The Protective Effects of GPR55 Against Hippocampal Neuroinflammation and Neurogenic Damage in CSDS Mice

Shiyu Shen
Fudan University School of Basic Medical Sciences
Rui Yu
Fudan University School of Basic Medical Sciences
Wei Li
Fudan University School of Basic Medical Sciences
Ling-Feng Liang
Fudan University School of Basic Medical Sciences
Qiuqin Han
Fudan University School of Basic Medical Sciences
Huijie Huang
Fudan University School of Basic Medical Sciences
Bing Li
Jinshan Hospital of Fudan University
Shifen Xu
Shanghai University of TCM: Shanghai University of Traditional Chinese Medicine
Gencheng Wu
Fudan University School of Basic Medical Sciences
Yu-Qiu Zhang
Fudan University School of Basic Medical Sciences
Jin Yu (yujin@shmu.edu.cn)
Fudan University School of Basic Medical Sciences https://orcid.org/0000-0001-7223-105X

Research

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Abstract

Background

Depression is one of the most prevalent mental illnesses in the world today, the onset of depression is usually accompanied by neuroinflammation and neurogenic damage. Recently, G protein coupled receptor 55 (GPR55) has been associated with mood regulation as a third kind of cannabinoid receptor, and the activation of GPR55 was demonstrated recently to have a neuroprotective effect on hippocampal neurogenesis against inflammatory insult. However, its role in regulating depression and anxiety remains poorly understood.

Methods

10-day chronic social defeat stress (CSDS) was utilized as an animal model of depression to explore the potential antidepressant effect of GPR55 agonist and electroacupuncture (EA), a common therapy for depression in China. Several behavioral tests i.e. forced swimming test, open field test and elevated plus maze were performed to evaluate the depression- and anxiety-like behaviors in mice. Expression of GPR55, hippocampal neuroinflammation, and neurogenesis were detected using immunohistochemistry, western blot and RT-PCR. Then, the effect of a GPR55 agonist, O-1602 on depression- and anxiety-like behaviors as well as hippocampal neuroinflammation and neurogenesis was examined. Furthermore, the potential antidepressant effect of 3-week EA treatment was also evaluated in another independent experiment. In the experiment, the behaviors and hippocampal neuroinflammation and neurogenesis were also subsequently examined.

Results

After CSDS, the protein level of GPR55 was decreased only in susceptible mice but the mRNA expression was not significantly different in all CSDS mice. Additionally, depression- and anxiety-like behaviors are accompanied by neuroinflammation and reduced neurogenesis in the hippocampus. And the activation of GPR55 during the process of CSDS prevented the development of depression- and anxiety- like behaviors, as well as hippocampal neuroinflammation and neurogenetic damage. Similarly, EA alleviated the depression- and anxiety-like behaviors, hippocampal neuroinflammation, and neurogenetic damage as well as decreased protein level of GPR55 in hippocampus induced by CSDS.

Conclusions

Our research demonstrated that activation of hippocampal GPR55 could rescue depression and anxiety phenotypes, reduce hippocampal neuroinflammation, and enhance hippocampal neurogenesis. Moreover, GPR55 might be involved in the beneficial effect of EA on depression.

Background

Depression is one of the most prevalent mental illnesses in the world today, and yet its etiologies remain unclear and current treatments are not wholly effective(1, 2). Numerous studies have employed chronic social defeat stress (CSDS) to study the neurobiological mechanisms of depression in rodents(3). A growing body of evidence implicatd the critical role of the endocannabinoid (eCB) system in emotional homeostasis and the pathophysiology of depression(4, 5). Recently, one type of G protein-coupled receptor, G protein coupled receptor 55 (GPR55), was identified as a novel CB receptor owing to its high affinity for cannabinoid ligands, such as Δ9-tetrahydrocannabinol, 2-arachidonoylglycerol, anandamide and rimonabant(6). It is highly expressed in the central nervous system (CNS), including hippocampus, striatum, amygdala and cortex, as well as in peripheral tissues such as endothelial cell, and the gastrointestinal tract. Reportedly, it is related to many pathophysiological and physiological processes and plays an important role in maintaining proper brain function(7). A recent study has clarified that GPR55 mediates anxiolytic-like effects in the medial orbital cortex of mice experiencing acute stress(8). Moreover, GPR55 is mainly expressed on microglia(9). However, the exact role of GPR55 in the modulation of depression is still unknown.

The hippocampus is a limbic system structure implicated in mood- and emotion-related processes. Hence, hippocampal dysfunction, including impaired adult hippocampal neurogenesis, has been implicated in the development of a number of psychiatric and neurological pathologies, such as depression(10–12). Our previous studies have also clarified that impaired neurogenesis in adult hippocampus is associated with the expression of depression-like symptoms, while electro-acupuncture and a tricyclic antidepressant, clomipramine may increase neurogenesis in adult hippocampus and alleviate depression-like behaviors simultaneously(13–15). But how treatments influence hippocampal neurogenesis is still obscure. A recent study gives some cues, suggesting that activation of GPR55 induced neuroprotection of hippocampal neurogenesis following chronic, systemic inflammation(16). Moreover, the endogenous agonist for GPR55 showed neuroprotective effects after excitotoxic lesion in microglial and GPR55-dependent manner in CNS(17). Furthermore, *Gpr55* mRNA expression in microglia is downregulated after treatment with LPS or IFNγ, suggesting that GPR55 is involved in neuroinflammation(18). In fact, LPS is sometimes used to build an animal model of inflammation-induced depression(19). Our previous study also found that microglial activation and neuroinflammation in hippocampus contribute to the onset of depression-like behaviors(20, 21). Thus, we decided to investigate if the hippocampal GPR55 mediates the stress-induced depression.

Given the findings in the abovementioned studies, we aimed to explore whether chronic social defeat produces depression- and anxiety-like behaviors and influences the expression of GPR55 in hippocampus. Based on these results, we further utilized pretreatment with an agonist of GPR55, O-1602 before every direct social attack in the 10-day CSDS procedure, to observe its effect on the CSDS-induced depression- and anxiety-like behaviors. Then, we gave CSDS mice EA treatment, a considerable therapy for depression patients in China, to observe the potential antidepressant effect of EA and its influence on the hippocampal GPR55. In addition, we investigated the possible role of GPR55 in the beneficial therapeutic effect of EA on depression using concomitant GPR55 antagonist and EA treatment in the

CSDS-induced depression model. To our knowledge; this is the first study ever focusing on the role of GPR55 in the depression- and anxiety-like behaviors induced by CSDS procedure.

Materials And Methods

Animals

Male C57BL/6J mice (7–9 weeks, Shanghai SLAC Laboratory Animal Co. Ltd.) and male retired CD1 (8–9 months, Vital River Laboratories, Beijing, China) were kept under a temperature- and humidity-conditioned environment with 12 h light/dark cycle (lights on from 7:00 A.M. to 7:00 P.M.). C57BL/6J mice were housed in groups, and CD1 aggressors were single-housed in the half of the cage which was separated by the perforated Plexiglas separators. All mice were fed ad libitum, and allowed to adjust to the new environment for at least 1 week before experimental manipulation. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Experimental Animal Ethics Committee of Shanghai Medical College, Fudan University.

CSDS

The CSDS paradigm was conducted as previously described (22) (Fig. 1A). In brief, CD1 retired breeders with reliable attack latencies were housed in the right side of the cage divided using perforated Plexiglas separators. The C57BL/6J mice were exposed a CD1 aggressor for 5 min every day, and the CD1 mice were novel and different for 10 consecutive days. When the daily social defeat procedure ended, the defeated mice were placed in a left side to the respective aggressive CD1 and were allowed visual, olfactory and auditory contact for 24 h. Control mice were housed separately in pairs in one cage, and changed to 10 different C57BL/6J mice for 10 consecutive days.

EA treatment

During EA treatment, the mice were restrained on a fixed shelf without anesthesia with their heads being able to move freely. Two acupuncture points were selected: Bai-Hui (Du-20) and Yang-Ling-Quan (GB-34, the right side). Du-20 is located above the apex auriculate, on the midline of the head. GB-34 is inferior and anterior to the head of the fibula. These two acupuncture points have been employed in previous studies, showing antidepressant effects. A pair of stainless-steel needles with diameters of about 0.3 mm were subcutaneously inserted into Du-20 (+) and GB-34 (−) to a depth of about 5 mm. The needles were connected to the output terminal of an EA apparatus (HANS Electric Stimulator, LH202H, 2/100 Hz, 0.1 mA) with the stimulation lasting for 30 min. The EA was administered once daily for five consecutive days every week, for three weeks (from day 12 to day 33, Fig. 6A). The mice in the vehicle group were restrained in the same way as the ones in the EA group.

Chemicals and Reagents

The following drugs were used in the study: O-1602 (Cayman Chemical, No.317321-41-8) and CID16020046 (Cayman Chemical, No.834903-43-4), the specific agonist and antagonist of GPR55 were dissolved in DMSO and then diluted in saline solution. Mice were administered different doses of CID16020046: low dose (0.3 mg/kg) and high dose (3 mg/kg). The drugs were administered via intraperitoneal (*i.p.*) injection. The agonist and antagonist were administered 30 min before every social defeat session and EA treatment.

Behavioral tests

Social interaction test (SIT)

On day 11 and day 34, the social interaction test was conducted to assess the social behavior of C57BL/6J mice (Fig. 1A)(22). The test was conducted by individually placing mice in a previously cleaned open field arena (42 cm×42 cm×42 cm). In the first 2.5 min session (“no target”), each C57BL/6J mice were permitted free exploration of the arena with an empty cage (10 cm length × 10 cm width × 42 cm height), which allowed for visual, olfactory and auditory contact. During the second 2.5 min session (“target”), the empty cage was replaced with a cage with a target, an unfamiliar aggressive CD1 mouse for each defeated C57BL/6J mouse in it. Time spent in the interaction zone (10 cm around the cage) by the C57BL/6J mouse was measured in two sessions. Based on this, the social interaction ratio (SIR) was defined as the ratio of time spent in the interaction zone with a target (CD1 mouse), to the time spent in the interaction zone with no target(22). Historically, if one mice spent more time in the interaction zone during with target than with no target, it would be defined as “resilient” mice (SIR ≥ 1), and “susceptible” mice were the ones with SIR < 1 (23, 24).

