Patchouli alcohol inhibits the NLRP3 inflammasome in microglia to enhance neurogenesis in male mice exposed to chronic mild stress

hui he[1], fan xie[2], jin Zhang[3], Li Mo[1], xi Kang[1], Lei Xie[1], Yue Zhang[1], Lu Wang[1], nan hu[1], cheng peng[2], and Zili You[1]

[1]University of Electronic Science and Technology of China
[2]Chengdu University of Traditional Chinese Medicine
[3]Guizhou University of Traditional Chinese Medicine

March 07, 2024

Abstract

Abstract Background and Purpose: Microglia-mediated neuroinflammation contributes to major depressive disorder (MDD). Targeting microglia is a promising strategy for treating MDD. Patchouli alcohol (PA), a active component of Pogostemon cablin, has anti-inflammatory and neuroprotective effects. Here, we investigate the microglia-mediated neurogenesis pathway in which PA ameliorates depressive-like behaviors in stress-induced animal model of depression. Experimental Approach: C57BL/6 male mice were exposed to chronic mild stress (CMS) for 4 weeks, then administered PA intraperitoneally at 10, 20 or 40 mg/kg once per day for 3 weeks. The antidepressant effects of PA were evaluated in the sucrose preference test, forced swimming test, and tail suspension test. Microglial phenotypes and activation of the NLRP3 inflammation were analyzed using RT-PCR, western blotting and immunofluorescence staining. Effects of PA on neurogenesis were analyzed in vitro and in vivo using immunofluorescence staining. Key Results: Behavioral assessments showed that PA alleviated depressive-like behaviors in CMS-exposed mice. CMS induced microglial activation and pro-inflammatory profiles, which were blocked by PA treatment. PA attenuated the activation of NLRP3 inflammasome, leading to decreases in the levels of caspase-1, ASC, IL-1β, and IL-18 in the hippocampus of CMS-exposed mice. In primary microglia cultures, PA inhibited LPS-induced NLRP3 inflammasome activation. PA rescued inflammation-inhibited neurogenesis in vivo and in vitro. Conclusion and implications: Our results suggest that PA inhibits the NLRP3 inflammasome and promotes microglia-mediated neurogenesis, leading to antidepressant effects. Thus, PA may be a novel treatment for inflammation-driven mental disorders.

1. Introduction

Major depressive disorder (MDD), a neuropsychiatric disorder with multi-factorial origins, affects more than 300 million people worldwide (Jo et al., 2015; Y. M. Zhang et al., 2021). Symptoms of depression include loss of interest in activities, loss of energy and "grit", and even thoughts of suicide (Krishnan & Nestler, 2010; Nestler et al., 2002). The heterogeneity of MDD has hampered efforts to elucidate its mechanisms and to develop effective treatments (Tan et al., 2020). Most drugs against MDD follow the "monoamine strategy", based on increasing serotonergic and noradrenergic signaling (Spellman & Liston, 2020), but these drugs take longer to show efficacy, they can cause substantial side effects, and they are ineffective in about one third of patients (Maes et al., 2011).

Microglia are the innate immune cells of the central nervous system (CNS), they actively respond to challenges, and they maintain brain homeostasis (Dani et al., 2018; Rustenhoven et al., 2018; L. Zhang et al., 2021). Specifically expressed in microglia in the CNS, the nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3) acts as a sensor and regulator of innate immune cells and plays a crucial role in stress responses (Arioz et al., 2019; Heneka et al., 2018). The NLRP3 inflammasome triggers

hyperactivation of microglia and mediates the pathogenesis of MDD (Su et al., 2017; Yirmiya et al., 2015). Microglia is an important population of cells within in the neurogenic niche, which releases various mediators that maintain the proliferation and differentiation of neuronal progenitor cells (NPCs) (Cope & Gould, 2019; J. Zhang et al., 2017). Microglial hyperactivation impairs neurogenesis in the hippocampus, and this impairment has been linked to depression (Kreisel et al., 2014; Samuels & Hen, 2011; Tang et al., 2016).

Traditional medicines that inhibit inflammation have long been used in China and elsewhere for the treatment of neuropsychiatric disease (J. Zhang et al., 2017; L. Zhang et al., 2021). *Pogostemon cablin* , which belongs to the Lamiaceae family and is broadly distributed throughout Southeast Asia, has been widely used in traditional health foods and medical practice (Junren et al., 2021; X. Lu et al., 2016). Patchouli alcohol (PA), a tricyclic sesquiterpene from *Pogostemon cablin* , exerts anti-inflammatory effects, as well as antibacterial and oxidative activities (Lian et al., 2018; Q. Lu et al., 2021; Xu et al., 2017), while triggering minimal toxicity (G. Hu et al., 2017; Y. Zhang et al., 2021). PA can switch macrophages from a pro- to anti-inflammatory phenotype and inhibit multiple inflammatory processes (Leong et al., 2019). PA also plays a neuroprotective role by repairing the blood-brain barrier and inhibiting inflammatory responses in a model of ischemic injury (Wei et al., 2018). PA exerts antidepressant effects in rat models of depression (G. Hu et al., 2017; Zhuo et al., 2020). However, much more work is needed in order to clarify how PA alleviates depression and to guide its clinical use.

We hypothesize that the antidepressant effects of PA are associated with the inhibition of NLRP3 inflammasome-dependent microglial activation and therefore the promotion of adult hippocampus neurogenesis. To test this hypothesis, we evaluated the effects of PA on depressive-like behaviors in mice exposed to chronic mild stress (CMS). Then, we investigated the ability of PA to modulate stress-induced NLRP3 inflammasome responses and microglial phenotypes. We also examined the effects of PA on neurogenesis *in vivo* and *in vitro* . Our results suggest that PA indeed restores stress-inhibited neurogenesis by blocking NLRP3-mediated microglial activation.

2. Material and methods

2.1. Animals

C57/BL male mice were purchased from Chengdu Dossy Experimental Animal Co. Ltd. (Chengdu, China). The mice were housed individually in cages, with *ad libitum* access to food and water, under specific pathogen-free conditions at a temperature of 25 ± 1.5 °C and relative humidity of 65 ± 5%, on a 12-h light/dark cycle (19:00 to 07:00 h). Mice were allowed to acclimate for 2 weeks, then they were grouped by weight and sucrose preference. All experimental procedures were approved by the Ethics Committee of the University of Electronic Science and Technology of China (1420200407-2) and carried out in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (8th edition, revised 2010).

2.2. CMS

Animals were exposed to CMS in the form of the following complex, unpredictable, and cooperative stressors (J. Zhang et al., 2021): food and water deprivation (12 h), reversal of day-night cycle (12 h), tail pinch (5 min), wet cage (24 h), empty cage (24 h), tilted cage (45°, 24 h), soiled bedding (24 h), restraint (2 h), and swimming in ice water (10 min). Mice were subjected to different stressors each day to prevent habituation.

2.3. Pharmacological treatments

PA (Fig. S1) was prepared to [?] 94% purity as described (G. Y. Hu et al., 2018) and dissolved in 1% DMSO to concentrations of 1, 2 or 4 mg/ml before use. Imipramine hydrochloride (IMI, Sigma-Aldrich, Germany) was dissolved in 0.9% saline to a final concentration of 2 mg/ml. Mice were randomly divided into the following groups: control + DMSO (CON), control + 20 mg/kg PA (PA), CMS + DMSO (CMS), CMS + 10 mg/kg PA (CMS+PA10), CMS + 20 mg/kg PA (CMS+PA20), CMS + 40 mg/kg PA (CMS+PA40), and CMS + 20 mg/kg IMI (CMS+IMI). CMS animals were exposed to stressors for 4 weeks, then administered PA or IMI by intraperitoneal injection for 3 weeks. The IMI dose was based on previous studies (J. Q. Zhang et al., 2016).

