Asperosaponin VI Ameliorates The CMS-Induced Depressive-Like Behaviors By Inducing A Neuroprotective Microglial Phenotype To Restore Hippocampal Synaptic Plasticity Via PPAR-γ Pathway

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Research

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Abstract

Background: The natural compound asperosaponin VI has shown potential as an antidepressant, but how it works is unclear. Here we explored its effects on mice exposed to chronic mild stress (CMS) and the underlying molecular pathways.

Methods: Mice were exposed to CMS for three weeks followed by asperosaponin VI (40 mg/kg) or imipramine (20 mg/kg) for another three weeks. Depression-like behaviors were assessed in the forced swimming test, sucrose preference test, tail suspension test, open field test and novelty-suppressed feeding test. Microglial phenotype and synaptic plasticity were evaluated using immunofluorescence staining, real-time quantitative PCR and enzyme-linked immunosorbent assays in hippocampus of mice. In some experiments, stressed animals were treated with the PPAR-γ antagonist GW9622 to examine its involvement in the effects of asperosaponin VI.

Results: Asperosaponin VI ameliorated depression-like behaviors of CMS mice based on all three behavioral tests, and this was associated with a switch of hippocampal microglia from a pro-inflammatory (iNOS⁺-Iba1⁺) to neuroprotective (Arg-1⁺-Iba1⁺) phenotype. The natural compound also promoted interactions between hippocampal microglia and neurons by enhancing CX3CL1/CX3CR1 and CD200/CD200R, and preserved synaptic plasticity based on PSD95 and CamKIIα levels. These effects of asperosaponin VI were blocked by GW9662.

Conclusion: CMS in mice induces a proinflammatory microglial phenotype, disrupting neuron-microglia communication and synaptic function in hippocampus, ultimately leading to depression-like behaviors. Asperosaponin VI may ameliorate the effects of CMS by inducing microglia to adopt a PPAR-γ-dependent neuroprotective phenotype.

1. Introduction

Major depressive disorder (MDD) is a pervasive neuropsychiatric disorder and a significant contributor to the global burden of disease [1]. MDD has heterogeneous causes and clinical manifestations, which has impeded an understanding of its pathogenesis and design of effective treatments [2, 3]. Antidepressants based on monoamine neurotransmitters can reduce symptoms, but they are effective in only one-third to half of patients [4–6]. Even when effective, current drugs take 3-6 weeks to begin working, and they often produce adverse effects [7]. Therefore, developing more effective antidepressants with fewer side effects is urgently needed.

MDD is associated with neuroinflammation, such as the increased concentrations of pro-inflammatory cytokines [8, 9]. Such neuroinflammation can be driven by microglia, the innate immune cells of the central nervous system [10, 11]. When continuously stimulated by immune responses, microglia can adopt a pro-inflammatory phenotype, secreting pro-inflammatory cytokines and neurotoxic substances, which damage synapses, induce apoptosis, inhibit neurogenesis, and eventually lead to depression symptoms [12–14]. Conversely, certain signals can induce microglia to adopt a protective phenotype, in
which scavenger receptors on their surface recognize metabolic wastes and nerve cell debris, which the microglia phagocytose [15, 16]. The microglia also releases anti-inflammatory cytokines and neurotrophic factors that protect and repair neurons, ultimately alleviating depression symptoms [17, 18]. Therefore, regulating the phenotype of activated microglia is an attractive strategy for treating depression.

The triterpenoid saponin asperosaponin VI, one of the active components of the traditional Chinese medicine *Radix Dipsaci*, exerts anti-osteoporotic and anti-inflammatory effects, and it can pass through the blood-brain barrier to protect neurons and improve neurological diseases [19–22]. In previous work, we showed that asperosaponin VI, delivered at a dose of 40 mg/kg, can inhibit neuroinflammatory responses by hippocampal microglia and mitigate depression-like behaviors induced by lipopolysaccharide in mice [23]. These findings suggested that asperosaponin VI exerts its antidepressant effects at least in part by regulating hippocampal microglial function.

To verify and extend these previous findings, we examined the effects of asperosaponin VI on microglial phenotype and behaviors of mice exposed to chronic mild stress (CMS), a classical model of MDD. We also examined the potential involvement of the PPAR-γ pathway in mediating the effects of asperosaponin VI.