Elevated plus-maze test (EPM)

On day 11 and day 34, each mouse was first subjected to SIT and then to the EPM test, the equipment was consisted of two closed arms (20 cm length × 12 cm height × 4 cm width) crossed with two similar open arms. The test was used to determine the percentage of time spent in the open arms to the time spent in either type of arms in 5 min, which serves as an assay of anxiety-like behavior of mice. The test took place in dim light (15 lux) and the apparatus was cleaned using 1% acetic acid to clear any remaining olfactory cues left by the previous mice.

Open field test (OFT)

On day 12 and day 35, OFT was conducted to evaluate anxiety and exploration behavior of the mice. The apparatus consisted of a square arena (50 cm × 50 cm) with opaque plastic walls, 40 cm in height. During the test, each mouse was gently placed in the center of the square and facing the same direction, and allowed to explore the arena for 5 min. The measured variables were the travelled distance and the number of rearing times (standing upright on hind legs). The test took place in dim light (15 lux) and after each test 1% acetic acid was used to remove the residual odor.

Forced swimming test (FST)

FST is widely used to assess the depression-like behavior of mice. During this test, each mouse was gently placed in a glass cylinder (16 cm in diameter and 32 cm in height) containing 20 cm of water (25 ± 1 °C) for 5 min and total duration of struggling time was recorded. In this test, the decrease of struggling time implies an increase in depression-like behavior.

Western blot analysis (WB)

Hippocampus was isolated for western blot analysis. The hippocampus was firstly lysed in RIPA buffer (Millipore, USA) and the protein was obtained by centrifugation at 12000 g for 15 min at 4 °C. Supernatants were collected, and the protein concentration of each sample was determined using bicinchoninic acid (BCA) assay (Thermo Fisher, USA). For gel electrophoresis, the same level of total protein was separated on 12% SDS-PAGE and electro-transferred to PVDF membranes (Millipore, USA). Blots were then blocked for 2 h with blocking buffer at RT. Following washing, PVDF membranes with protein were incubated overnight at 4 °C with primary antibody. After washing 3 times with TBST for a total of 1 h, PVDF membranes were then incubated for 2 h at 4 °C with secondary antibody and then washed 3 times with TBST, each time for 10 min. Immunoreactivity was visualized using ECL reagent (Millipore, USA). The membranes were probed with Tubulin antibody (Rabbit mAb, 1:5000, Proteintech, 10094-1-AP, USA) as an internal control. The proteins were probed with respective primary antibodies including anti-GPR55 (Rabbit mAb, 1:1000, Proteintech, 55249-1-AP, USA), anti-NLRP3 (Rabbit mAb, 1:2000, Cell Signaling Technology, 15101, USA), anti-ASC (Rabbit mAb, 1:1000, Cell Signaling Technology, 67824, USA), and anti-Caspase-1 (Rabbit mAb, 1:1000, Cell Signaling Technology, AB-P-R001, USA). Image J analysis software was used for densitometric analysis of immunoblots.

RNA extraction and RT-PCR analysis

The hippocampus was homogenized in Trizol reagent (Takara, Kusatsu, Shiga, Japan) for total RNA extraction. The purity and concentration of the samples were assessed by the Nanodrop Spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, USA). The adoptable ratios of the absorbances at wavelengths of 260 and 280 nm were 1.8-2.0, and the unqualified samples were eliminated. And then, about 1 μg of RNA was used for RT-PCR assay, performed using TB green Premix Ex TaqTM (Takara, Kusatsu, Shiga, Japan) on a Real-Time PCR Detection System (Roche, Basel, Switzerland) according to the manufacturer's protocol. Data were then processed using the $2^{-\Delta\Delta CT}$ method. The sequences of genes including *Gapdh, Gpr55, interleukin-1β (Il-1β), Il-6, Il-4, Il-10, iNOS, Tnfα* and their primer pairs were listed followed:

Gapdh:

Forward: AGGTCGGTGTGAACGGATTTG

Reverse: GGGGTCGTTGATGGCAACA

Gpr55:

Forward: CTGGCAGTCCATATCCCCAC

Reverse: GCACCAGCAGTAAATCGAAAACA

Il-1β:

Forward: GAAATGCCACCTTTTGACAGTG

Reverse: TGGATGCTCTCATCAGGACAG

Il-6:

Forward: CTGCAAGAGACTTCCATCCAG

Reverse: AGTGGTATAGACAGGTCTGTTGG

Il-4:

Forward: CCCCAGCTAGTTGTCATCCTG

Reverse: CAAGTGATTTTTGTCGCATCCG

iNOS:

Forward: GGAGTGACGGCAAACATGACT

Reverse: TCGATGCACAACTGGGTGAAC

Tnfα:

Forward: CCTGTAGCCCACGTCGTAG

Reverse: GGGAGTAGACAAGGTACAACCC

RNA scope

For the preparation of hippocampal slices, animals were anesthetized with 4% pentobarbital sodium, and brain samples were dissected out and fixed in 4% neutral buffered formalin for 16 to 32 h at 4°C, and then the brains were dehydration with a gradient of 10%, 20% and 30% sucrose-PB solution sections. After the brains were cut to 20 μm thickness through Cryostat Microtome (Leica, CM1850, Germany), the brain sections were placed at 50°C for 30 min using a hot plate. Then, hydrogen peroxide was used to incubated for 10 min and washed by distilled water for 3–5 time. And immediately treated with 10 μg/mL protease (Advanced Cell Diagnostics, Hayward, CA, USA) at 40°C for 30 min in a HybEZ hybridization oven (Advanced Cell Diagnostics, Hayward, CA, USA). Subsequently, hybridization with target probes for 2h in 40℃ HybEZ hybridization oven, and washed by washing buffer for 2 min. And added AMP1, AMP2 and AMP3 according the order, and incubated each probe for 30 min in 40℃ HybEZ hybridization oven, and then washed by washing buffer for 2 min each. The probe signal is labeled according to the channel

of the target probe, and corresponding chromogenic detection were added. Images were obtained and analyzed using Olympus FV10 confocal microscope (Olympus, FV10, Japan).

Immunohistochemistry (IHC)

Mice were deeply anesthetized with 4% pentobarbital sodium and were perfused transcardially with 20 mL 0.9% saline before the brain collection. Then the whole brain was placed in 4% paraformaldehyde overnight at 4°C. After dehydration with a gradient of 20% and 30% sucrose-PBS solution, 35um brain slices were prepared through Cryostat Microtome (Leica, CM1850, Germany). Briefly, brain sections were blocked with 10% goat serum and incubated for 2 h at RT with primary antibodies DCX (Rabbit mAb, 1:500, Cell Signaling Technology, 14802, USA) and Nestin (Rabbit mAb, 1:500, Abcam, ab221660, USA). Then the brain sections were incubated with Alexa Fluor 488 (1:400, Thermo Fisher Scientific, A-11034, USA) secondary antibody for 2 h at RT and then washed 3 times for 15 min with PBS. Images were obtained and analyzed using Olympus FV10 confocal microscope (Olympus, FV10, Japan). At least three sections were analyzed per mouse, and the average of the individual measurements was used to calculate group means.

Statistical analysis

All data were analyzed using GraphPad Prism (USA), version 6.0c and were presented as (mean ± S.E.M). The data were normally distributed and showed homogeneity of variance. Thus, differences among groups were examined using one-way ANOVA and two-way ANOVA, followed by Turkey's multiple comparison test, where a P value below 0.05 was considered statistically significant, and a P value below 0.08 was considered a significance trend.

Results

CSDS induced significant depression- and anxiety-like behaviors and the decrease of GPR55 in hippocampus

After exposure to 10 days of CSDS (Fig. 1a, b), almost thirty percent of stressed mice exhibited social avoidance behavior (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(3, 95)} = 13.15$, $P < 0.001$) (SIR < 1, Fig. 1c, d, e) in SIT and were considered susceptible (Sus), while the remaining mice did not show substantial avoidance behavior and were considered resilient (Res). Despite this, all the mice exposed to CSDS showed some depression-like manifestations, such as behavioral despair indicated by spending less time struggling in FST (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(3, 92)} = 58.86$, $P < 0.001$) (Fig. 1f). Meanwhile, all the mice exhibited anxiety-like manifestation, such as spending significantly less time exploring the open arms in EPM (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(3, 95)} = 21.48$, $P < 0.001$) (Fig. 1g, h) and lower exploration indicated by rearing less (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(3, 95)} = 34.52$, $P < 0.001$) (Fig. 1i, k). However, the locomotor activity was not influenced (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(3, 56)}$

= 0.5310, $P = 0.6629$) (Fig. 1j, k). These results affirmed that CSDS can induce depression- and anxiety-like phenotypes in mice.

Next, we observed the protein level of GPR55 in hippocampus of socially defeated mice (including resilient and susceptible mice). A significant decrease in the protein levels of GPR55 was observed in hippocampus of susceptible mice, but not that of resilient mice (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(2, 21)} = 10.73$, $P < 0.001$) (Fig. 2a, b). However, the *Gpr55* mRNA did not show significant difference between control mice and defeated mice (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(2, 17)} = 0.02096$, $P = 0.9793$) (Fig. 2c). This finding suggests that it is the expression of GPR55 protein, and not the transcription of *Gpr55* mRNA that was affected by CSDS and related to social avoidance behavior. Meanwhile, GPR55 has been reported to modulate inflammation and is expressed in immune cells, such as monocytes and microglia (9). Thus, we further confirmed whether GPR55 is mainly expressed in microglia in hippocampus by using RNA Scope. The results from in situ RNA analysis of the hippocampus showed that most fluorescence signaling of *Gpr55* mRNA was colocalized with Iba1, and did not colocalize with Nestin, which indicates that GPR55 is mainly expressed in microglia (Fig. 2d, e).

