity [63].

Perhaps most remarkably, both compounds demonstrated significant effects on synaptic plasticity and BDNF signaling [64, 65]. The restoration of key synaptic proteins (PSD-95, MAP2, SYP) suggests a fundamental mechanism for improving neural circuit function. This was coupled with enhanced BDNF-TrkB signaling, a pathway crucial for neuroplasticity and antidepressant response [66]. Recent advances in our understanding of rapid-acting antidepressants have highlighted the critical role of synaptic plasticity in therapeutic response [67]. The parallel improvement in both structural and molecular markers of plasticity indicates a coordinated effect on neural circuit remodeling, aligning with current perspectives on antidepressant mechanisms [12].

The integration of these multiple pathways appears crucial for the compounds' therapeutic efficacy. Current research emphasizes that depression involves dysfunction across multiple interacting systems [61], making multi-target approaches particularly valuable. This is especially relevant given recent evidence suggesting that treatment resistance may result from the failure to address multiple pathological mechanisms simultaneously [25]. The ability of ERP and Macelignan to simultaneously modulate these various pathways may explain their robust antidepressant effects and suggests potential advantages over single-target treatments.

The region-specific effects observed in our study provide important insights into the circuit-level actions of these compounds. Recent neuroimaging and molecular studies have highlighted distinct roles of different brain regions in depression pathophysiology [63]. The differential responses between hippocampus and prefrontal cortex in our study align with their established roles in emotional processing and stress response [68]. For example, the more pronounced effects on BDNF signaling in the hippocampus correlate with recent findings on region-specific neuroplasticity mechanisms [69], while the selective modulation of inflammatory markers in the prefrontal cortex aligns with current understanding of mood regulation circuits [70].

Our findings have substantial translational implications, particularly in the context of treatment-resistant depression [25]. The identification of Macelignan as an active antidepressant component provides a scientific basis for standardizing and optimizing ERP formulation, addressing a critical need in traditional medicine development [62, 71]. Moreover, the multi-pathway effects observed suggest potential utility in treatment-resistant cases, where single-mechanism drugs often fail [72]. Recent clinical guidelines have emphasized the importance of novel therapeutic approaches for treatment-resistant depression [73], making our findings particularly relevant for this challenging patient population.

The comparative analysis between ERP and Macelignan offers valuable insights into traditional medicine optimization. While Macelignan appears to be a key active ingredient, the broader effects of ERP in some parameters suggest that other components contribute through complementary mechanisms. This synergistic action aligns with emerging evidence supporting multi-component approaches in complex psychiatric disorders [74]. Furthermore, our findings contribute to the growing understanding of biomarkers in depression treatment. The consistent effects on oxidative stress markers, inflammatory factors, and BDNF signaling suggest potential biomarkers for treatment response [75], which could help identify patient subgroups most likely to benefit from these compounds.

Compared to other reported antidepressant treatments, our findings reveal several unique advantages of ERP and Macelignan. Traditional monoamine-based antidepressants typically show a response rate of 50–70% and require 6–8 weeks to achieve therapeutic effects [76]. In contrast, our results demonstrated that both ERP and Macelignan exhibited significant antidepressant effects within 30 days of administration, with efficacy comparable to conventional antidepressants in behavioral tests. Notably, while ketamine and its derivatives show rapid antidepressant effects within hours, concerns about their side effects and long-term safety remain [77]. Our compounds showed no obvious adverse effects during the treatment period, suggesting better safety profiles. Emerging innovative approaches have shown promise, including music therapy's protective effects against oxidative stress and inflammation [78], rabies virus glycoprotein-modified exosomes carrying BDNF for enhanced brain targeting and neurogenesis [79], and intranasal delivery of BDNF or antagomir-miR-10a-5p as a practical strategy combining therapeutic efficacy with ease of administration [80]. However, these approaches either require specialized delivery systems or face limitations in practical application. Our compounds showed no obvious adverse effects during the treatment period, suggesting better safety profiles. Recent studies on natural compounds like curcumin and resveratrol have shown promise in treating depression, with reported efficacy rates of 60% in clinical trials [81, 82]. The multi-target effects of ERP and Macelignan observed in our study, particularly their ability to simultaneously modulate oxidative stress, neuroinflammation, and synaptic plasticity, suggest potentially broader therapeutic benefits compared to single-target natural compounds. Furthermore, unlike some traditional Chinese medicines that require complex preparation processes, ERP offers the advantage of standardized formulation while maintaining multiple active components.

Several limitations of our study should be acknowledged. First, while we demonstrated multiple mechanisms of action, the temporal sequence of these effects remains unclear. Future studies using time-course analyses could help distinguish primary mechanisms from secondary consequences [23]. Second, our study focused primarily on male mice, and future investigations should examine potential sex-specific differences in treatment response [83], particularly given recent evidence of sex-dependent variations in antidepressant efficacy [70]. Third, while we observed promising acute effects, longer-term studies are needed to evaluate sustained efficacy and potential adaptation mechanisms [19].Looking forward, several key questions emerge for future investigation. First, the potential interactions between Macelignan and other ERP components require systematic evaluation using advanced pharmacological approaches. Second, the development of biomarker-guided treatment strategies could help optimize therapeutic outcomes [49]. Third, clinical studies are needed to validate these promising preclinical findings and establish optimal therapeutic protocols [73]. Additionally, research into the impact of these compounds on epigenetic regulation and synaptic homeostasis could provide further mechanistic insights.

In conclusion, our study provides comprehensive evidence for the antidepressant effects of both ERP and Macelignan while elucidating their underlying mechanisms involving oxidative stress, neuroinflammation, and neuroplasticity pathways. The multi-target approach demonstrated here represents a promising strategy for developing more effective treatments for this complex psychiatric disorder. These findings not only validate the traditional use of ERP but also identify Macelignan as a promising compound for antidepressant drug development, potentially opening new avenues for treating this devastating disorder. Future research should focus on translating these findings into clinical applications while further exploring the complex interactions between the multiple pathways involved in antidepressant response.

DATA AVAILABILITY

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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AUTHOR CONTRIBUTIONS

YD and YC conceived and designed this study; YLW, LC and XLZ performed the experiments; YLW, YD, WQL and QSL analyzed and interpreted the data; YD drafted the manuscript with critical revisions from all authors. all the authors interpreted the data and provided critical revisions for the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS

All animal experiments in this study were conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work the author(s) used Chatgpt in order to improve language and readability, with caution. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

ADDITIONAL INFORMATION

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