2. Materials And Methods

2.1 Animals

Male 8-week-old C57BL/6 mice were purchased from Changsha Tianqin Biotechnology (Changsha, China) and allowed to acclimate for one week prior to experiments. All mice were housed individually under a standard 12-h light-to-dark cycle in temperature- and humidity-controlled rooms throughout the experiments. Mice were randomly assigned to experimental or control groups and an observer blinded to treatment conditions performed the behavioral tests and collected and analyzed the data. All experiments were approved by the Institutional Animal Care and Use Committee of the Guizhou University of Traditional Chinese Medicine.

2.2 Chronic mild stress (CMS)

Mice 9 weeks old at the start of experiments were caged individually and subjected to CMS as described [17] for three weeks. Each day, animals were exposed to three of the following stressors in random order: empty water bottles (12 h), food deprivation (12 h), tail clipping (10 min), restraint (2 h), lights-off for 3 h during the daylight phase, cage shaking (1 h), cage tilting (45°, 24 h), reversal of the light–dark cycle (24 h), strobe lighting (12 h), damp bedding (24 h) and a soiled cage (24 h).

2.3 Treatment with asperosaponin VI or the PPAR-γ antagonist GW9622

Asperosaponin VI (99.92% pure; Chengdu Alfa Biotechnology Chengdu, China) was dissolved in 0.9% saline to a concentration of 2 mg/mL. After the 3-week CMS procedure, mice received a single daily
intraperitoneal injection of asperosaponin VI (40 mg/kg/d), imipramine (20 mg/kg/d; Sigma-Aldrich, St.Louis, MO, USA) or GWP662 (1 mg/kg; Sigma, St.Louis, MO, USA) for another three weeks.

2.4 Behavioral testing

2.4.1 Sucrose preference test (SPT)

The SPT was performed as described [17]. Mice were individually housed, deprived of food and water for 12 h, and then given access to 1% sucrose solution (A) and water (B) for 2 h. The bottle positions were switched daily to avoid a side bias. The sucrose preference was calculated each week for each mouse using the formula: 100 \times \left[ \frac{\text{VolA}}{\text{VolA} + \text{VolB}} \right]$. Sucrose consumption was normalized to the body weight of each mouse.

2.4.2 Tail suspension test (TST)

Each mouse was individually suspended by applying adhesive tape to the tip of the tail and connecting the tape to a ledge 30 cm above the cage floor. The animal was recorded for 6 min using a high-definition camera. An observer masked to treatment conditions recorded the latency between suspension and first abandonment of struggle as well as the time spent immobile during the 6-min period.

2.4.3 Forced swimming test (FST)

At 24 h before the test, mice were placed individually in a glass cylinder of height 25 cm and diameter 15 cm that was filled with water at 26°C to a depth of 15 cm. The next day, the mice were placed again in the same situation for 6 min and recorded using a high-definition camera. An observer blinded to treatment conditions recorded the time spent immobile during the last 4 min.

2.4.4 Open field test (OFT)

Mice were placed into an open field (50 × 50 cm) and allowed to explore freely for 15 min. Total distance and time spent in the center (25 × 25 cm) were determined using video-tracking software (OFT100, Taimeng Tech. Chengdu, China).

2.4.5 Novelty-suppressed feeding test (NSFT)

Mice were deprived of food and water for 12 h before the test, then each mouse was placed for 5 min in a rectangular chamber (40 × 40 × 30 cm) containing a sugar pill in the center of the chamber. The time it took for a mouse to pick up the sugar with its forelimb was recorded as latency using a camera system.