GPR55 agonist, O-1602, ameliorated social avoidance, as well as depression- and anxiety-like behaviors induced by CSDS

Due to the fact that CSDS induced a significant decrease in the protein level of GPR55 in susceptible mice, but not in resilient mice, we speculated if the impaired function of GPR55 was related to social avoidance behavior of susceptible mice. Hence, we further investigated, if activation of GPR55 can prevent CSDS-induced social avoidance behavior. To this end, a GPR55 agonist, O-1602 was injected intraperitoneally (10 mg/kg, *i.p.*) 30 min before each direct attack everyday (Fig. 3a). We found that pretreatment with O-1602 significantly blocked the deleterious effect of CSDS on the social avoidance behavior (two-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(1, 24)} = 7.022$, P 0.01) (Fig. 3b, c). Unexpectedly, we also observed that pretreatment with O-1602 significantly increased struggling time in FST (two-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(1, 24)} = 10.51$, $P < 0.01$) (Fig. 3d), the percentage of time spent in the open arms in EPM ($F_{(1, 24)} = 4.166$, $P = 0.0524$) (Fig. 3e), and rearing frequency in OFT (two-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(1, 24)} = 17.49$, $P < 0.001$) (Fig. 3f) compared with mice in the vehicle group. However, administration of O-1602 did not alter the motor function of mice (two-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(1, 24)} = 0.7581$, $P = 0.3926$) (Fig. 3g). These results suggest that impaired function of GPR55 might have a major influence on mediating the development of social avoidance behavior, also affecting other depression- and anxiety-like phenotypes.

Activation of GPR55 alleviated neuroinflammation and restored impaired neurogenesis

As previously mentioned, depression is usually accompanied by upregulated neuroinflammation and neurogenesis deficits (25, 26). Considering that Lysophosphatidylinositol (LPI), the main endogenous agonist for GPR55, exhibited anti-inflammatory effects in cultured microglia (27) and showed neuroprotective effects after excitotoxic lesion in a microglial and GPR55-dependent manner in CNS(17), we further investigated the effect of O-1602 on neuroinflammation and neurogenesis in hippocampus. In line with previous studies(20, 21), the results showed that the three parts of NLRP3 inflammasome, NLRP3(two-way ANOVA followed by Turkey's post-hoc of NLRP3. Treatment $F_{(1, 8)} = 18.3$, $P < 0.01$), ASC (two-way ANOVA followed by Turkey's post-hoc of ASC. Treatment $F_{(1, 8)} = 11.29$, $P < 0.01$), and Caspase-1 (two-way ANOVA followed by Turkey's post-hoc of Caspase-1. Treatment $F_{(1, 8)} = 12.97$, $P < 0.01$) were significantly increased in hippocampus of defeated mice (Fig. 4a-d). As we expected, O-1602 was able to significantly block the activation of NLRP3 inflammasome in defeated mice (Fig. 4a-d). We also examined several cytokines and chemokines related to neuroinflammatory processes in hippocampus and found that levels of *Il-1β* (two-way ANOVA followed by Turkey's post-hoc of *Il-1β*, Treatment $F_{(1, 12)} = 6.980$, $P = 0.0215$, Fig. 4e), *Il-6* (two-way ANOVA followed by Turkey's post-hoc of *Il-6*, Treatment $F_{(1, 12)} = 32.83$, $P < 0.001$, Fig. 4f), *iNOS* (two-way ANOVA followed by Turkey's post-hoc of *iNOS*, Treatment $F_{(1, 12)} = 3.512$, $P < 0.001$, Fig. 4g), and *Tnfa* (two-way ANOVA followed by Turkey's post-hoc of *Tnfa*, Treatment $F_{(1, 12)} = 8.088$, $P = 0.0148$, Fig. 4h) mRNA were elevated, while *Il-4* (two-way ANOVA followed by Turkey's post-hoc of *Il-4*, Treatment $F_{(1, 12)} = 3.757$, $P = 0.0137$, Fig. 4i) and *Il-10* mRNA levels (two-way ANOVA followed by Turkey's post-hoc of *Il-10*, Treatment $F_{(1, 12)} = 7.280$, $P = 0.0194$, Fig. 4j) were lowered in defeated mice as compared to the vehicle control group. O-1602 pretreatment also reduced the expression of pro-inflammatory cytokines at transcription level (Fig. 4e-j).

Meanwhile, to understand whether O-1602 would have neuroprotective effect, we utilized Nestin and doublecortin (DCX) immunostaining to assess hippocampal neurogenesis. Nestin is a well-known marker of neural stem progenitor cells (NSPCs), and DCX is mainly expressed in neural precursor cells and immature neurons. Consistent with the previous studies(14, 15), DCX^+ cells (Fig. 5a-b) and $Nestin^+$ (Fig. 5c-d) in the dentate gyrus (DG) were significantly reduced in defeated mice as compared to control mice. Meanwhile, administration of O-1602 partly reversed the CSDS-induced decrease in the abundance of DCX^+ cells (two-way ANOVA followed by Turkey's post-hoc, Treatment $F_{(1, 12)} = 5.722$, $P = 0.0340$) (Fig. 5a-b) and $Nestin^+$ (two-way ANOVA followed by Turkey's post-hoc, Treatment $F_{(1, 12)} = 12.42$, $P < 0.001$) (Fig. 5c-d) in DG.

EA treatment markedly ameliorated depression- and anxiety-like behaviors of the stressed mice

Due to limited efficacy and serious side effects of many antidepressants, many patients in China tend to turn to various types of Traditional Chinese Medicine to deal with depression. Acupuncture is one of the major representative TCM treatments. The underlying mechanism of the antidepressant effect of EA remains unclear. Our previous study found that EA can improve the behavioral deficits of rats exposed to

chronic unpredictable stress (CUS), including behavioral despair and anxiety-like behaviors, as well as alleviated the activation of neuroinflammation-related markers in hippocampus, such as NLRP3 inflammasome, microglia, and pro-inflammatory cytokines (28). In the present study, after exposure to 10 days of CSDS, defeated mice were treated with EA for 3 weeks (Fig. 6a). EA showed significant beneficial effects on social interaction, as indicated by the increased SIR as compared to defeated mice (one-way ANOVA followed by Turkey's post-hoc, Treatment $F_{(2, 33)} = 14.12$, $P < 0.001$) (Fig. 6b-c). Three weeks after the end of CSDS, defeated mice still exhibited significant depression- and anxiety-like behaviors (Fig. 6d-g). EA treatment induced significant antidepressant- and anxiolytic-like effects as indicated by increased struggling time in FST (one-way ANOVA followed by Turkey's post-hoc, Treatment $F_{(2, 30)} = 13.17$, $P < 0.001$) (Fig. 6d), increased open arm time percentage in EPM (one-way ANOVA followed by Turkey's post-hoc, Treatment $F_{(2, 33)} = 10.66$, $P < 0.001$) (Fig. 6e), as well as increased rearing frequency in OFT (one-way ANOVA followed by Turkey's post-hoc, Treatment $F_{(2, 21)} = 11.50$, $P < 0.001$) (Fig. 6f). However, there was still no significantly difference in the total distance travelled in OFT between the groups (one-way ANOVA followed by Turkey's post-hoc, Treatment $F_{(2, 33)} = 1.247$, $P = 0.3006$) (Fig. 6g). All in all, the results display that EA markedly ameliorated the depression- and anxiety-like behaviors of the stressed mice.

EA regulated GPR55 expression, alleviated neuroinflammation, and decreased neurogenesis deficits caused by CSDS

Because EA is able to alleviate CSDS-induced anxiety- and depression-like behaviors in mice, we wondered if the antidepressant effects of EA also attenuated the reduction of GPR55 protein in the hippocampus of defeated mice. Thus, we examined GPR55 expression by using WB, and found that 3 weeks of EA treatment significantly restored the protein levels of GPR55 in defeated mice (one-way ANOVA followed by Turkey's post-hoc, Treatment $F_{(2, 9)} = 34.26$, $P < 0.001$) (Fig. 7a-b). As we expected, treatment with EA effectively reduced the activation of NLRP3 (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(2, 9)} = 82.27$, $P < 0.001$), ASC (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(2, 9)} = 12.54$, $P < 0.001$), Caspase-1 (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(2, 9)} = 22.64$, $P < 0.0001$) (Fig. 7c-f), significantly reduced the RNA level of pro-inflammatory cytokines, which were upregulated in the hippocampus of defeated mice, such as *Il-1β* (one-way ANOVA followed by Turkey's post-hoc of *Il-1β*, Treatment $F_{(2, 9)} = 24.40$, $P < 0.001$), *Il-6 (*one-way ANOVA followed by Turkey's post-hoc of *Il-6*, Treatment $F_{(2, 9)} = 15.36$, $P < 0.01$); *Tnfa* (one-way ANOVA followed by Turkey's post-hoc of *Tnfa*, Treatment $F_{(2, 9)} = 21.12$, $P < 0.001$); *iNOS* (one-way ANOVA followed by Turkey's post-hoc of *iNOS*, Treatment $F_{(2, 9)} = 32.19$, $P < 0.001$) (Fig. 7g-j), and upregulated the anti-inflammatory cytokines, which were reduced in defeated mice (one-way ANOVA followed by Turkey's post-hoc of *Il-4*, Treatment $F_{(2, 9)} = 6.553$, $P = 0.0175$; one-way ANOVA followed by Turkey's post-hoc of *Il-10*, Treatment $F_{(2, 9)} = 29.09$, $P < 0.001$) (Fig. 7k, l). Furthermore, we examined hippocampal neurogenesis after EA treatment and found that EA significantly reversed the CSDS-induced decrease in the mean fluorescence intensity of DCX^+ (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(2, 9)} = 51.68$, $P < 0.001$) (Fig. 8a-b) and

Nestin$^+$ cells (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(2, 9)} = 134.5$, $P < 0.001$) (Fig. 8c-d) in DG. These results suggest, that the restored GPR55 expression, the reduced neuroinflammation, and the improved neurogenesis might mediate the potential beneficial effect of EA on depression.