2.4. Behavioral measurements

2.4.1. Sucrose preference test (SPT)

After the animals had acclimated to their housing for 2 weeks, they were trained for the SPT during 48 h as described (J. Q. Zhang et al., 2016). During the first 24 h, mice were provided two bottles of 1% sucrose in water; during the second 24 h, one bottle of sucrose was replaced by drinking water. During the SPT, the mice were deprived of food and water for 24 h, then given one bottle of 1% sucrose and one bottle of drinking water. Both bottles were weighed prior to the test and again 2 h later. Sucrose preference was calculated using the equation: Sucrose preference (%) = sugar consumption (g) / [sugar consumption (g) + water consumption (g)] x 100%. Once each week for 7 weeks, animals were subjected to the SPT and weighed.

2.4.2 Tail suspension test (TST)

The TST was performed as described (Zomkowski et al., 2006), with minor adjustments. Mice whose sight and hearing were blocked were suspended by the tail at a height of 30 cm from the floor using tape. Absolute lack of movement by the animal was defined as immobility, and the time spent immobile during 6 min was recorded.

2.4.3 Forced swim test (FST)

The forced swimming test was performed as before (J. Q. Zhang et al., 2016). Mice were placed into a beaker (25 cm high, 12 cm in diameter) containing 18 cm of water. The temperature of water was kept around 25degC. A pre-test was performed 24 h before the actual test. Immobility time was recorded during the last 4 min of the entire 6-min test. To avoid residual odor interference, water in the beaker was replaced every time a test finished.

2.4.4 Open field test (OFT)

Mice were placed gently in the center of a rectangular chamber (50 x 50 x 50 cm) and allowed to explore the area for 5 min. Digitized images of the path of each mouse were stored, and the locomotion activity and time spent in the center were analyzed using OFT100 software (Taimeng Tech, China).

2.4.5 Coat score

The coat score can provide behavioral insights into the behavior of stressed animals because it reflects self-scratching or biting linked to mood disorders (Cao et al., 2013). The physical state of the fur was assessed after the 4-week CMS period and again after the 3-week drug administration period. The total coat score was calculated as the sum of individual scores for the head, neck, forepaws, dorsal coat, ventral coat, hind paws, and tail. A score of 0 was given if the coat was unkempt, 1 if it was well-groomed.

2.4.5 Locomotor activity test (LAT)

The locomotor activity test was performed to evaluate spontaneous activity in mice after the last administration of PA or IMI. Animals were placed in six separate areas and allowed to acclimate for 1 min, then the times spent standing and moving were recorded for 10 min (Techman Software zz6, China). In order to avoid odor interference, the apparatus was cleaned with 75% alcohol after each use.

2.5. Cell culture

2.5.1. Primary microglia culture

Primary microglia were collected and cultured as described (Zhao et al., 2019). Brains were collected from C57BL/6J mice aged 0-3 days old and placed into cold, sterile phosphate-buffered saline (PBS; catalog no. G4202, Servicebio, China). The meninges was carefully removed but the parenchyma was retained, and the brains were digested for 5 min at 37 degC with 0.25% pancreatin (catalog no. 25200056, Gibco, USA). The digestion was halted by adding one volume of microglial medium containing 10% fetal bovine serum (catalog no. 10099141C, Gibco) in DMEM/F12 (catalog no. C11330500BT, Gibco), then filtered through a 70-μm

cell strainer. The suspension was centrifuged at 300 g for 10 min, and the pellet was washed with PBS, then centrifuged again at 300 g for 10 min, and finally resuspended in 15 ml of microglial medium. The cells were seeded into a 75-cm^2 square culture flask, which was placed for 48 h at 37 °C in an atmosphere of 5% CO_2. The medium was changed after 48 h, and primary microglia were collected on day 14 for experiments. The isolated microglia were treated for 3 h with 100 ng/ml LPS (Sigma-Aldrich, Germany) or 0.05% DMSO (catalog no. D2650, Sigma-Aldrich, Germany), then with PA for 1 h, and finally with ATP (5 μM) for 30 min. The treated microglia were seeded into 6-well plates at 1 × 10^5cells/cm^2 for experiments ending in RT-PCR analysis and protein assay, or into 24-well plates at 5 ×10^4cells/cm^2 for experiments ending in immunocytochemistry.

2.5.2. NPC culture

NPCs were isolated from C57/BL mice aged 0-3 days and cultured as described (L. Zhang et al., 2021). Brains were removed as described in section 2.5.1 and the hippocampal regions were quickly removed, placed into high-glucose DMEM/F12, digested with enzyme A at 37 °C for 10 min, then incubated for 3 days in high-glucose DMEM/F12 containing B27 supplement (× 200), N2 (× 100), 20 ng/ml FGF2, and 20 ng/ml EGF at 37 °C in an atmosphere of 5% CO_2. Differentiated cells were cultured in differentiation medium (high-glucose DMEM/F12, 40 ng/ml N2, 80 ng/ml B27 supplement, and 10% fetal bovine serum).

2.5.3. Conditioned microglial medium for NPC culture

Primary microglia were treated with PBS or 100 ng/mL LPS for 3 h, followed by PA or not for 30 min, then finally 5 μM ATP for 30 min. Next, the medium was replaced with high-glucose DMEM/F12 and cultured at 37 °C in an atmosphere of 5% CO_2. After 24 h, the culture medium was collected for use as NPC differentiation medium. The various differentiation media were labeled as follows to indicate how the primary cultures had been treated before the conditioned medium was harvested: CM was harvested from microglia cultured in the presence of PBS; PA-M-CM, from microglia cultured in PA; LPS-M-CM, from microglia cultured in the presence of LPS followed by ATP; and PA+LPS-CM, from microglia cultured in the presence of LPS, then PA and finally ATP.

2.6. RNA extraction and reverse transcription-quantitative PCR (RT-PCR)

Mice were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital (R&D Systems, USA) that had been diluted in 0.9% saline. Mice were perfused with saline to eliminate the influence of peripheral cytokine secretion on the CNS, then the hippocampus was quickly dissociated on ice. Total RNA was extracted with Trizol (Invitrogen Life Technologies, USA) as described (31078757) and reverse-transcribed with the First Strand cDNA Synthesis Kit (Takara Biomedical Technology, Japan). The cDNA was stored at 80 °C, and later analyzed by RT-qPCR using TaKaRa reagents (catalog no. 220 6210A, Japan) and a Bio-Rad CFX 96 cycler (Hercules, USA) with the following cycling conditions: 95 °C for 10 min, followed by 38 cycles at 95 °C for 3 s, at the annealing temperature for 30 s, and 72 °C for 5 min extension. Gene expression was analyzed using the 2^{-Ct} method relative to the expression of β-actin.

The following primer pairs were used for gene amplification: β-actin, 5'-CCG TGA AAA GAT GAC CCA GAT C-3' and 5'-CAC AGC CTG GAT GGC TAC GT-3'; IL-1β, 5'-GAA ATG CCA CCT TTT GAC AGT GAT-3' and 5'-TCT CCA CAG CCA CAA TGA GTG-3'; NLRP3, 5'-GAC CGT GAG GAA AGG ACC AG-3' and 5'-GGC CAA AGA GGA ATC GGA CA-3'; IL-18, 5'-GAC TCT TGC GTC AAC TTC AAG G-3' and 5'-CAG GCT GTC TTT TGT CAA CGA-3'; TNF-α, 5'-TAC TGA ACT TCG GGG TGA TTG GTC C-3' and 5'-CAG CCT TGT CCC TTG AAG AGA ACC-3'; and IL-6, 5'-TAG TCC TTC CTA CCC CAA TTT CC-3' and 5'-TTG GTC CTT AGC CAC TCC TTC-3'.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Hippocampal sections of mice were sonicated in RIPA buffer (catalog no. D2650, Solarbio, China) containing protease inhibitors. Cytokine concentrations were quantified using ELISA kits (catalog no. EMC001b.48, QuantiCyto, China) according to the manufacturer's protocol.