2.5 RNA extraction and real-time PCR

At the end of experiments, mice were sacrificed, the whole brain was removed, and the hippocampus and cortex were isolated and placed into separate enzyme-free 1.5-mL microcentrifuge tubes. Total RNA was extracted separately from the hippocampus and cortex using Trizol (Invitrogen Life Technologies, Shanghai, China), then reverse-transcribed into cDNA using the high-capacity cDNA conversion kit (Takara, Tokyo, Japan) in strict accordance with the manufacturer's instructions. RT-PCR reaction mixture
contains 1 µL of template cDNA, 5 µL MasterMix, and 1 µL primer (Sangon Biotech, Sichuan, China); add DEPC water to a total reaction volume of 10 µL. The PCR was performed in a CFX 96 system (Bio-Rad, Hercules, California, USA) using the following steps: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, then 39 cycles of annealing at the appropriate temperature for 34 s, followed by extension. Each sample was analyzed in three replicates. Expression level was determined using the 2^−ΔΔCt method with reference to the β-actin gene. The primers of each gene were listed in Supplementary Table 1.

2.6 Immunocytochemistry

Whole brain from mice was perfused and fixed in 4% paraformaldehyde for 48 h, dehydrated, frozen, cut into thin sections, thoroughly cleaned with 0.5% Triton X-100 for 15 min, blocked with 10% donkey serum for 1 h, and incubated overnight at 4°C with the following primary antibodies (Abcam, Cambridge, UK): mouse anti-Iba1 (1:400), mouse anti-Gfap (1:500), rabbit anti-iNOS (1:100), rabbit anti-Arg-1 (1:200), and rabbit anti-NeuN (1:200). On the next day, slices were washed three times with phosphate-buffered saline (PBS) and incubated in the dark for 2 h with secondary antibodies. DAPI was added to stain the nuclei, and slices were observed under a fluorescence microscope (Olympus BX 51, Japan). Images were imported into Image J software (version 1.45J; National Institutes of Health, Bethesda, MD), and an intensity threshold was defined to differentiate positive staining from background.

2.7 Enzyme-linked immunosorbent assay (ELISA)

Hippocampus and cortex from mice were placed into separate 1.5-mL microcentrifuge tubes and completely homogenized. The concentration of total protein in the supernatant was determined using the BCA kit (Boster, Shanghai, China), then aliquots of the supernatants were diluted to the same total protein concentration. These diluted samples were assayed for interleukin (IL)-1β, IL-10, brain-derived neurotrophic factor (BDNF) and tumor necrosis factor (Tnf)-α using a commercial ELISA kit (Boster, Shanghai, China) in strict accordance with the manufacturer's instructions.

2.8 Western blotting

The hippocampus and cortex of the mice were lysed by RIPA lysis buffer (Solarbio, Beijing, China) then centrifuged at 1000×g for 30 min. The concentration of total protein was measured by the BCA method. Equal amount of protein was resolved using 12% SDS polyacrylamide gel. Fractionated proteins were transferred onto PVDF membranes at 300 mA for 30 min, then the membrane was washed in TBST, blocked in skim milk for 30 min, and incubated overnight on a shaker at 4°C with primary antibody. The membrane was again washed three times with TBST, incubated with secondary antibody for 30 min, washed three times with TBST, and Bands were visualized using the BM Chemiluminescence Western Blotting Kit (Roche Diagnostics GmbH, Mannheim, Germany). Membranes were analyzed using the ChemiDoc Touch system (Bio-Rad, Hercules, California, USA), and band intensity was quantified using Alpha software (version 1.45J; National Institutes of Health, Bethesda, MD, USA).

2.9 Statistical analysis
All statistical analyses were performed using GraphPad Prism software (version 8.0, SPSS Inc., Chicago, USA). Data were presented as mean ± SEM. Pairwise comparisons were assessed for significance using Student's two-tailed t-test, and comparisons among three or more values were assessed using one- or two-way ANOVA and Tukey’s post hoc tests. Levels of significance are marked in figures as * p < 0.05, significant; ** p < 0.01, very significant; and *** p < 0.001, highly significant.

3. Results

3.1 Asperosaponin VI ameliorates depression-like behaviors induced by CMS in mice

CMS for three weeks reduced sucrose preference (Fig. 1A), which was partially reversed by a subsequent 3-week treatment with asperosaponin VI or imipramine (Fig. 1B). Analysis at the level of individual mice showed that the 3-week treatment with asperosaponin VI improved sucrose preference in nearly 90% of CMS mice, while imipramine improved it in just over 60% (Fig. 1C).