GPR55 antagonist, CID16020046 reversed the antidepressant and anxiolytic effects of EA

As mentioned above, EA treatment has similar effect as GPR55 agonist. Thus, we speculated that GPR55 might be involved in the antidepressant and anxiolytic effect of EA. To investigate the role of GPR55 in the therapeutic effect of EA, we employed an antagonist of GPR55, CID16020046. After 10 days of CSDS procedure, defeated mice were divided into three groups, one group was given EA treatment, while the remaining two groups received combined treatment with EA and CID16020046 (low dose: 0.3 mg/kg and high dose: 3 mg/kg). All of the treatments were given for 3 weeks, and the behavioral tests were performed in the same order as previously (Fig. 9a).

As shown in Fig. 9, EA exhibited significant beneficial effect on social avoidance behavior, as well as anxiety- and depression-like behavior. However, the administration of CID16020046 attenuated the effects of EA on social avoidance behavior (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(4, 55)} = 8.735$, $P < 0.001$) (Fig. 9b-c), both in the high-dose group and the low-dose group. Interestingly, CID16020046 also significantly attenuated the beneficial effects of EA on struggling time in FST (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(4, 56)} = 54.21$, $P < 0.001$) (Fig. 9d), the open arm time percentage in EPM (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(4, 56)} = 16.61$, $P < 0.001$) (Fig. 9e), and rearing frequency in OFT (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(4, 56)} = 28.94$, $P < 0.001$) (Fig. 9f). Moreover, neither 0.3 mg/kg nor 3 mg/kg of CID16020046 significantly influenced the effect of EA on total distance travelled in OFT (one-way ANOVA followed by Turkey's post-hoc. Treatment $F_{(4, 56)} = 1.988$, $P = 0.1088$) (Fig. 9g). These results hint that the GPR55 antagonist reversed most of the antidepressant and anxiolytic effects of EA. Taken together, these findings support the contention that GPR55 plays an important role in the antidepressant- and anxiolytic-like impact induced by EA.

Discussion

The results exhibited that CSDS caused significant depression- and anxiety-like manifestations and decreased the protein level of GPR55, which was mainly expressed on microglia in hippocampus. In addition, O-1602, an agonist of GPR55, was able to attenuate some depression- and anxiety-like behaviors, simultaneously reducing neuroinflammation and protecting neurogenesis in defeated mice (Fig. 10). Meanwhile, EA as a potential antidepressant therapy, exhibited antidepressant- and anxiolytic-like, as well as neuroprotective effects in CSDS mice and restored the GPR55 expression Moreover, the utilization of GPR55 antagonist significantly blocked the antidepressant- and anxiolytic-like effects of EA.

These results shed some light onto the role of GPR55 in etiology of depression and anxiety, hinting that upregulating GPR55 signaling might be a promising therapy for depression.

An increasing number of studies have employed CSDS to study the neurobiological mechanisms of depression in rodents(29, 30). In this model, C57 mice are repeatedly exposed to CSDS induced by CD1 aggressor mice for 10 consecutive days. Subsequently, these mice tend to display robust depression- and anxiety-like phenotypes, as corroborated by the findings of the present study(22). Specifically, after exposure to CD1 aggressor mice for 10 consecutive days, nearly thirty percent of C57 mice exhibited social avoidance behavior in social interaction test, thus being classified into susceptible group, while the other part of C57 mice did not, which placed them in the resilient group. However, in other behavioral tests, there were no significant differences between susceptible and resilient mice. Both of the groups exhibited significant behavioral despair, decreased exploration and locomotor activity, as well as more anxiety-like behaviors.

Perhaps mood regulation is a field with limited information about the physiological functions of GPR55. Although, expression of *Gpr55* mRNA in brain structures related to mood regulation, such as hippocampus, cortex, striatum and limbic system has been reported(6, 31), the description of its role in the process is in the best of cases incipient and remains to be comprehensively elucidated. However, some isolated reports have shed some light on the process and suggest that this receptor may carry important behavioral consequences. For example, intracerebroventricular administration of a GPR55 agonist, O-1602, induced an anxiolytic-like effect, while a GPR55 antagonist, ML193, increased anxiety-like behaviors in rats(32). Another study also provides similar results where activation of GPR55 was able to alleviate anxiety-like behaviors in acutely stressed mice, while a selective knockdown of GPR55 in the medial orbital cortex abolished this anxiolytic effect of O-1602 and induced anxiety-like behaviors(8). Interestingly, GPR55 knockout mice were also reported to have similar anxiety-like behaviors as wild-type mice(31). In concordance with the three studies, the present study further confirmed the anxiolytic effect as well as the antidepressant-like effect of GPR55 agonist in CSDS-induced animal model of depression.

EA is similar to acupuncture, which is a popular method within Traditional Chinese Medicine and includes a wide range of practices in the treatment of depression in Asian populations, especially in China. Our current results show that EA has significant antidepressant and anxiolytic effects. In previous studies, we have also observed neuroprotective effect of EA after chronic unpredictable stress(28, 33). The present study showed that EA induced antidepressant effect and upregulated the protein level of GPR55 in hippocampus. Interestingly, Kallendrusch et al. found that administration of GPR55 agonists in the adult rat hippocampus induced a (microglia-dependent) neuroprotective effect after excitotoxic lesions(34). Their study bridges the antidepressant and protective effect of GPR55 agonist and EA. Combined with their results, as well as those from our previous studies, the present findings further suggest that EA may induce antidepressant and neuroprotective effect via upregulating GPR55.

Moreover, the studies of GPR55 expression after exposure to stress are rare. Marco et al. observed increased expression of GPR55 in the frontal cortex of male and the hippocampus of female rats after

exposure to experimental early life stress (35). Interestingly, in a rat model of autism induced by prenatal exposure to valproic acid, a decrease in the expression of GPR55 in the frontal cortex and hippocampus was reported (36, 37). This scarce information suggests that alterations in GPR55 expression may subserve certain pathologies, such as autism and anxiety. However, density of GPR55 was significantly downregulated only in susceptible mice, which exhibited obvious social avoidance behavior, while O-1602 was able to significantly prevent the social avoidance behavior induced by CSDS. This raises the question, whether GPR55 works as a major factor in social anxiety disorders.

In summary, the present study shows that hippocampal GPR55 reduction is linked to CSDS-induced depression- and anxiety-like behaviors and that GPR55 agonists reverse the detrimental effects of CSDS. Evidently, GPR55 plays an important role in depression and anxiety, thus encouraging our further efforts to reveal the exact mechanism.

Conclusions

Our research found that CSDS model decreased the protein level of GPR55 in microglia of hippocampus, and GPR55 agonist reversed the depression- and anxiety-like behaviors, inhibited hippocampal neuroinflammation and neurogenetic damage induced by CSDS. These revealed the positive regulation effect of GPR55 in hippocampus on depression- and anxiety-like behaviors. In the meantime, GPR55 antagonist impeded the beneficial effect of EA, which revealed how GPR55 contributed to EA in depression. It could be concluded that GPR55 not only regulate depression- and anxiety- like behaviors, but also a key mechanism for EA treatment.

Abbreviations

GPR55: G protein-coupled receptor 55

eCB: Endocannabinoid

CSDS: Chronic social defeat stress

EA: Electroacupuncture

CNS: Central nervous system

SIT: Social interaction test

EPM: Elevated plus-maze test

OFT: Open field test

FST: Forced swimming test

Res: Resilient

Sus: Susceptible

LPI: Lysophosphatidylinositol

DCX: Doublecortin

NSPCs: Neural stem progenitor cells

DG: Dentate gyrus

CUS: Chronic unpredictable stress

Declarations

Availability of data and materials

The data used in this study can be reasonably requested from the corresponding authors.

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Author information

Affiliations

Department of Integrative Medicine and Neurobiology, Institutes of Brain Science, State Key Laboratory of Medical Neurobiology, and Collaborative Innovation Center for Brain Science, School of Basic Medical Sciences, Shanghai Medical College, Fudan University, Shanghai 200032, China

Shi-Yu Shen, Rui Yu, Wei Li, Ling-Feng Liang, Qiu-Qin Han, Hui-Jie Huang, Gen-Cheng Wu, Yu-Qiu Zhang, Jin Yu

Center Laboratories, Jinshan Hospital of Fudan University, Shanghai 201508, China

Bing Li

Department of Acupuncture, Shanghai Municipal Hospital of Traditional Chinese Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Shi-Fen Xu

Contributions

SYS, RY, WL: Investigation, Formal analysis, Writing – original draft. LFL, QQH: Conceptualization, Writing – review & editing. HJH, BL: Formal analysis, Writing – review & editing. SFX, GCW, YQZ: Conceptualization, Methodology – review & editing, Funding acquisition. JY: Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Corresponding authors

Correspondence to Yu-Qiu Zhang or Jin Yu.

Ethics declarations

Ethics approval and consent to participate

All experiments involved in this study were conducted in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals, and were approved by the School of Basic Medicine of Fudan University.