2.8. Western blotting

Hippocampal tissue or isolated hippocampal microglia were sonicated in RIPA buffer containing protease inhibitors, and total soluble protein concentration was assayed using a BCA kit (catalog no. P0012S, Beyotime, China). Equal amounts of protein (30 μg at 2 μg/μl) were fractionated on 12% Tris-glycine SDS/PAGE gels and transferred to a PVDF membrane (0.22 or 0.45 μm). Membranes were blocked with 5% milk, then incubated overnight at 4 °C with primary antibodies against the following proteins: IL-6 (1:3,000; catalog no. 66146-1-Ig, Proteintech, China), TGF-β (1:1000; catalog no. ER65189, HUABIO, China), IL-1β (1:500; catalog no. bs-20449R, Bioss, China), NLRP3 (1:1000; catalog no. AG-20B-0014, AdipoGen, USA), caspase-1 (1:1000; catalog no. AG-20B-0042-C100, AdipoGen), ASC (1:1000; catalog no. sc-514414, Santa Cruz Biotechnology, USA), GAPDH (1:1000; catalog no. GB11002, Servicebio) and β-actin (1:2000; catalog no. GB11001, Servicebio). Then membranes were incubated for 2 h at room temperature with horseradish peroxidase-conjugated goat secondary antibodies against rabbit IgG (1:10000; catalog no. ab6721, Abcam, UK) or mouse IgG (1:10000; catalog no. ab6789, Abcam). Antibody binding was detected using the BeyoECL Plus kit (catalog no. P0018S, Beyotime) and quantitated using Image J software (version 1.45 J; National Institutes of Health, USA).

2.9. Immunofluorescence

To examine the differentiation of NPCs, BrdU (50 mg/kg) was injected on three consecutive days. Mice were deeply anesthetized with 1% pentobarbital (10 ml/kg; R&D Systems, USA), then slowly infused with PBS, and finally fixed with 4% paraformaldehyde (pH 7.2). Brains were collected and post-fixed with 4% paraformaldehyde for 24 h, then dehydrated by sequential immersion for 12 h each in 10%, then 20%, and finally 30% sucrose. The dehydrated tissue was stored at -20 °C and later sliced into 30-μm sections on a freezing microtome (CM1900; Leica Microsystems, Wetzlar, Germany). All slices were collected into PBS containing 0.02% NaN_3 and stored at 4 °C.

For immunofluorescence staining, slices were first washed with PBS 3 times, each time for 5 min, then treated with Immunol Staining Wash Buffer (5 min; catalog no. P0106C, Beyotime), Quick Antigen Retrieval Solution for Frozen Sections (catalog no. P0090, Beyotime) (5 min), and QuickBlock Blocking Buffer for Immunol Staining (catalog no. P0252, Beyotime) (1 h). Then slices were incubated with primary antibody overnight at 4 degC, warmed to 37 degC for 1 h, washed with PBS, incubated with secondary antibody at 37 degC for 1 h, washed again and stained with DAPI. Primary antibodies were against the following proteins: Iba1 (1:400; catalog no. ab5076, Abcam), NLRP3 (1:100; catalog no. AG-20B-0014, AdipoGen), ASC (1:100; catalog no. sc-514414, Santa Cruz Biotechnology), BrdU (1:500; catalog no. ab6326, Abcam), DCX (1:400; catalog no. 4604S, Cell Signaling Technology, USA), and GFAP (1:400; catalog no. 3670, Cell Signaling Technology). The secondary antibodies were as follows: donkey anti-mouse IgG (1:1000; catalog no. A21202, Invitrogen Life Technologies, USA), donkey anti-rabbit IgG (1:1000; catalog no. A21207, Invitrogen Life Technologies), goat anti-rabbit IgG (1:1000; catalog no. A32731, Invitrogen Life Technologies), and donkey anti-goat IgG (1:500; catalog no. 705-585-003, Jackson ImmunoResearch, USA).

The sections were mounted on slides and imaged under a fluorescence microscope (Carl Zeiss, Germany).

2.10. Statistical analysis

All quantitative results were expressed as mean +- standard error of the mean (SEM), n[?] 5. Data were plotted and analyzed statistically using GraphPad Prism 7.0 (Solvusoft, USA). Normal distribution of data was checked using the Shapiro-Wilk test, and two groups differences were assessed for significance using unpaired t-test and Welch's correction or a non-parametric Mann-Whitney test. Differences among three or more groups were assessed using one- or two-way analysis of variance and Tukey's *post hoc* tests.

3. Results

3.1 PA ameliorated depressive-like behaviors in CMS mice

The effects of PA on depressive-like behaviors were examined in a mouse model of CMS-induced depression.

Mice were exposed to CMS, then treated for 3 weeks with DMSO or PA at 10, 20, or 40 mg/kg (Fig. 1A). In the sucrose preference test, which can assess anhedonia in mice, CMS-exposed animals displayed lower sucrose preference than the CON group, while PA at all three doses remarkably increased such preference, similar to the licensed tricyclic antidepressant IMI (Fig. 1B).CMS prolonged the time that animals were immobile in the TST and FST, reflecting desperation behavior. These stress-induced, depressive-like behaviors were partially reversed by PA at the two higher doses, similar to the effect of IMI (Fig. 1C-1D). At the same time, CMS led to weight loss and coat deterioration in the mice. PA improved the physical state of the coat and markedly increased body weight of CMS-exposed animals (Fig. 1H and Fig. S2A).

These effects likely reflect a purely antidepressant mechanism, since PA did not significantly affect distance travelled in the OFT (Fig. 1F) or locomotor activity in the LAT (Fig. S2B). PA did not affect behavior, spontaneous activity or body weight of unstressed control animals. Since PA significantly improved all abnormal behaviors in CMS-exposed mice at a dose of 20 mg/kg, but not 10 mg/kg or 40 mg/kg, we used the dose of 20 mg/kg in subsequent experiments.

3.2. PA suppressed CMS-induced microglial activation in hippocampus

To explore the effects of PA on microglia, we used immunohistochemical staining to detect the morphological profiles of microglia in the dentate gyrus and hippocampal regions CA1 and CA3. CMS increased the number and area of microglia, with the microglia appearing amoeboid and featuring many larger branches and shorter branches (Fig. 2A-B). PA partially reversed these CMS-induced changes (Fig. 2A-B). Sholl analysis showed that PA treatment also reversed the CMS-induced increase in number of hippocampal microglia intersections (Fig. 2C). CMS stimulated secretion of the pro-inflammatory cytokines IL-1β, L-18, IL-6, and TNF-α in the hippocampus, which PA suppressed (Fig. 2D -2E). PA alone showed no obvious effects on microglia in unstressed control mice.

These findings suggest that PA significantly relieves stress-induced neuroinflammation by inhibiting microglial activation in hippocampus.

3.3 PA inhibited activation of the NLRP3 inflammasome in microglia

Depression linked to neuroinflammation appears to involve activation of the NLRP3 inflammasome in microglia (Franklin et al., 2018). Therefore, we wondered whether PA might inhibit such activation, so we examined the effects of PA on NLRP3 inflammasome in the microglial cytoplasm. Indeed, PA substantially reversed the CMS-induced increases in levels of NLRP3 protein and in numbers of $NLRP3^{+}$-$Iba1^{+}$ cells in the hippocampus (Fig. 3A). PA also significantly suppressed CMS-induced increases in levels of ASC protein and in numbers of ASC^{+}-$Iba1^{+}$ cells in the hippocampus (Fig. 3B).