CMS shortened latency and led to longer time spent immobile in the TST (Fig. 1D) and FST (Fig. 1E). Asperosaponin VI, but not imipramine, prolonged latency in the FST, while both compounds shortened the time spent immobile (Fig. 1F). Neither compound affected the distance travelled in the OFT (Fig. 1G), indicating that asperosaponin VI does not affect nerve transmission. Asperosaponin VI significantly improved feeding latency in the NSFT, and it partially reversed the weight loss induced by CMS (Fig. 1H).

3.2 Asperosaponin VI induces hippocampal microglia to switch from a pro-inflammatory to neuroprotective phenotype after CMS

CMS increased expression of the microglial marker Iba1 and the marker of microglial activation Cd11b in the hippocampus, as well as expression of Cd11b in cortex (Fig. 2A). Microscopy of tissue slices showed that CMS induced soma enlargement, thickening and shortening of processes and loss of branching in hippocampal microglia (Fig. 2B and 2C). These findings indicate stress-induced microglial activation.

Asperosaponin VI did not cause obvious changes in morphology or CD11b expression in microglia in the hippocampus or cortex, nor did it reverse the increase in Iba1⁺ area, increase in cell number or decrease in microglial branching induced by CMS (Fig. 2A-2C). Similarly, the compound did not alter the CMS-induced radial morphology of astrocytes in the hippocampus and cortex (Fig. 2D-2F).

These results suggest that asperosaponin VI does not inhibit CMS-induced activation of hippocampal microglia; instead, it may regulate the type of microglial activation to influence depression-like behaviors. Consistent with this hypothesis, we found that CMS increased the proportions of pro-inflammatory (iNOS⁺·Iba1⁺) microglia and decreased the proportions of anti-inflammatory (Arg-1⁺·Iba1⁺) in hippocampus but not in cortex (Fig. 3A and 3B). Asperosaponin VI partially reversed these changes.
Similarly, CMS increased levels of the pro-inflammatory cytokines IL-1β, IL-6, iNOS and Tnf-α in cortex and hippocampus (Fig. 3C and 3D). Asperosaponin VI partially reversed these changes while also upregulating the protective molecules arginase (Arg)-1, (interleukin) IL-10, transforming growth factor (Tgf)-β and BDNF in hippocampus (Fig. 3A-3G). These results suggest that asperosaponin VI switches the phenotype of activated microglia in hippocampus from pro-inflammatory to neuroprotective.

### 3.3 Asperosaponin VI acts via the PPAR-γ pathway to exert its anti-inflammatory and antidepressant effects

Since PPAR-γ signaling plays a key role in anti-inflammatory microglial phenotypes, we asked whether asperosaponin VI acts via such signaling to exert its “phenotype switching” effect. Indeed, CMS reduced expression of PPAR-γ-1 and PPAR-γ-2 as well as levels of phosphorylated PPAR-γ in hippocampus, which asperosaponin VI partially reversed (Fig. 4A). PPAR-γ localized in cytoplasm and nucleus of microglia in the hippocampus of mice that were exposed to CMS and then treated with asperosaponin VI (Fig. 4B).

To confirm the role of PPAR-γ in mediating the anti-inflammatory effects of asperosaponin VI, we repeated the above experiments in the presence of the PPAR-γ antagonist GW9662 (Fig. 4C), which effectively blocked the PPAR-γ pathway in hippocampus (Fig. 4D). Such blockade abolished the antidepressant effects of asperosaponin VI in the SPT, TST and FST (Fig. 4E), as well as its ability to increase numbers of Arg-1+ microglia and decrease numbers of iNOS+ microglia in the hippocampus of CMS mice (Fig. 5A and 5B). In contrast, blockade did not alter the morphology or Cd11b expression of hippocampal microglia in CMS mice.

Blockade of PPAR-γ signaling also abolished the ability of asperosaponin VI to suppress pro-inflammatory cytokines and elevate anti-inflammatory cytokines in the hippocampus of CMS mice (Fig. 5E-5I). These results suggest that asperosaponin VI exerts its antidepressant and anti-inflammatory effects via the PPAR-γ signaling pathway.