Consent for publication

Not applicable

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

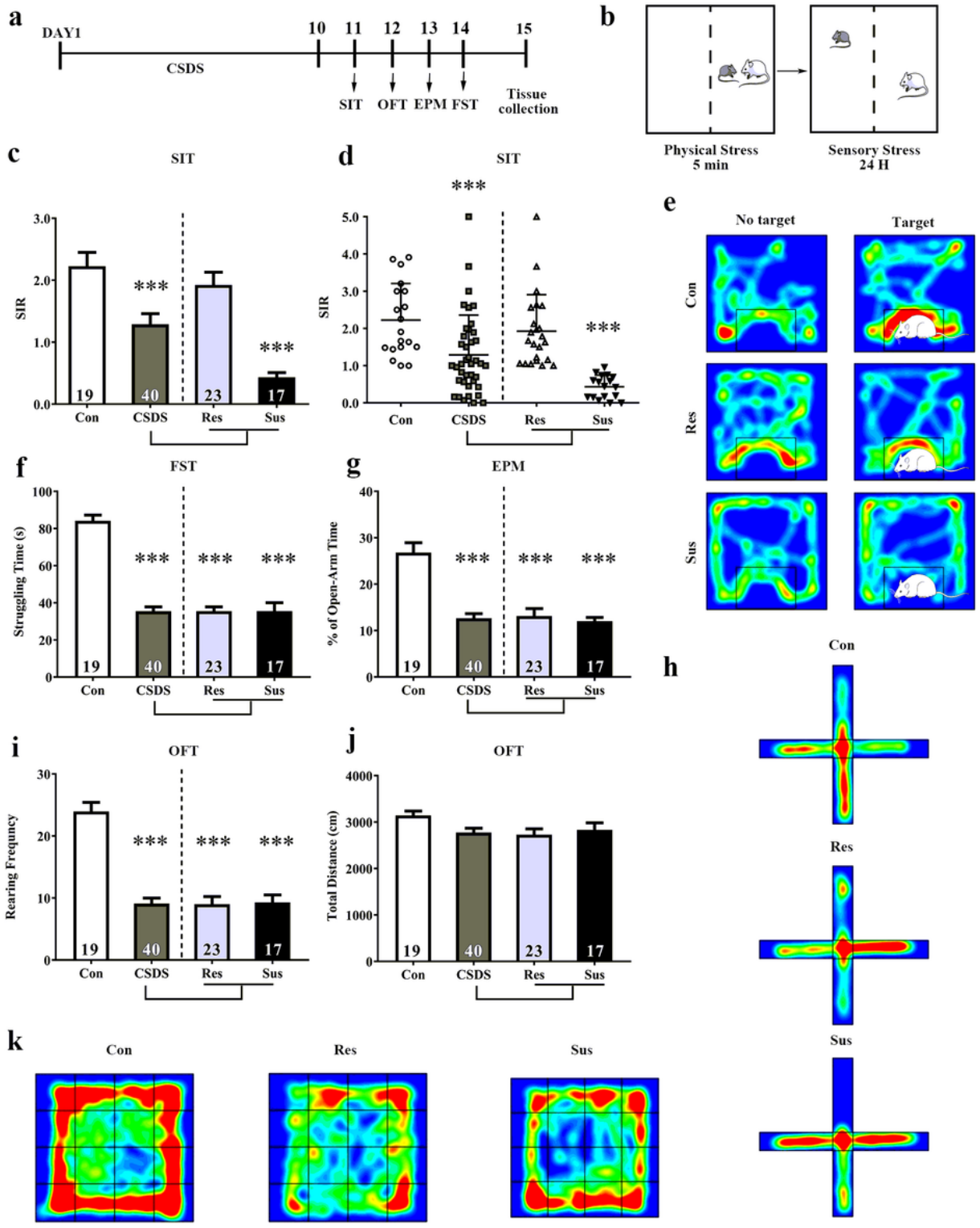

Figure 1

Chronic social defeat stress induced depression- and anxiety-like behaviors in C57BL/6 male mice. After 10 consecutive days of social defeat stress (a, b), social interaction ratio (c, d), struggling time in FST (f), open-arm time percentage in EPM (g), rearing frequency (i), and total distance in OFT(j), were tested to evaluate the establishment of depression- and anxiety-like model induced by social defeat stress. Heat maps of behavioral tests of SIT (e), EPM (h), OFT (k) were showed respectively. Con: control mice, Res:

resilient mice, Sus: susceptible mice. Data are presented as the mean±S.E.M. (n=17-23 in each group). ** P < 0.01, ***P < 0.001.

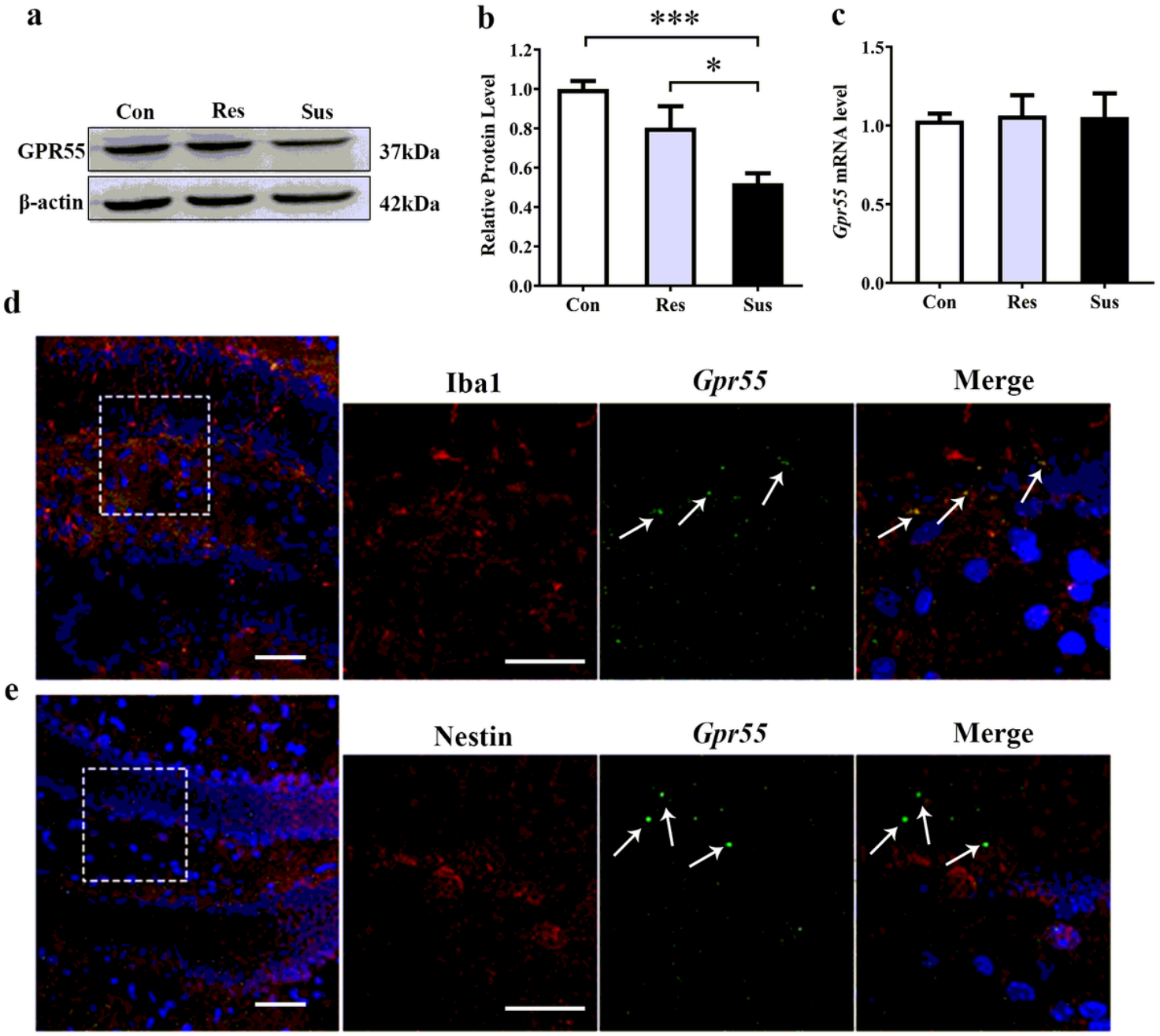

Figure 2

Regulation of protein and mRNA levels of GPR55 in hippocampus by CSDS, and the colocalization of Gpr55 RNA with IBA1 and Nestin. Western blots (a, b) and RT-PCR (c) quantification of GPR55 in hippocampus. RNA scope and immunofluorescence co-staining of hippocampal sections of Iba1 and Gpr55 mRNA(d), and Nestin and Gpr55 mRNA (e). Representative images shown Nestin/Iba-1 (red), Gpr55 RNA (green), and DAPI (blue). Data are presented as the mean±S.E.M. (n=5-7 in each group). *P < 0.05, ***P < 0.001. The arrows indicated the Gpr55 RNA. Scale bar = 50μm.