Activation of the NLRP3 inflammasome requires the cleavage of pro-caspase-1 into its active form, which then cleaves pro-IL-1β and pro-IL-18 into their functional forms, ultimately initiating inflammatory responses (Gordon et al., 2018; Heneka et al., 2018). CMS elevated levels of cleaved caspase-1 and cleaved IL-1β in hippocampus, which PA partially reversed. However, PA did not alter levels of pro-caspase-1 or pro-IL-1β in the hippocampus of CMS-exposed mice (Fig. 3C). These findings suggest that PA prevents the NLRP3 inflammasome from activating microglia in the hippocampus of CMS-exposed mice.

To further examine the apparent effects of PA on the NLRP3 inflammasome, we switched to primary cultures of microglia. PA did not significantly affect primary microglial survival (Fig. 4A). We found that 40 μM PA downregulated NLRP3, IL-β, TNF-α and IL-18 at the mRNA level (Fig. 4B). ELISA showed that IL-1β protein levels decreased significantly after treatment with 40 μM PA (Fig. 4C). In primary microglia treated with LPS and ATP, PA reduced the formation of ASC puncta (Fig. 4D) and decreased the fluorescence intensity of NLRP3 signal in the cytoplasm (Fig. 4E). Consistent with *in vivo* results, PA significantly suppressed NLRP3 and ASC protein levels in primary microglia, as well as secretion of cleaved caspase-1 and cleaved IL-1β into the culture medium (Fig. 4F).

In contrast, PA did not obviously affect activation of the NLRP3 inflammasome in primary microglia in the absence of LPS or ATP. These results suggest that PA prevents the NLRP3 inflammasome from activating

primary microglia by reducing the formation of ASC specks.

3.4 PA reversed CMS-induced inhibition of neurogenesis in hippocampus

Chronic stress stimulates the hippocampal microglia secretion of IL-1β, which suppresses neurogenesis and causes depression-like behaviors (Kreisel et al., 2014). We wondered whether PA might restore hippocampal neurogenesis in CMS mice by suppressing activation of the NLRP3 inflammasome and downregulating IL-1β. After injecting BrdU into animals to label proliferating cells and DCX to label immature neurons, we found that the numbers of $BrdU^+$, DCX^+ and $BrdU^+$-DCX^+ proliferating cells in dentate gyrus were significantly lower in CMS-treated animals than in CON animals (Fig. 5A). PA partially reversed these effects. Similarly, the number of DCX^+ cells in dentate gyrus was significantly decreased in CMS-exposed mice, which PA restored (Fig. 5B). At the same time, PA significantly increased the volume of the granular cell layer (Fig. 5C). These results suggest that PA can restore neurogenesis in CMS animals. PA did not, however, affect neurogenesis in unstressed control mice.

These results are consistent with the idea that PA enhances neurogenesis by inhibiting microglial activation, but other explanations are possible. In order to gain more direct evidence for microglial activation, we examined the numbers of $MAP2^+$ and $GFAP^+$ cells in primary cultures of NPCs incubated with PA alone, or with conditioned medium from microglia that had been cultured under different conditions. PA on its own did not obviously affect numbers of $MAP2^+$ or $GFAP^+$cells (Fig. 6A). Conditioned medium from microglia treated with LPS and ATP significantly reduced the number of $MAP2^+$ cells, which PA partially reversed. Notably, treating NPCs with conditioned medium from LPS+ATP+PA microglia did not significantly alter the number of $GFAP^+$ cells (Fig. 6B-6C). These results further support that PA enhances neurogenesis by inhibiting microglial activation.

4. Discussion

Under stress, the NLRP3 inflammasome is activated in microglia, leading to the release of inflammatory mediators and to the inhibition of neurogenesis in the hippocampus, and these changes are associated with depressive-like behaviors (Colonna & Butovsky, 2017; Ndoja et al., 2020). In this study, we found that PA ameliorates depressive-like behaviors by inhibiting activation of the microglial NLRP3 inflammasome and enhancing neurogenesis in the hippocampus of CMS-exposed mice.

Our work provides the molecular analyses justifying the longstanding use of *Pogostemon cablin* in traditional medicine as a treatment against mental disorders (X. Lu et al., 2016). Our demonstration of therapeutic efficacy, coupled with safety information from the long-term clinical use of *P. cablin* (Junren et al., 2021) and from experimental assays of PA toxicity (G. Hu et al., 2017), rationalizes the use of PA as an antidepressant and may help guide future efforts to develop effective treatments against MDD. We assessed the antidepressant effects of PA and its mechanism of action in CMS mice. The CMS model of depression is widely used to elucidate the neurobiology of depression as well as explore potential treatments (Willner, 2017). CMS-exposed mice showed a variety of abnormal behaviors, including decreased response to rewards in the SPT and longer immobility time in the FST and TST. PA or IMI reversed the anhedonia and desperate behaviors. Our results suggest that PA is not only effective but also safe in a preclinical animal model. In fact, PA reversed the weight loss and coat degradation induced by CMS, suggesting better survival status, consistent with the drug's tolerability and gastroprotective effects (Zheng et al., 2014). These results indicate that PA is a potentially safe and effective drug candidate for the treatment of depression in animal models, but further study is needed in advance of clinical application.

Chronic neuroinflammation mediated by microglial activation is considered a vital pathological hallmark of MDD, and such activation manifests as an increase in the number of microglia and changes in their morphology (Colonna & Butovsky, 2017; Ndoja et al., 2020). CMS-exposed mice showed hyperactivation of microglia, as detected in substantial enlargement of the soma, increased number of branches, elongation of branches, and increased number of $Iba1^+$ cells. PA reversed these changes and decreased the release of pro-inflammatory factors in the hippocampus, leading to milder behavioral defects, consistent with previous work in which PA alleviated depression-like behavior in rats, potentially via the mTOR pathway, but there

is insufficient evidence to prove the potential action target of PA (Zhuo et al., 2020). Our finding shows, for the first time, that PA regulates microglial activation and plays an antidepressant role by alleviating neuroinflammation. We focused on microglia in the hippocampus, because this brain region is involved in behavioral responses to stress, and it has been tightly linked to the pathophysiology of MDD (Anacker & Hen, 2017). In the absence of CMS, PA did not obviously affect microglia, confirming its tolerability and safety. Our results justify further study to clarify how PA suppresses microglial activation.

The NLRP3 inflammasome acts as a sensor and effector activated by chronic stress, which in turn leads to depression-like behaviors (Han et al., 2021; Y. Zhang et al., 2015). In the CMS model, stress elevated levels of NRLP3 protein and numbers of NRLP3$^+$ microglia in hippocampus, and PA partially reversed these effects. Consistent with the effects of PA *in vivo* , we found that PA directly inhibited activation of the microglial NLRP3 inflammasome in primary cultures of microglia. In these cells, the activated NLRP3 inflammasome activates caspase-1, which promotes the cleavage of pro-IL-1β and pro-IL-18 to their mature and functional states, ultimately initiating inflammatory responses, impairing neurons and exacerbating disease progression (Gordon et al., 2018; Heneka et al., 2018). CMS increased levels of cleaved-caspase-1 and cleaved-IL-1β in the hippocampus via activation of the NLRP3 inflammasome. PA decreased the levels of caspase-1 and IL-1β by inhibiting the microglial NLRP3 inflammasome. Whether PA binds directly to NLRP3 is unclear: we found that PA downregulated ASC expression, implying that it prevents the binding of ASC to NLRP3, and this binding is important for inflammasome activation (Hara et al., 2013; A. Lu et al., 2014). Treating primary microglia with PA reduced the formation of ASC spots. Co-immunoprecipitation and other experiments are needed to explore in detail how PA inhibits NLRP3 inflammasome activation. Taken together, our results suggest that PA exerts direct anti-inflammatory effects that inhibit activation of the NLRP3 inflammasome.