### 3.4 Asperosaponin VI protects hippocampal synaptic plasticity from CMS

Dysfunctional microglia can communicate abnormally with neurons, which disrupts synaptic function and may help to explain MDD behaviors. In our mice, CMS reduced hippocampal expression of Cx3cl1 and its receptor Cx3cr1 as well as expression of CD200 and its receptor CD200R; these pairs mediate communication between neurons and microglia. Asperosaponin VI partially reversed these CMS-induced changes, while GW9662 abolished the effects of asperosaponin VI (Fig. 6A).

CMS downregulated PSD95, CamKII α and CamKII β as well as decreased levels of phosphorylated GluA 2 in hippocampus, but did not affect the number of NeuN+ cells in the hippocampal dentate gyrus, all of which suggest inhibition of synaptic plasticity but not apoptosis of mature neurons (Fig. 6B and 6C). These changes in synaptic plasticity were partially reversed by asperosaponin VI, but not in the presence of GW9662 (Fig. 6C and 6D). Levels of PPAR-γ positively correlated with levels of PSD95, CamKII α,
CamKII β and phosphorylated GluA 2 (Fig. 6E). These results suggest that asperosaponin VI requires PPAR-γ to induce neuroprotective microglia and repair CMS-induced damage to synaptic plasticity in hippocampus.

4. Discussion

We showed in previous work that asperosaponin VI inhibits NF-κB signaling to mitigate lipopolysaccharide-induced depression-like behaviors in mice by reducing microglia-mediated acute neuroinflammation [23]. Here, using a classical animal model of depression, we showed that asperosaponin VI induces a PPAR-γ-dependent neuroprotective microglial phenotype that mitigates depression-like behaviors induced by CMS, which is associated with restoration of hippocampal synaptic function. Our work extends the list of conditions where asperosaponin VI can exert therapeutic anti-inflammatory and neuroprotective effects in the brain, a list that already includes Alzheimer’s disease and optic nerve damage [19, 24].

Depression usually manifests as diverse debilitating symptoms, including hopelessness and anhedonia [25]. Anhedonia, a core symptom of MDD, can be assessed in the SPT [26]. In addition to the SPT, we used the FST and TST to assess passive stress-coping behavioral despair [27, 28]. As expected, CMS caused depression-like behaviors in all these tests, which subsequent asperosaponin VI treatment improved, to an even greater extent than the classic monoamine antidepressant imipramine. In fact, asperosaponin VI but not imipramine partially restored weight loss caused by CMS, suggesting that the former may lack serious side effects at the dose of 40 mg/kg. We believe that these results indicate genuine antidepressant effects of asperosaponin VI, because the compound did not significantly alter performance in the OFT.

Our previous research indicated that the dysregulation of pro- and anti-inflammatory cytokines plays a crucial role in depression [8]. In the present study, CMS upregulated the pro-inflammatory IL-1β, IL-6, iNOS and Tnf-α in hippocampus of mice, and asperosaponin VI reversed these changes while also upregulating the anti-inflammatory cytokines Arg-1, IL-10, and Tgf-β as well as BDNF. These results establish a link between the neuroprotective and anti-inflammatory effects of asperosaponin VI.

Stress or immunostimulation has been shown to induce neuroinflammation, which appears to involve microglial activation, particularly in the hippocampus [29, 30]. Consistent with these previous studies, we found here that CMS caused morphological changes in hippocampal microglia indicative of microglial activation. Asperosaponin VI did not reduce the extent of overall microglial activation in hippocampus of CMS mice; instead, it altered the type of such activation, from a pro-inflammatory to neuroprotective phenotype. The decrease in proportion of pro-inflammatory microglia translates to lower production of pro-inflammatory cytokines and neurotoxic products (such as nitric oxide and quinolinic acid) [31], which increase neuropathic pain and inhibit hippocampal neurogenesis, contributing to cognitive deficits and depression-like behaviors [10, 32, 33].
We found that the anti-inflammatory effects of asperosaponin VI are mediated by PPAR-γ, a ligand-dependent transcription factor belonging to the nuclear hormone receptor superfamily [34]. PPAR-γ regulates the expression of anti-inflammatory cytokines [35], and the PPAR-γ agonists pioglitazone or rosiglitazone can switch activated microglia cells from a pro-inflammatory to anti-inflammatory state [36, 37]. Our previous research showed that asperosaponin VI acts via PPAR-γ to switch activated microglia from a pro-inflammatory to anti-inflammatory phenotype in vitro [38]. In present study, we further demonstrated that asperosaponin VI acts via PPAR-γ to induce a neuroprotective phenotype in hippocampal microglia of CMS-exposed mice and mitigate depressive-like mouse behaviors. Conversely, blocking the PPAR-γ signaling pathway abolished the neuroprotective microglia in induced by asperosaponin VI in hippocampus of CMS-exposed mice, as well as the antidepressant effect of asperosaponin VI. Thus, we speculated that asperosaponin VI exerts its antidepressant and anti-inflammatory effects via the PPAR-γ signaling pathway to regulating the phenotype of microglia.