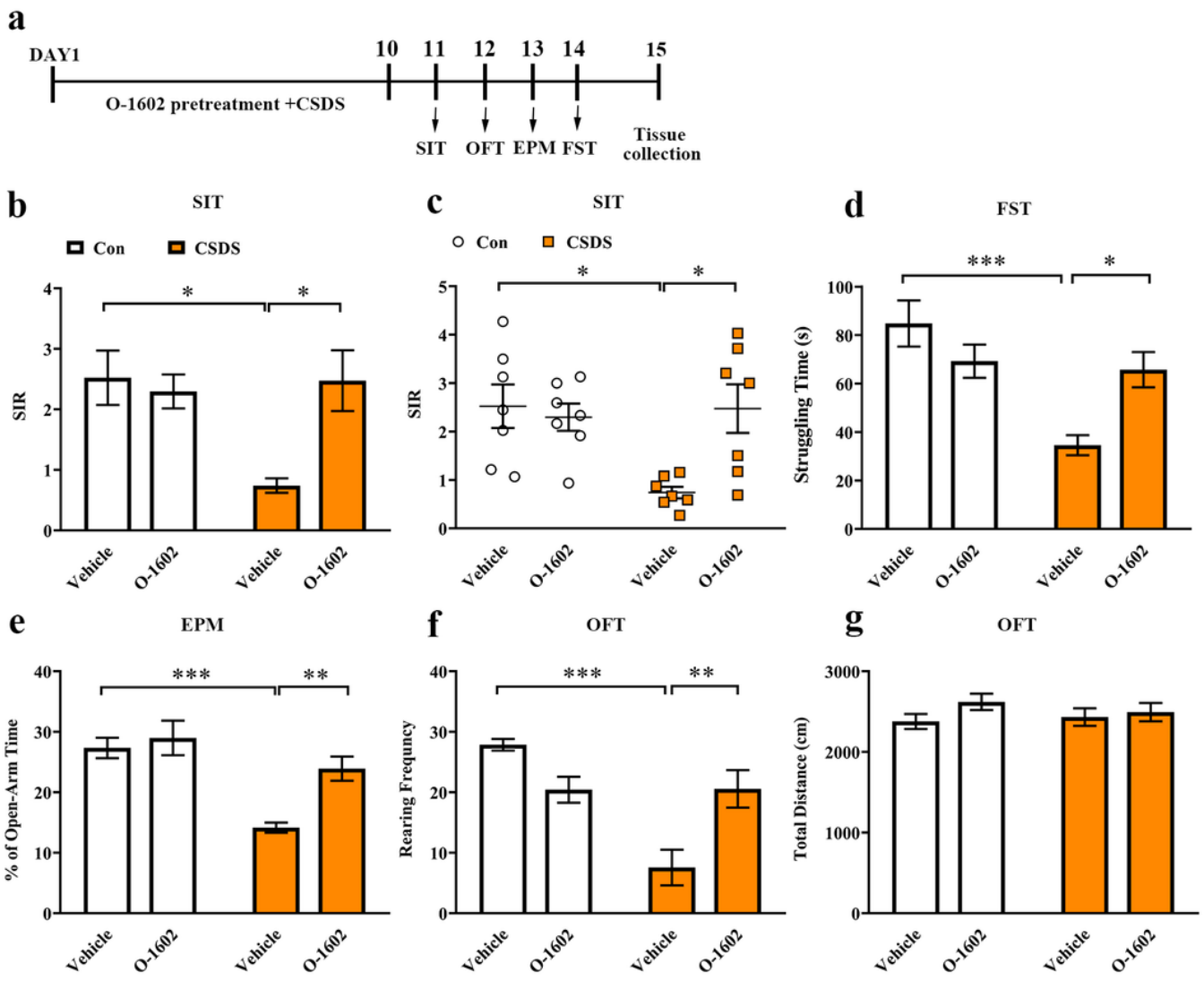

Figure 3

GPR55 agonist, O-1602 reversed depression- and anxiety- behaviors induced by CSDS. The mice were randomly divided into 4 groups: vehicle group, vehicle+ CSDS group, O-1602 group, and O-1602+ CSDS group. After CSDS stress and O-1602 treatment for 10 days (a), social interaction ratio (b, c), struggling time in FST (d), open-arm time percentage in EPM (e), rearing frequency (f) and total distance (g) in OFT were examined to quantify the effect of O-1602 on the behaviors induced by CSDS. All data are shown as mean±S.E.M, n=7-10 in each group, *P < 0.05, ** P < 0.01, ***P < 0.001.

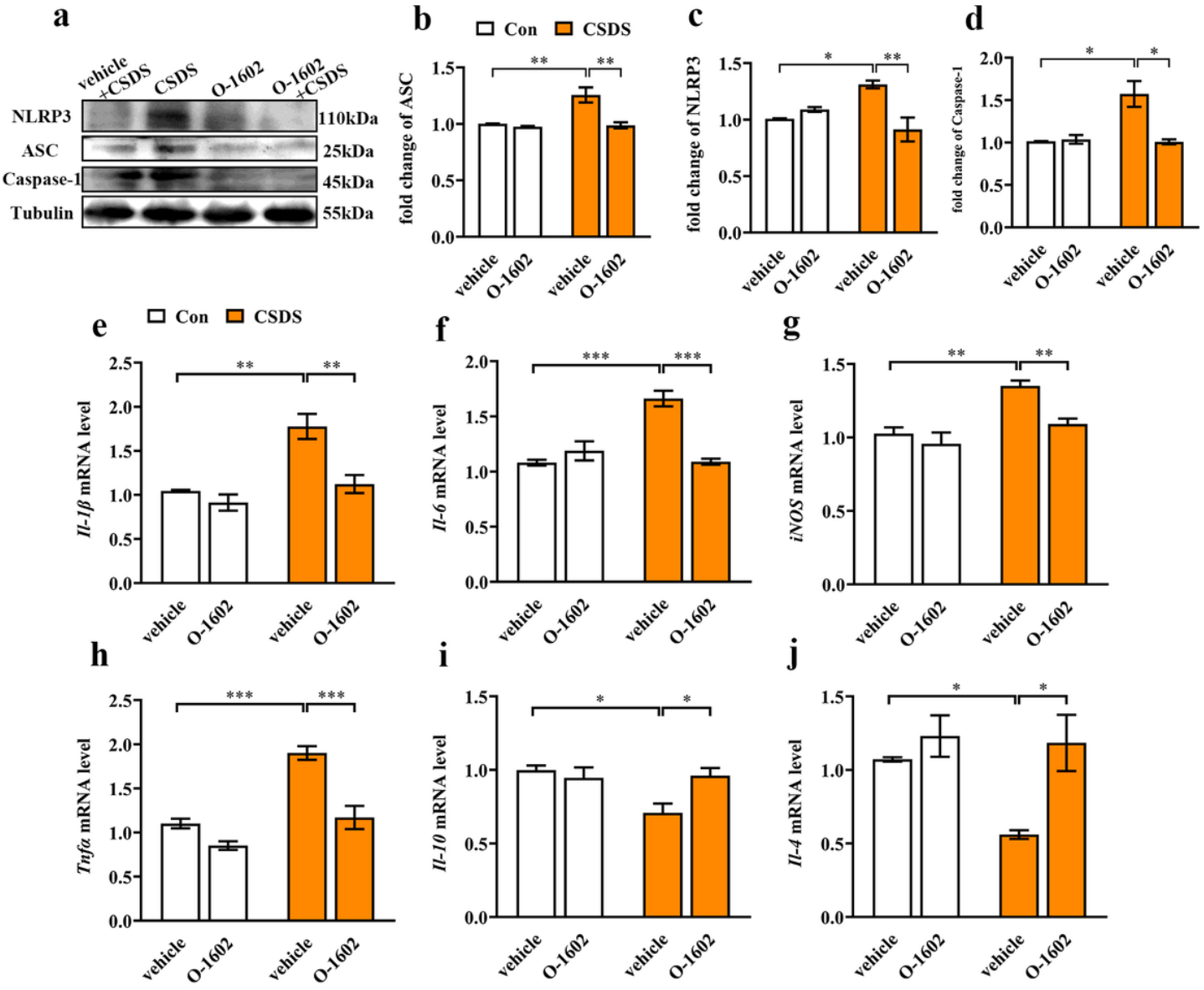

Figure 4

O-1602 inhibited neuroinflammation induced by CSDS. After behavior tests, hippocampus were collected and analyzed for protein levels of NLRP3, ASC and Caspase-1 using western blotting (a-d). And the expression of the pro-inflammatory (such as Il-1β, Il-6, iNOS, TnfFα) (e-h) and anti- inflammatory genes (such as Il-4, Il-10) (i-j) was measured by RT-PCR in the hippocampus of mice. All data are shown as mean±S.E.M, n=5 in each group, *P < 0.05, ** P < 0.01, ***P < 0.001.