Stress-induced microglia release abundant proinflammatory cytokines that initiate neuroinflammation and impair neurogenesis, which may drive MDD pathogenesis and progression (Egeland et al., 2017; Yi et al., 2021). Depressed patients show reduced hippocampal neurogenesis and even hippocampal atrophy (Cameron & Schoenfeld, 2018; Roddy et al., 2019; Schoenfeld et al., 2017). Antidepressants can exert their effects by restoring hippocampal neurogenesis (Czéh et al., 2001; Koo & Duman, 2008; Ramirez et al., 2015). The activated NLRP3 inflammasome appears to contribute to depressive-like behaviors, at least in part, by inhibiting neurogenesis (Komleva et al., 2021). We found that PA significantly enhanced neurogenesis in CMS animals. We also found that PA may reduce or even reverse hippocampal atrophy, based on measurement of DG and GCL volumes. Enhanced neurogenesis was sufficient to produce antidepressant effects and was effective at alleviating depressive symptoms (Ramirez et al., 2015). Accordingly, drugs that enhance neurogenesis may be promising antidepressant treatments. Our experiments suggest that PA stimulates neurogenesis not directly by acting on NPCs, but indirectly by inhibiting microglial activation and thereby the secretion of neuroinflammatory factors, which rescues impaired neurogenesis.

In conclusion, PA acts as an effective anti-inflammatory compound by attenuating NLRP3-dependent microglial activation in CMS-exposed mice. Our study provides the first evidence that PA exerts antidepressant effects by alleviating neuroinflammation and promoting neurogenesis in an animal model of depression. The findings indicate that PA might be a novel therapy to treat depressive disorders.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (U1808204), Sichuan Science and Technology Program (2020YJ0225, 2019JDPT0010), the Open Research Fund, Key Laboratory of Systematic Research, Distinctive Chinese Medicine Resources in Southwest China (2019003), and CAS Key Laboratory of Mental Health, Institute of Psychology (KLMH2019K04). We are grateful to A. Chapin Rodríguez for help in revising the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Declaration of Competing Interest

The authors declare no conflicts of interest.

References

Anacker, C., & Hen, R. (2017). Adult hippocampal neurogenesis and cognitive flexibility - linking memory and mood. *Nat Rev Neurosci, 18* (6), 335-346. doi:10.1038/nrn.2017.45

Arioz, B. I., Tastan, B., Tarakcioglu, E., Tufekci, K. U., Olcum, M., Ersoy, N., Bagriyanik, A., Genc, K., & Genc, S. (2019). Melatonin Attenuates LPS-Induced Acute Depressive-Like Behaviors and Microglial NLRP3 Inflammasome Activation Through the SIRT1/Nrf2 Pathway.*Front Immunol, 10* , 1511. doi:10.3389/fimmu.2019.01511

Cameron, H. A., & Schoenfeld, T. J. (2018). Behavioral and structural adaptations to stress. *Front Neuroendocrinol, 49* , 106-113. doi:10.1016/j.yfrne.2018.02.002

Cao, X., Li, L. P., Wang, Q., Wu, Q., Hu, H. H., Zhang, M., Fang, Y. Y., Zhang, J., Li, S. J., Xiong, W. C., Yan, H. C., Gao, Y. B., Liu, J. H., Li, X. W., Sun, L. R., Zeng, Y. N., Zhu, X. H., & Gao, T. M. (2013). Astrocyte-derived ATP modulates depressive-like behaviors. *Nat Med, 19* (6), 773-777. doi:10.1038/nm.3162

Colonna, M., & Butovsky, O. (2017). Microglia Function in the Central Nervous System During Health and Neurodegeneration. *Annu Rev Immunol, 35* , 441-468. doi:10.1146/annurev-immunol-051116-052358

Cope, E. C., & Gould, E. (2019). Adult Neurogenesis, Glia, and the Extracellular Matrix. *Cell Stem Cell, 24* (5), 690-705. doi:10.1016/j.stem.2019.03.023

Czéh, B., Michaelis, T., Watanabe, T., Frahm, J., de Biurrun, G., van Kampen, M., Bartolomucci, A., & Fuchs, E. (2001). Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine.*Proc Natl Acad Sci U S A, 98* (22), 12796-12801. doi:10.1073/pnas.211427898

Dani, M., Wood, M., Mizoguchi, R., Fan, Z., Walker, Z., Morgan, R., Hinz, R., Biju, M., Kuruvilla, T., Brooks, D. J., & Edison, P. (2018). Microglial activation correlates in vivo with both tau and amyloid in Alzheimer's disease. *Brain, 141* (9), 2740-2754. doi:10.1093/brain/awy188

Egeland, M., Guinaudie, C., Du Preez, A., Musaelyan, K., Zunszain, P. A., Fernandes, C., Pariante, C. M., & Thuret, S. (2017). Depletion of adult neurogenesis using the chemotherapy drug temozolomide in mice induces behavioural and biological changes relevant to depression.*Transl Psychiatry, 7* (4), e1101. doi:10.1038/tp.2017.68

Franklin, T. C., Wohleb, E. S., Zhang, Y., Fogaça, M., Hare, B., & Duman, R. S. (2018). Persistent Increase in Microglial RAGE Contributes to Chronic Stress-Induced Priming of Depressive-like Behavior.*Biol Psychiatry, 83* (1), 50-60. doi:10.1016/j.biopsych.2017.06.034

Gordon, R., Albornoz, E. A., Christie, D. C., Langley, M. R., Kumar, V., Mantovani, S., Robertson, A. A. B., Butler, M. S., Rowe, D. B., O'Neill, L. A., Kanthasamy, A. G., Schroder, K., Cooper, M. A., & Woodruff, T. M. (2018). Inflammasome inhibition prevents α-synuclein pathology and dopaminergic neurodegeneration in mice. *Sci Transl Med, 10* (465). doi:10.1126/scitranslmed.aah4066

Han, X., Xu, T., Fang, Q., Zhang, H., Yue, L., Hu, G., & Sun, L. (2021). Quercetin hinders microglial activation to alleviate neurotoxicity via the interplay between NLRP3 inflammasome and mitophagy. *Redox Biol, 44* , 102010. doi:10.1016/j.redox.2021.102010

Hara, H., Tsuchiya, K., Kawamura, I., Fang, R., Hernandez-Cuellar, E., Shen, Y., Mizuguchi, J., Schweighoffer, E., Tybulewicz, V., & Mitsuyama, M. (2013). Phosphorylation of the adaptor ASC acts as a molecular switch that controls the formation of speck-like aggregates and inflammasome activity. *Nat Immunol, 14* (12), 1247-1255. doi:10.1038/ni.2749

Heneka, M. T., McManus, R. M., & Latz, E. (2018). Inflammasome signalling in brain function and neurodegenerative disease. *Nat Rev Neurosci, 19* (10), 610-621. doi:10.1038/s41583-018-0055-7

Hu, G., Peng, C., Xie, X., Zhang, S., & Cao, X. (2017). Availability, Pharmaceutics, Security, Pharmacokinetics, and Pharmacological Activities of Patchouli Alcohol. *Evid Based Complement Alternat Med, 2017* , 4850612. doi:10.1155/2017/4850612

Hu, G. Y., Peng, C., Xie, X. F., Xiong, L., Zhang, S. Y., & Cao, X. Y. (2018). Patchouli alcohol isolated from Pogostemon cablin mediates endothelium-independent vasorelaxation by blockade of Ca(2+) channels in rat isolated thoracic aorta. *J Ethnopharmacol, 220* , 188-196. doi:10.1016/j.jep.2017.09.036