How proinflammatory microglia lead to depression is a hot topic of current research. A great deal of researches showed the dysfunctional microglia can lead to abnormal neuron-microglia communication and disrupt synaptic function [39–41]. In this study, we found CMS-induced decrease in the intercommunicating molecules between neuron and microglia (Cx3cl1 / Cx3cr1 and CD200 / CD200R) in hippocampus of CMS-exposed mice was reversed by ASA VI treatment. Meanwhile, the PSD-95, CamKII α and CamKII β as well as decreased levels of phosphorylated GluA 2 in hippocampus of CMS-exposed mice, these are thought to be crucial for morphological maturation and synaptic development of hippocampal neurons [42–47], were partially reversed by asperosaponin VI via PPAR-γ-dependent pathway. These results reveal for the first time the role of asperosaponin VI in maintaining normal communication between neurons and microglia as well as in repairing CMS-induced damage to synaptic plasticity in hippocampus.

5. Conclusion

In summary, our experiments in mice suggest that CMS induces depression-like behaviors by inducing a pro-inflammatory microglial phenotype that disrupts neuron-microglia communication and synaptic function. Asperosaponin VI ameliorates the effects of CMS by inducing, via PPAR-γ, a neuroprotective microglial phenotype that partially restores hippocampal synaptic function (Fig. 7). These findings may provide further insights into the pathogenesis of depression and the development of natural antidepressants. Our study provides further evidence for asperosaponin VI as a potential antidepressant and a reference for research on depression.

Abbreviations

**AMPA**, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionicacid receptor; **Arg-1**, arginase-1; **ASA VI**, Asperosaponin VI; **BDNF**, brain derived neurotrophic factor; **CamKIIα**, α subunit of calcium/calmodulin-dependent protein kinase II; **CamKIIβ**, β subunit of calcium/calmodulin-dependent protein kinase II; **CMS**, chronic mild stress; **CNS**, central nervous system; **DAPI**, 4',6-diamidino-2-phenylindole; **DG**, dentate
Declarations

Ethics approval and consent to participate

The animal study was reviewed and approved by Guizhou University of Traditional Chinese Medicine.

Consent for publication

All authors agree to the publication of this manuscript.

Data availability statement

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

Competing interests

The authors declare no conflicts of interest related to this research.

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Authors’ contributions

Jinqiang Zhang designed the research and revised the manuscript. Xue Jiang and Saini Yi wrote the manuscript. Saini Yi and Qin Liu carried out the experiments and performed data analysis. Dapeng Su, Liangyuan Li, Chenghong Xiao, Weike Jiang and Changgui Yang gave methodological support and conceptual advice. All of the authors have read and approved the final manuscript.

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Figures

Figure 1

Asperosaponin VI ameliorates CMS-induced depression-like behaviors (A) Timeline of the experimental process. (B) Sucrose preference during 6 weeks in Ctrl mice and mice subjected to CMS, followed by treatment with ASA VI or IMI. *P < 0.05, **P < 0.015, ***P < 0.005 vs Ctrl group, #P < 0.05, ##P < 0.015, ###P < 0.005 vs CMS group. (C) Sucrose preference of individual mice before treatment (3-week) or after treatment (6-week) with ASA VI or IMI. (D) Latency and time spent immobile in the TST. (E) Latency and time spent immobile in the FST. (F) Latency to feed in the NSFT. (G) Distance travelled in the OFT. (H) Body weight of Ctrl or CMS mice. Individual data are shown (n=8-12 per group). *P<0.05, **P<0.01, ***P<0.005 (two-way ANOVA with Tukey’s multiple-comparisons test). Abbreviations: ASA VI,
asperosaponin VI; CMS, chronic mild stress; Ctrl, the control; IMI, imipramine; TST, tail suspension test; FST, forced swimming test; NSFT, novelty-suppressed feeding test; OFT, open field test.