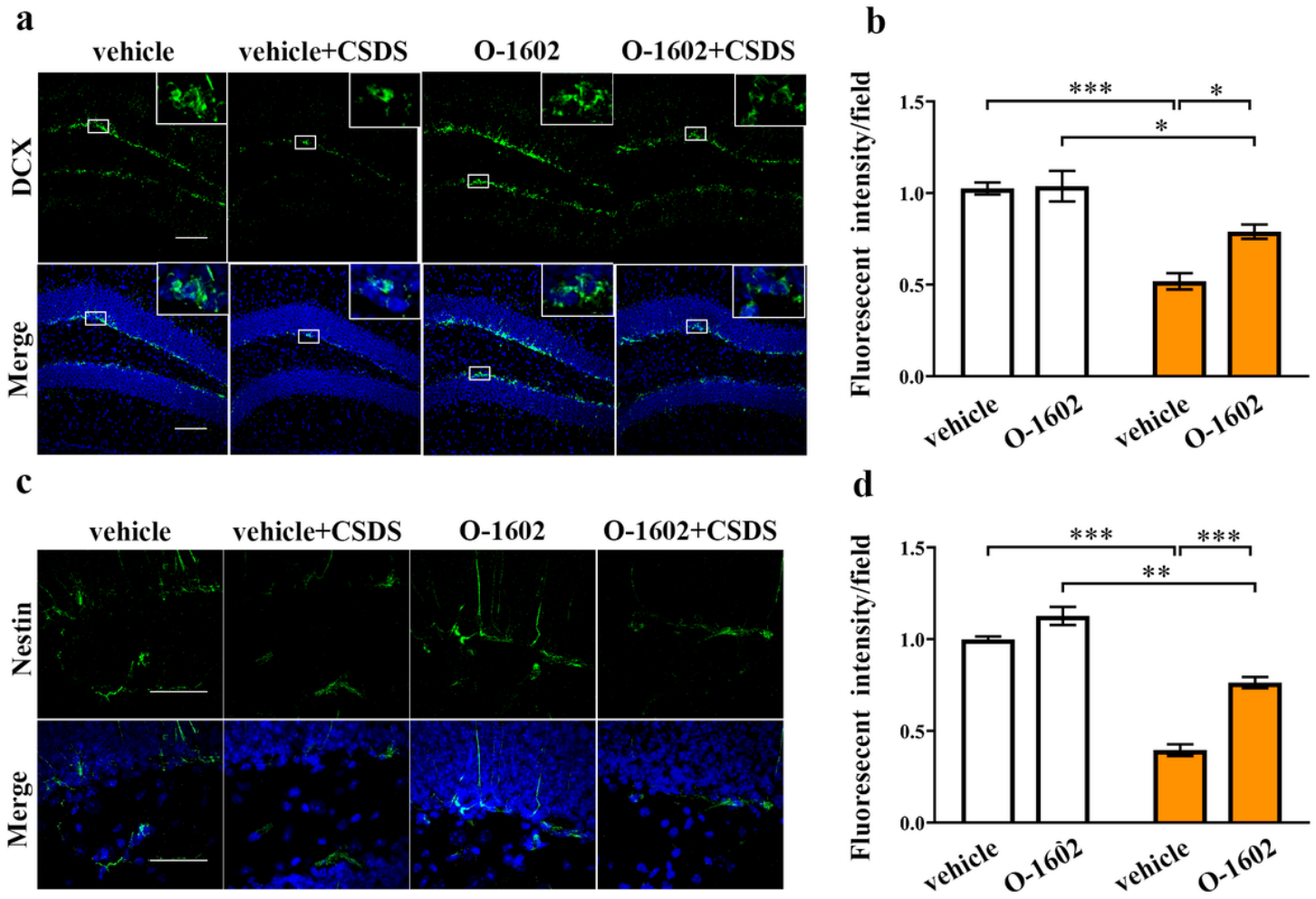

Figure 5

O-1602 enhanced hippocampal neurogenesis reduced by CSDS. Immunofluorescence staining of hippocampal sections. DCX (a, b) and Nestin (c, d) expression in the dentate gyrus (DG) of hippocampus in vehicle mice and mice exposed to CSDS with/without O-1602 or O-1602 treatment. Representative images shown Nestin/DCX (green), and DAPI (blue). All data are shown as mean±S.E.M, n=5 in each group, *P < 0.05, ** P < 0.01, ***P < 0.001. Scale bar = 50μm.

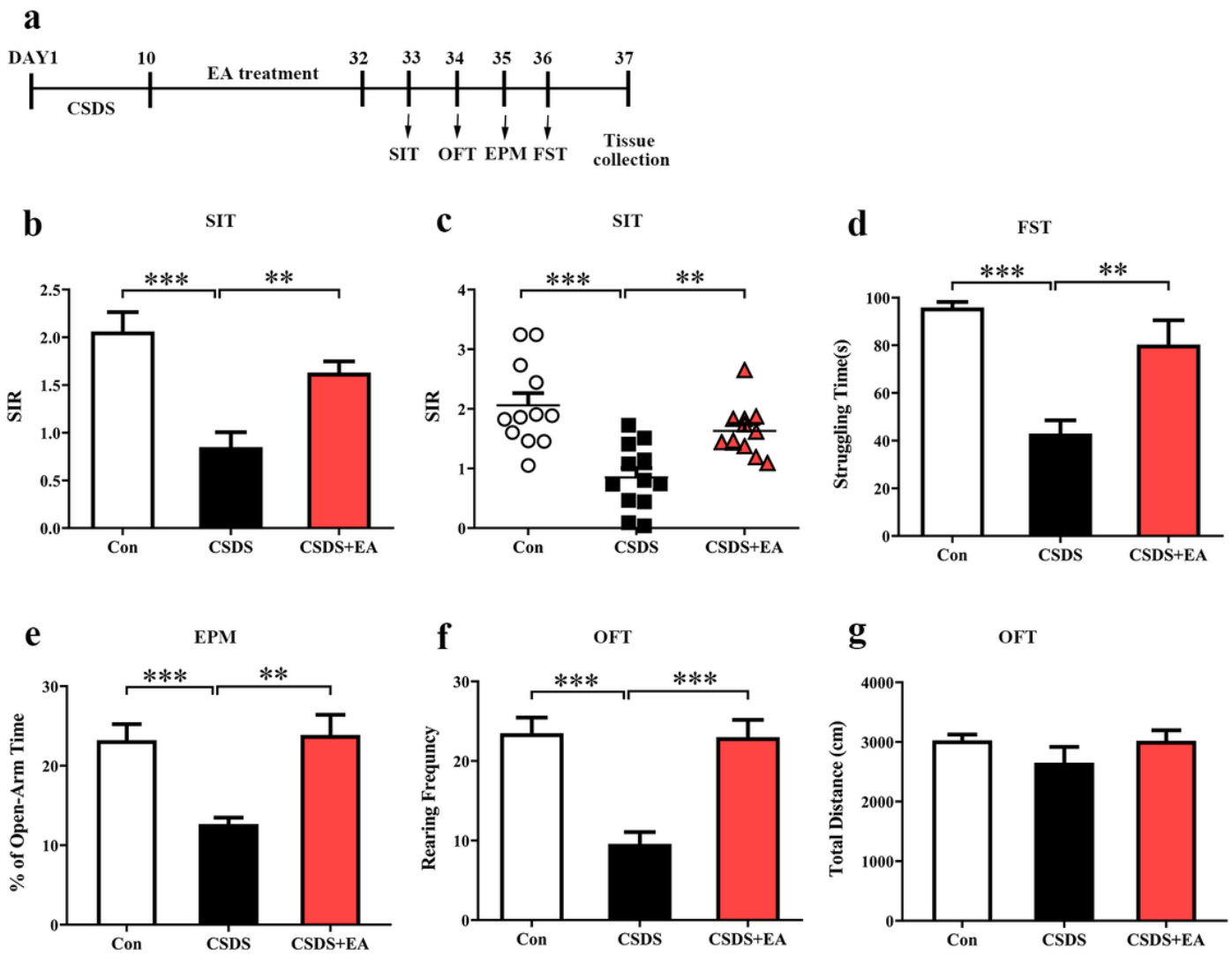

Figure 6

EA alleviated CSDS-induced depression- and anxiety-like behaviors. The mice were randomly divided into 3 groups: control group, CSDS group, and CSDS + EA group. Mice received EA treatment for 3 weeks following the 10-day CSDS (a). And social interaction test was conducted on the 43th day (b, c), struggling time in FST(d), open-arm time percentage in EPM (e) rearing frequency (f), total distance in OFT(g) were tested after EA treatment to evaluate the effect of EA on the behaviors induced by CSDS. All data are shown as mean±S.E.M, n=7-10 in each group, ** P < 0.01, ***P < 0.001.

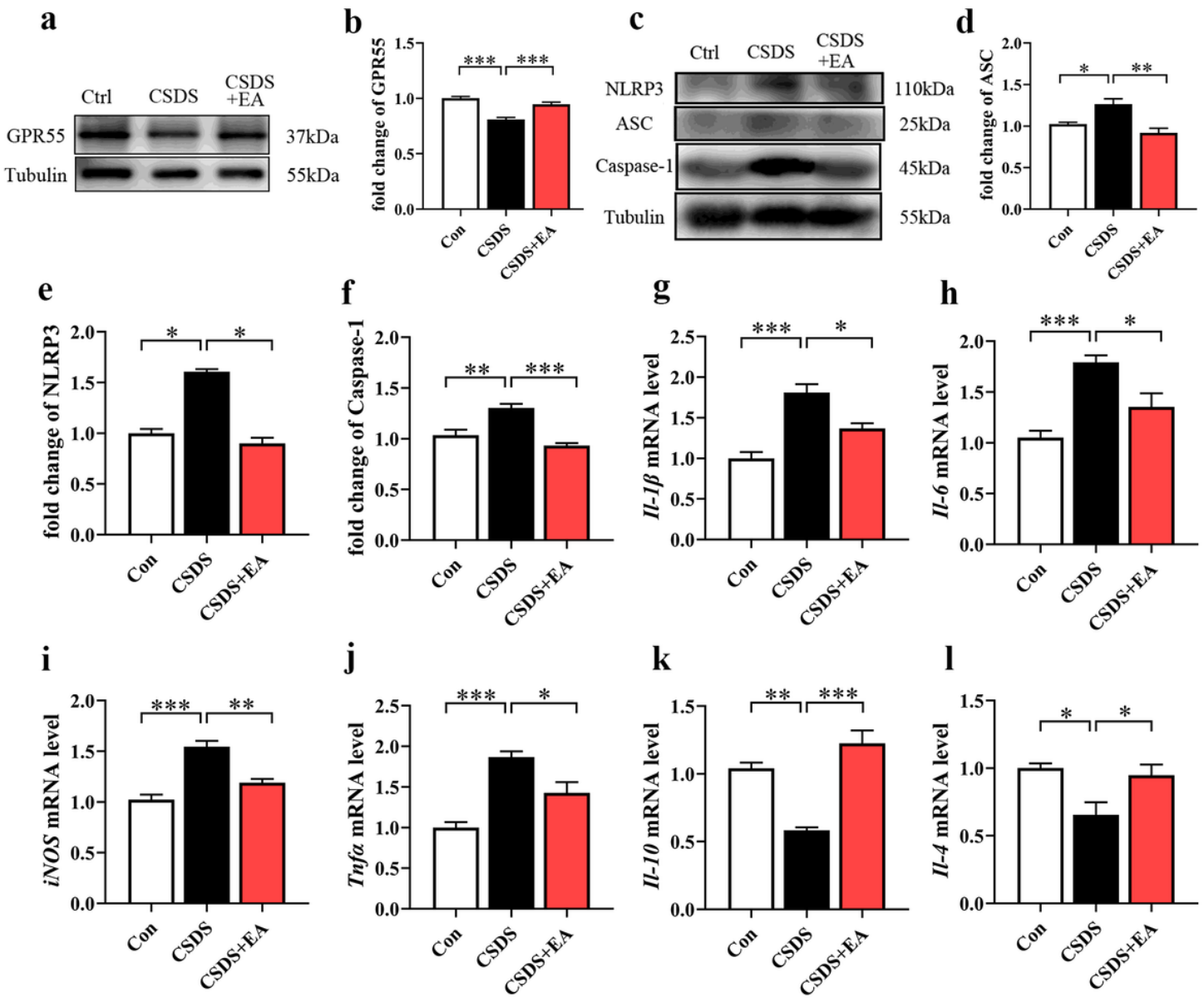

Figure 7

EA treatment upregulates GPR55 expression, and ameliorates neuroinflammation induced by CSDS. After behavior tests, hippocampus were collected and analyzed for protein levels of GPR55 (a, b), NLRP3, ASC and Caspase-1 using western blotting (c, d). And the expression of the pro-inflammatory (such as Il-1β, I-6, iNOS, Tnfα) (e-j) and anti- inflammatory genes (such as Il-4, Il-10) (k, l) were measured by RT-PCR in the hippocampus of mice. All data are shown as mean±S.E.M, n=5 in each group, *P < 0.05, ** P < 0.01, ***P < 0.001.