Jo, W. K., Zhang, Y., Emrich, H. M., & Dietrich, D. E. (2015). Glia in the cytokine-mediated onset of depression: fine tuning the immune response. *Front Cell Neurosci, 9* , 268. doi:10.3389/fncel.2015.00268

Junren, C., Xiaofang, X., Mengting, L., Qiuyun, X., Gangmin, L., Huiqiong, Z., Guanru, C., Xin, X., Yanpeng, Y., Fu, P., & Cheng, P. (2021). Pharmacological activities and mechanisms of action of Pogostemon cablin Benth: a review. *Chin Med, 16* (1), 5. doi:10.1186/s13020-020-00413-y

Komleva, Y. K., Lopatina, O. L., Gorina, Y. V., Chernykh, A. I., Trufanova, L. V., Vais, E. F., Kharitonova, E. V., Zhukov, E. L., Vahtina, L. Y., Medvedeva, N. N., & Salmina, A. B. (2021). Expression of NLRP3 Inflammasomes in Neurogenic Niche Contributes to the Effect of Spatial Learning in Physiological Conditions but Not in Alzheimer's Type Neurodegeneration. *Cell Mol Neurobiol* . doi:10.1007/s10571-020-01021-y

Koo, J. W., & Duman, R. S. (2008). IL-1beta is an essential mediator of the antineurogenic and anhedonic effects of stress. *Proc Natl Acad Sci U S A, 105* (2), 751-756. doi:10.1073/pnas.0708092105

Kreisel, T., Frank, M. G., Licht, T., Reshef, R., Ben-Menachem-Zidon, O., Baratta, M. V., Maier, S. F., & Yirmiya, R. (2014). Dynamic microglial alterations underlie stress-induced depressive-like behavior and suppressed neurogenesis. *Mol Psychiatry, 19* (6), 699-709. doi:10.1038/mp.2013.155

Krishnan, V., & Nestler, E. J. (2010). Linking molecules to mood: new insight into the biology of depression. *Am J Psychiatry, 167* (11), 1305-1320. doi:10.1176/appi.ajp.2009.10030434

Leong, W., Huang, G., Khan, I., Xia, W., Li, Y., Liu, Y., Li, X., Han, R., Su, Z., & Hsiao, W. L. W. (2019). Patchouli Essential Oil and Its Derived Compounds Revealed Prebiotic-Like Effects in C57BL/6J Mice.*Front Pharmacol, 10* , 1229. doi:10.3389/fphar.2019.01229

Lian, D. W., Xu, Y. F., Ren, W. K., Fu, L. J., Chen, F. J., Tang, L. Y., Zhuang, H. L., Cao, H. Y., & Huang, P. (2018). Unraveling the Novel Protective Effect of Patchouli Alcohol Against Helicobacter pylori-Induced Gastritis: Insights Into the Molecular Mechanism in vitro and in vivo. *Front Pharmacol, 9* , 1347. doi:10.3389/fphar.2018.01347

Lu, A., Magupalli, V. G., Ruan, J., Yin, Q., Atianand, M. K., Vos, M. R., Schröder, G. F., Fitzgerald, K. A., Wu, H., & Egelman, E. H. (2014). Unified polymerization mechanism for the assembly of ASC-dependent inflammasomes. *Cell, 156* (6), 1193-1206. doi:10.1016/j.cell.2014.02.008

Lu, Q., Jiang, C., Hou, J., Qian, H., Chu, F., Zhang, W., Ye, M., Chen, Z., Liu, J., Yao, H., Zhang, J., Xu, J., Wang, T., Fan, S., & Wang, Q. (2021). Patchouli Alcohol Modulates the Pregnancy X Receptor/Toll-like Receptor 4/Nuclear Factor Kappa B Axis to Suppress Osteoclastogenesis.*Front Pharmacol, 12* , 684976. doi:10.3389/fphar.2021.684976

Lu, X., Yang, L., Lu, C., Xu, Z., Qiu, H., Wu, J., Wang, J., Tong, J., Zhu, Y., & Shen, J. (2016). Molecular Role of EGFR-MAPK Pathway in Patchouli Alcohol-Induced Apoptosis and Cell Cycle Arrest on A549 Cells In Vitro and In Vivo. *Biomed Res Int, 2016* , 4567580. doi:10.1155/2016/4567580

Maes, M., Leonard, B., Fernandez, A., Kubera, M., Nowak, G., Veerhuis, R., Gardner, A., Ruckoanich, P., Geffard, M., Altamura, C., Galecki, P., & Berk, M. (2011). (Neuro)inflammation and neuroprogression as new

pathways and drug targets in depression: from antioxidants to kinase inhibitors. *Prog Neuropsychopharmacol Biol Psychiatry, 35* (3), 659-663. doi:10.1016/j.pnpbp.2011.02.019

Ndoja, A., Reja, R., Lee, S. H., Webster, J. D., Ngu, H., Rose, C. M., Kirkpatrick, D. S., Modrusan, Z., Chen, Y. J., Dugger, D. L., Gandham, V., Xie, L., Newton, K., & Dixit, V. M. (2020). Ubiquitin Ligase COP1 Suppresses Neuroinflammation by Degrading c/EBPβ in Microglia.*Cell, 182* (5), 1156-1169.e1112. doi:10.1016/j.cell.2020.07.011

Nestler, E. J., Barrot, M., DiLeone, R. J., Eisch, A. J., Gold, S. J., & Monteggia, L. M. (2002). Neurobiology of depression. *Neuron, 34* (1), 13-25. doi:10.1016/s0896-6273(02)00653-0

Ramirez, S., Liu, X., MacDonald, C. J., Moffa, A., Zhou, J., Redondo, R. L., & Tonegawa, S. (2015). Activating positive memory engrams suppresses depression-like behaviour. *Nature, 522* (7556), 335-339. doi:10.1038/nature14514

Roddy, D. W., Farrell, C., Doolin, K., Roman, E., Tozzi, L., Frodl, T., O'Keane, V., & O'Hanlon, E. (2019). The Hippocampus in Depression: More Than the Sum of Its Parts? Advanced Hippocampal Substructure Segmentation in Depression. *Biol Psychiatry, 85* (6), 487-497. doi:10.1016/j.biopsych.2018.08.021

Rustenhoven, J., Smith, A. M., Smyth, L. C., Jansson, D., Scotter, E. L., Swanson, M. E. V., Aalderink, M., Coppieters, N., Narayan, P., Handley, R., Overall, C., Park, T. I. H., Schweder, P., Heppner, P., Curtis, M. A., Faull, R. L. M., & Dragunow, M. (2018). PU.1 regulates Alzheimer's disease-associated genes in primary human microglia.*Mol Neurodegener, 13* (1), 44. doi:10.1186/s13024-018-0277-1

Samuels, B. A., & Hen, R. (2011). Neurogenesis and affective disorders.*Eur J Neurosci, 33* (6), 1152-1159. doi:10.1111/j.1460-9568.2011.07614.x

Schoenfeld, T. J., McCausland, H. C., Morris, H. D., Padmanaban, V., & Cameron, H. A. (2017). Stress and Loss of Adult Neurogenesis Differentially Reduce Hippocampal Volume. *Biol Psychiatry, 82* (12), 914-923. doi:10.1016/j.biopsych.2017.05.013

Spellman, T., & Liston, C. (2020). Toward Circuit Mechanisms of Pathophysiology in Depression. *Am J Psychiatry, 177* (5), 381-390. doi:10.1176/appi.ajp.2020.20030280