**Figure 2**

Effects of asperosaponin VI on the morphology of microglia and astrocytes in hippocampus of CMS mice (A) Levels of mRNA encoding Iba1 and Cd11b in hippocampus and cortex of Ctrl or CMS mice after treatment with saline or ASA VI (n = 4 animals). (B) Representative micrographs of microglia in hippocampus and cortex of Ctrl or CMS mice after treatment with saline or ASA VI. Microglia were stained with Iba1 (red) by immunocytochemistry, and nuclei were stained with DAPI (blue). (C) Quantification of the number, area and length of branches on Iba1+ cells in hippocampus and cortex of Ctrl or CMS mice after treatment with saline or ASA VI. Results of each group were obtained from 5 mice, and 5-6 micrographs (40×) containing hippocampus were collected from each sample. All Iba1+ cells in each micrograph were measured. Each dot in the bar graph represents the average of all Iba1+ cells analyzed for each mouse. (D) Levels of mRNA encoding Gfap in hippocampus and cortex of Ctrl or CMS mice after treatment with saline or ASA VI (n = 4 animals). (E) Representative micrographs of astrocytes in hippocampus and cortex of Ctrl or CMS mice after treatment with saline or ASA VI. Astrocytes were stained with Gfap (red) by immunocytochemistry, and nuclei were stained with DAPI (blue). (F) Quantification of the number, area and length of branches on Iba1+ cells in hippocampus and cortex of Ctrl or CMS mice after treatment with saline or ASA VI. Data for individual animals are displayed (mean ± SEM), *P <0.05, **P<0.01 (two-way ANOVA with Tukey's multiple-comparisons test).

**Figure 3**

Asperosaponin VI switches activated microglia from a pro-inflammatory to neuroprotective phenotype in hippocampus of CMS mice (A) Fluorescence micrographs of pro-inflammatory microglia (iNOS+-Iba1+ cells) in hippocampus of CMS mice. Pro-inflammatory cytokines were stained with an antibody against iNOS (green); microglia, with an antibody against Iba1 (red); and nuclei, with DAPI (blue). The cells positive for iNOS and Iba1 were considered pro-inflammatory microglia. At right is the quantification of the percentage of iNOS+-Iba1+ cells in hippocampus and cortex of Ctrl or CMS mice after treatment with saline or ASA VI. (B) Fluorescence micrographs of anti-inflammatory microglia (Arg-1+-Iba1+ cells) in hippocampus of CMS mice after treatment with ASA VI. At right is quantification of the percentage of Arg-1+-Iba1+ cells in hippocampus and cortex of Ctrl or CMS mice after treatment with saline or ASA VI. (C) Levels of mRNAs encoding the pro-inflammatory cytokines Tnf-α, iNOS, and IL-6 in hippocampus and cortex of Ctrl or CMS mice after treatment with saline or ASA VI. (D) Levels of IL-1β in hippocampus and cortex of Ctrl or CMS mice after treatment with saline or ASA VI. (E) Levels of mRNAs encoding the anti-inflammatory cytokines IL-10, Arg-1, and Tgf-β in hippocampus and cortex of Ctrl or CMS mice after treatment with saline or ASA VI. (F) Levels of BDNF in hippocampus and cortex of Ctrl or CMS mice after treatment with saline or ASA VI. (G) Ratio of the levels of mRNAs encoding anti-inflammatory cytokines...
(IL-10, Arg-1, Tgf-β) to the levels of mRNAs encoding pro-inflammatory cytokines (iNOS, Tnf-α, IL-6) in hippocampus and cortex of Ctrl or CMS mice after treatment with saline or ASA VI. (H) Schematic illustrating how ASA VI switches activated microglia from a pro-inflammatory to neuroprotective phenotype in hippocampus of CMS mice. Data for individual animals are displayed (mean ± SEM, n=3-4), *P<0.05, **P<0.01, ***P<0.005 (two-way ANOVA with Tukey’s multiple-comparisons test).

Figure 4

Asperosaponin VI ameliorates CMS-induced depression-like behaviors by activating the PPAR-γ pathway
(A) Levels of p-PPAR-γ, PPAR-γ 1 and PPAR-γ 2 in hippocampus of Ctrl, ASA VI, CMS, and CMS+ASA VI mice. Levels of PPAR-γ-1 and PPAR-γ-2 were normalized to those of β-actin. Levels of phosphorylated PPAR-γ were normalized to those of PPAR-γ-1 and PPAR-γ-2 respectively (n = 3, each sample in triplicate).
(B) Fluorescence micrographs showing PPAR-γ expression in microglia of hippocampus in CMS mice after treatment with ASA VI. PPAR-γ was stained with antibody (green), microglia were stained with an anti-Iba1 antibody (red), and nuclei were stained with DAPI (blue). (C) Timeline of the experimental process on the effect of GW9662 on CMS mice. (D) Effects of GW9662 treatment on levels of p-PPAR-γ, PPAR-γ-1, and PPAR-γ-2 in hippocampus of ASA VI + CMS mice (n = 3, each sample in triplicate). (E) Effects of GW9662 treatment on the sucrose preference, immobility time in tail suspension test and forced swimming test and the traveled distance in open field test of ASA VI + CMS mice. Data for individual animals are displayed (mean ± SEM), *P<0.05, **P<0.01, ***P<0.005, two-way (A and B) or one-way (C and D) ANOVA with Tukey's multiple-comparisons test).

Figure 5

Asperosaponin VI induces an anti-inflammatory microglial phenotype via PPAR-γ (A) Effects of GW9662 treatment on morphology of hippocampal microglial in ASA VI + CMS mice. (B) Effects of GW9662 treatment on levels of mRNA encoding Cd11b in hippocampus of ASA VI + CMS mice. (C and D) Effects of GW9662 treatment on microglia with pro- or anti-inflammatory phenotypes in hippocampus of ASA VI + CMS mice. (E-I) Effects of GW9662 treatment on mRNA or protein levels of pro-inflammatory cytokines (Tnf-α, iNOS, IL-6, and IL-1β) and anti-inflammatory cytokines (IL-10, Arg-1, and IL-4) in hippocampus of ASA VI + CMS mice. Data for individual animals are displayed individually (n=4-6), *P<0.05, **P<0.01, ***P<0.005 (one-way ANOVA with Tukey's multiple-comparisons test).

Figure 6

Asperosaponin VI partially restores synaptic function in hippocampus of CMS mice via a PPAR-γ-dependent pathway (A) Levels of mRNAs encoding ligand-receptor pairs that mediate communication between neurons and microglia (Cx3cl1 / Cx3cr1, CD200 / CD200R) in hippocampus. (B) Fluorescence
micrographs of mature neurons in the DG of hippocampus, identified through staining for NeuN. At right is quantification of the area of NeuN+ cells in the DG. Each dot in the bar graph represents the average of 5–6 micrographs for each mouse (n = 5). (C and D) Effects of asperosaponin VI on levels of PSD95, CamKII α, CamKII β, and p-GluA2 in hippocampus of CMS mice treated with GWP662 or not. Levels of proteins were normalized to those of β-actin (n = 5-6, each sample in triplicate). (E) Correlation of the levels of p-PPA-γ with the levels of PSD-95, CamKII α, CamKII β, and p-GluA2. Each circle represents one mouse (n = 5-6). Data for individual animals are displayed (mean ± SEM), *P<0.05, **P<0.01, ***P<0.005 (one-way ANOVA with Tukey's multiple-comparisons test).

Figure 7
Mechanism by which asperosaponin VI improve CMS-induced depression CMS in mice induces a proinflammatory microglial phenotype, disrupting neuron-microglia communication and synaptic function in hippocampus, ultimately leading to depression-like behaviors. Asperosaponin VI may ameliorate the effects of CMS by inducing microglia to adopt a PPAR-γ-dependent neuroprotective phenotype.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.doc
- Graphicalabstract.png