Su, W. J., Zhang, Y., Chen, Y., Gong, H., Lian, Y. J., Peng, W., Liu, Y. Z., Wang, Y. X., You, Z. L., Feng, S. J., Zong, Y., Lu, G. C., & Jiang, C. L. (2017). NLRP3 gene knockout blocks NF-ϰB and MAPK signaling pathway in CUMS-induced depression mouse model. *Behav Brain Res, 322* (Pt A), 1-8. doi:10.1016/j.bbr.2017.01.018

Tan, Y. L., Yuan, Y., & Tian, L. (2020). Microglial regional heterogeneity and its role in the brain. *Mol Psychiatry, 25* (2), 351-367. doi:10.1038/s41380-019-0609-8

Tang, M. M., Lin, W. J., Pan, Y. Q., Guan, X. T., & Li, Y. C. (2016). Hippocampal neurogenesis dysfunction linked to depressive-like behaviors in a neuroinflammation induced model of depression. *Physiol Behav, 161* , 166-173. doi:10.1016/j.physbeh.2016.04.034

Wei, L. L., Chen, Y., Yu, Q. Y., Wang, Y., & Liu, G. (2018). Patchouli alcohol protects against ischemia/reperfusion-induced brain injury via inhibiting neuroinflammation in normal and obese mice. *Brain Res, 1682* , 61-70. doi:10.1016/j.brainres.2017.12.039

Willner, P. (2017). The chronic mild stress (CMS) model of depression: History, evaluation and usage. *Neurobiol Stress, 6* , 78-93. doi:10.1016/j.ynstr.2016.08.002

Xu, Y. F., Lian, D. W., Chen, Y. Q., Cai, Y. F., Zheng, Y. F., Fan, P. L., Ren, W. K., Fu, L. J., Li, Y. C., Xie, J. H., Cao, H. Y., Tan, B., Su, Z. R., & Huang, P. (2017). In Vitro and In Vivo Antibacterial Activities of Patchouli Alcohol, a Naturally Occurring Tricyclic Sesquiterpene, against Helicobacter pylori Infection. *Antimicrob Agents Chemother, 61* (6). doi:10.1128/aac.00122-17

Yi, L. T., Zhang, M. M., Cheng, J., Wan, H. Q., Li, C. F., Zhu, J. X., Zhang, Q. P., Liu, Q., & Xu, G. H. (2021). Antidepressant-like Effects of Degraded Porphyran Isolated from Porphyra haitanensis. *Mol Nutr Food Res, 65* (9), e2000869. doi:10.1002/mnfr.202000869

Yirmiya, R., Rimmerman, N., & Reshef, R. (2015). Depression as a microglial disease. *Trends Neurosci, 38* (10), 637-658. doi:10.1016/j.tins.2015.08.001

Zhang, J., Rong, P., Zhang, L., He, H., Zhou, T., Fan, Y., Mo, L., Zhao, Q., Han, Y., Li, S., Wang, Y., Yan, W., Chen, H., & You, Z. (2021). IL4-driven microglia modulate stress resilience through BDNF-dependent neurogenesis. *Sci Adv, 7* (12). doi:10.1126/sciadv.abb9888

Zhang, J., Xie, X., Tang, M., Zhang, J., Zhang, B., Zhao, Q., Han, Y., Yan, W., Peng, C., & You, Z. (2017). Salvianolic acid B promotes microglial M2-polarization and rescues neurogenesis in stress-exposed mice. *Brain Behav Immun, 66* , 111-124. doi:10.1016/j.bbi.2017.07.012

Zhang, J. Q., Wu, X. H., Feng, Y., Xie, X. F., Fan, Y. H., Yan, S., Zhao, Q. Y., Peng, C., & You, Z. L. (2016). Salvianolic acid B ameliorates depressive-like behaviors in chronic mild stress-treated mice: involvement of the neuroinflammatory pathway. *Acta Pharmacol Sin, 37* (9), 1141-1153. doi:10.1038/aps.2016.63

Zhang, L., Tang, M., Xie, X., Zhao, Q., Hu, N., He, H., Liu, G., Huang, S., Peng, C., Xiao, Y., & You, Z. (2021). Ginsenoside Rb1 induces a pro-neurogenic microglial phenotype via PPARγ activation in male mice exposed to chronic mild stress. *J Neuroinflammation, 18* (1), 171. doi:10.1186/s12974-021-02185-0

Zhang, Y., Liu, L., Liu, Y. Z., Shen, X. L., Wu, T. Y., Zhang, T., Wang, W., Wang, Y. X., & Jiang, C. L. (2015). NLRP3 Inflammasome Mediates Chronic Mild Stress-Induced Depression in Mice via Neuroinflammation.*Int J Neuropsychopharmacol, 18* (8). doi:10.1093/ijnp/pyv006

Zhang, Y., Long, Y., Yu, S., Li, D., Yang, M., Guan, Y., Zhang, D., Wan, J., Liu, S., Shi, A., Li, N., & Peng, W. (2021). Natural volatile oils derived from herbal medicines: A promising therapy way for treating depressive disorder. *Pharmacol Res, 164* , 105376. doi:10.1016/j.phrs.2020.105376

Zhang, Y. M., Ye, L. Y., Li, T. Y., Guo, F., Guo, F., Li, Y., & Li, Y. F. (2021). New monoamine antidepressant, hypidone hydrochloride (YL-0919), enhances the excitability of medial prefrontal cortex in mice via a neural disinhibition mechanism. *Acta Pharmacol Sin* . doi:10.1038/s41401-021-00807-0

Zhao, Q., Wang, Q., Wang, J., Tang, M., Huang, S., Peng, K., Han, Y., Zhang, J., Liu, G., Fang, Q., & You, Z. (2019). Maternal immune activation-induced PPARγ-dependent dysfunction of microglia associated with neurogenic impairment and aberrant postnatal behaviors in offspring. *Neurobiol Dis, 125* , 1-13. doi:10.1016/j.nbd.2019.01.005

Zheng, Y. F., Xie, J. H., Xu, Y. F., Liang, Y. Z., Mo, Z. Z., Jiang, W. W., Chen, X. Y., Liu, Y. H., Yu, X. D., Huang, P., & Su, Z. R. (2014). Gastroprotective effect and mechanism of patchouli alcohol against ethanol, indomethacin and stress-induced ulcer in rats. *Chem Biol Interact, 222* , 27-36. doi:10.1016/j.cbi.2014.08.008

Zhuo, J., Chen, B., Sun, C., Jiang, T., Chen, Z., Liu, Y., Nie, J., Yang, H., Zheng, J., Lai, X., Su, Z., Li, C., & Li, Y. (2020). Patchouli alcohol protects against chronic unpredictable mild stress-induced depressant-like behavior through inhibiting excessive autophagy via activation of mTOR signaling pathway. *Biomed Pharmacother, 127* , 110115. doi:10.1016/j.biopha.2020.110115

Zomkowski, A. D., Santos, A. R., & Rodrigues, A. L. (2006). Putrescine produces antidepressant-like effects in the forced swimming test and in the tail suspension test in mice. *Prog Neuropsychopharmacol Biol Psychiatry, 30* (8), 1419-1425. doi:10.1016/j.pnpbp.2006.05.016

Figure 1. PA alleviates CMS-induced depressive-like behaviors in mice. (A) Experimental schedule. FST, forced swimming test; IMI, imipramine; OFT, open field test; SPT, sucrose preference test; TST, tail suspension test. (B) Changes in sucrose preference between the 4th week and the 7th week in individual animals. (C) Changes in latency and immobility time in the TST after treatment (week 7). (D) Changes in immobility time in the FST after treatment (week 7). (E) Changes in distance travelled in the OFT after

treatment (week 7). (F) Animal body weight. Data are mean ± SEM (n = 9 mice/group). *P < 0.05, **P < 0.01, ***P < 0.001 vs the control group; #P < 0.05, ##P < 0.01, ##P < 0.001 vs the CMS group.

Figure 2. PA inhibits microglial activation in the hippocampus of CMS-exposed mice. (A) Immunostaining against the microglia marker Iba1 in the dentate gyrus (DG) and in hippocampal regions CA1 and CA3. (B) Quantitation of numbers and morphology of Iba1+ cells in the three hippocampal regions. (C) Average intersections and number of intersections were evaluated by Sholl analysis in hippocampus. (D) Levels of mRNAs encoding IL-1β, IL-18, IL-6 and TNF-α in hippocampus. (E) Western blotting of IL-6 and TNF-α in hippocampus. β-actin served as the internal reference. Data are mean ± SEM (n = 5 mice/group). *P < 0.05, **P < 0.01, ***P < 0.001 vs the control group; #P < 0.05, ##P < 0.01, ##P < 0.001 vs the CMS group.

Figure 3. PA inhibits activation of the NLRP3 inflammasome in the hippocampus of CMS-exposed mice. (A) Left: western blotting of NLRP3 in hippocampus. GAPDH served as an internal reference. Middle: immunostaining against Iba1 (red) and NLRP3 (green) in hippocampus. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. Right: quantification of NLRP3+ microglia, where each dot represents the average of 5–6 micrographs for each mouse (n = 5 mice/group, each sample in triplicate). (B) Left: western blotting of ASC in hippocampus. Middle: immunostaining against Iba1 (red) and ASC (green) in hippocampus. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. Right: quantification of ASC+ microglia, where each dot represents the average of 5–6 micrographs for each mouse (n = 5 mice/group, each sample in triplicate). (C) Left: Schematic of how PA may block the NRP3 inflammasome from activating caspase-1. Middle: western blotting of pro-caspase-1, pro-IL-1β, cleaved caspase-1, and cleaved IL-1β in hippocampus. Right: quantitation of the western blots. Data are mean ± SEM (n = 5 mice/group, each sample in triplicate). *P < 0.05, **P < 0.01, ***P < 0.001 vs the control group; #P < 0.05, ##P < 0.01, ##P < 0.001 vs the CMS group.

Figure 4. PA inhibits activation of the NLRP3 inflammasome in primary microglia. (A) The CCK8 kit was used to measure the viability of primary microglia after treatment with PA. (B) Levels of mRNAs encoding IL-1β, NLRP3, TNF-α, and IL-18 in primary microglia, relative to levels in the PBS group. (C) An ELISA kit was used to assay the levels of intracellular IL-1β in primary microglia. (D) Left: immunostaining against ASC (green) and Iba1 (red) in primary microglia. Nuclei were stained with DAPI (blue). Scale bar, 10 μm. Right: number of ASC specks in primary microglia. (E) Left: immunostaining against NLRP3 (red) and ACS (green) in primary microglia. Nuclei were stained with DAPI (blue). Scale bar, 50 μm. Right: fluorescence intensity of $NLRP3^+$ primary microglia. (F) Left: western blotting of NLRP3, pro-caspase-1, pro-IL-1β, ASC, cleaved caspase-1, and cleaved IL-1β in primary microglia. β-actin served as internal reference. Right: quantitation of western blots. Data are mean ± SEM (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001 vs the PBS group; #P < 0.05, ##P < 0.01, ##P < 0.001 vs the LPS + ATP group.

Figure 5. PA partially reverses CMS-induced inhibition of hippocampal neurogenesis in mice. (A) Upper: immunostaining against BrdU (green) and doublecortin (DCX, red) in the dentate gyrus. Nuclei were stained with DAPI (blue). Scale bar, 50 μm. Lower: quantitation of $BrdU^+$ and $BrdU^+$-DCX^+ cells in the dentate gyrus. (B) Upper: immunostaining against DCX (red) in the hippocampus. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. Lower: number of DCX^+ cells in the dentate gyrus. (C) DAPI staining (blue) in the dentate gyrus, with quantification of the volume of the granule cell layer. Scale bar, 50 μm. Data are mean ± SEM (n = 5 mice/group). *P < 0.05, **P < 0.01, ***P < 0.001 vs the control group; #P < 0.05, ##P < 0.01, ##P < 0.001 vs the CMS group.

Figure 6. Effects of conditioned medium from PA-treated microglia on NPC differentiation. (A) Left: primary cultures of NPCs were treated with the indicated PA concentrations, then immunostained against GFAP (red) to label astrocytes or against MAP2 (green) to label neurons. Scale bar, 50 μm. Right: Percentages of $MAP2^+$or $GFAP^+$ cells. (B) Schematic of the experiment in which microglia were activated with LPS in the presence or absence of PA, then the conditioned medium from those cultures was transferred to NPC cultures. (C) Middle: primary cultures of NPCs after incubation in conditioned medium as described

in panel B. Cultures were immunostained as in panel A. Scale bar, 50 μm. Right: Percentages of MAP2+ or GFAP+ cells. Data are mean ± SEM (n=5). *P < 0.05, **P < 0.01, ***P < 0.001 vs the PBS group; #P < 0.05, ##P < 0.01, ##P < 0.001 vs the LPS + ATP group.

Figure S1 Patchouli alcohol (PA) structure. Patchouli alcohol (PA) structure.

Figure S2. The effect of PA on CMS-exposed mice. A. Changes in the number of movement and standing of mice in locomotor activity test (LAT) at the 7th week. B. The coat score of mice at the 7th week. Each value are shown as the mean ± SEM (n = 9 mice/group, *P < 0.05, **P < 0.01, ***P < 0.001 vs the control group, #P < 0.05, ##P < 0.01, ##P < 0.001 vs the CMS + Veh group).

Hosted file

Figure 1 .pptx available at https://authorea.com/users/740952/articles/713515-patchouli-alcohol-inhibits-the-nlrp3-inflammasome-in-microglia-to-enhance-neurogenesis-in-male-mice-exposed-to-chronic-mild-stress

Hosted file

Figure 2.pptx available at https://authorea.com/users/740952/articles/713515-patchouli-alcohol-inhibits-the-nlrp3-inflammasome-in-microglia-to-enhance-neurogenesis-in-male-mice-exposed-to-chronic-mild-stress

Hosted file

Figure 3.pptx available at https://authorea.com/users/740952/articles/713515-patchouli-alcohol-inhibits-the-nlrp3-inflammasome-in-microglia-to-enhance-neurogenesis-in-male-mice-exposed-to-chronic-mild-stress

Hosted file

Figure 4.pptx available at https://authorea.com/users/740952/articles/713515-patchouli-alcohol-inhibits-the-nlrp3-inflammasome-in-microglia-to-enhance-neurogenesis-in-male-mice-exposed-to-chronic-mild-stress

Hosted file

Figure 5.pptx available at https://authorea.com/users/740952/articles/713515-patchouli-alcohol-inhibits-the-nlrp3-inflammasome-in-microglia-to-enhance-neurogenesis-in-male-mice-exposed-to-chronic-mild-stress

Hosted file

Figure 6.pptx available at https://authorea.com/users/740952/articles/713515-patchouli-alcohol-inhibits-the-nlrp3-inflammasome-in-microglia-to-enhance-neurogenesis-in-male-mice-exposed-to-chronic-mild-stress

Hosted file

Figure S1.pptx available at https://authorea.com/users/740952/articles/713515-patchouli-alcohol-inhibits-the-nlrp3-inflammasome-in-microglia-to-enhance-neurogenesis-in-male-mice-exposed-to-chronic-mild-stress

Hosted file

Figure S2.pptx available at https://authorea.com/users/740952/articles/713515-patchouli-alcohol-inhibits-the-nlrp3-inflammasome-in-microglia-to-enhance-neurogenesis-in-male-mice-exposed-to-chronic-mild-stress