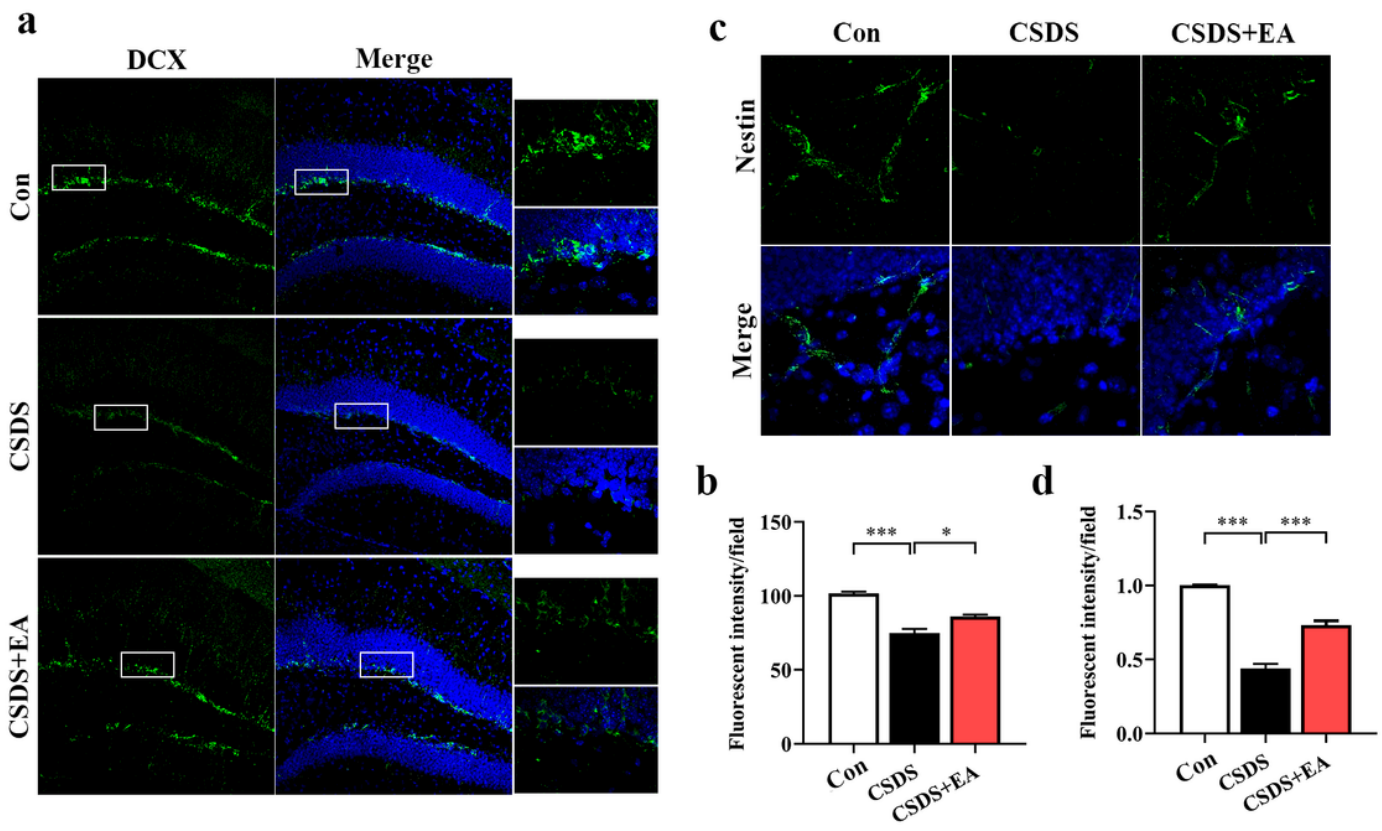

Figure 8

EA increased hippocampal neurogenesis decreased by CSDS. Immunofluorescence staining of hippocampal sections. DCX (a, b) and Nestin (c, d) expression in the dentate gyrus (DG) of hippocampus in vehicle mice and mice exposed to CSDS, and CSDS mice with EA treatment. Representative images shown Nestin /DCX (green), and DAPI (blue). All data are shown as mean±S.E.M, n=5 in each group, *P < 0.05, ** P < 0.01, ***P < 0.001. Scale bar = 50μm.

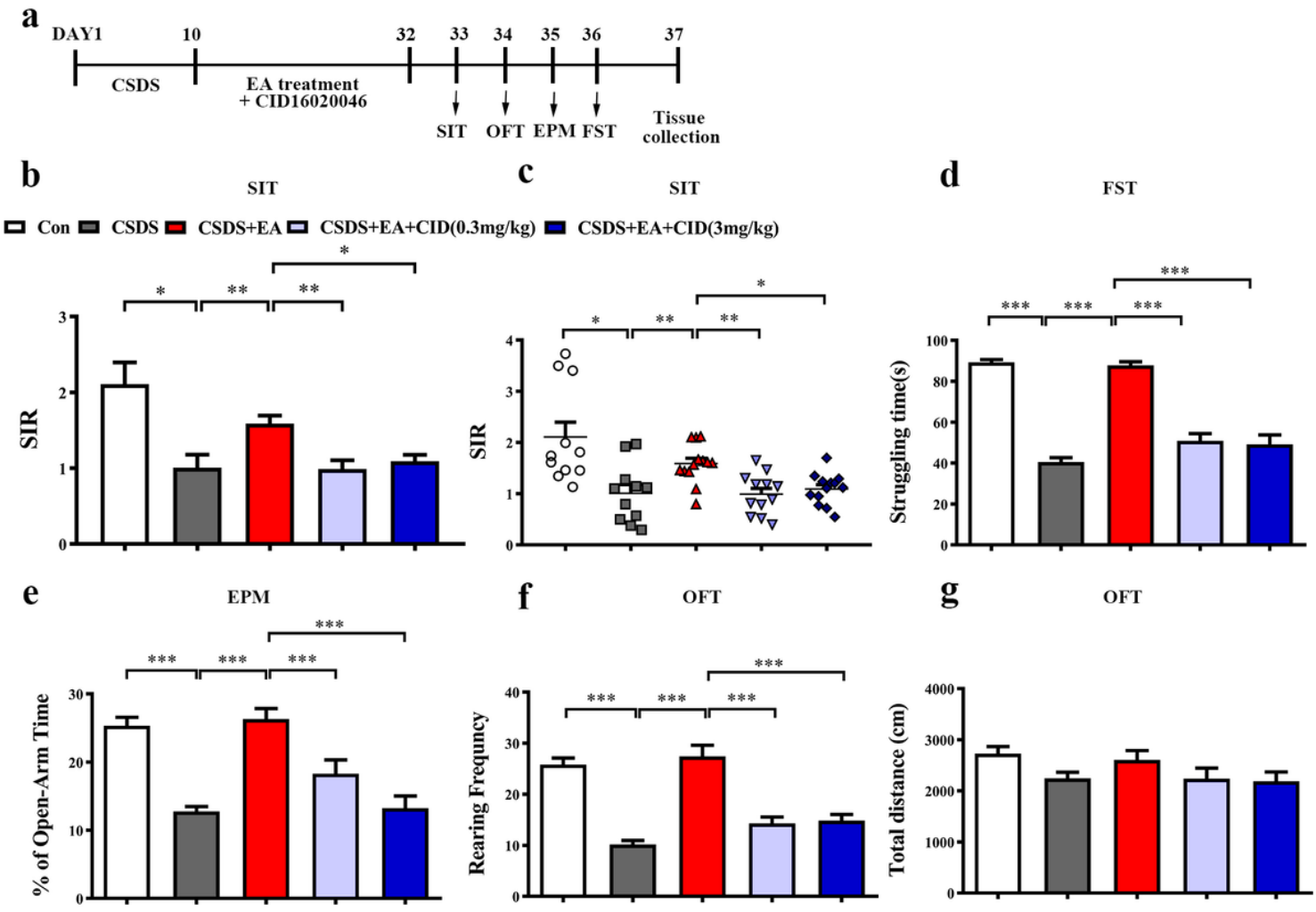

Figure 9

CID16020046 reversed the antidepressant and anxiolytic effects of EA treatment. The mice were randomly divided into 5 groups: control group, CSDS group, CSDS + EA group, CSDS+EA+CID16020046(0.3mg/kg), and CSDS+EA+CID16020046(3mg/kg). Mice received EA and CID16020046 treatment for 3 weeks following the 10-day CSDS (a). And social interaction test was conducted on the 43th day (b, c), struggling time in FST(d), open-arm time percentage in EPM (e), rearing frequency (f), total distance (g) in OFT were tested after all treatments. All data are shown as mean±S.E.M, n=7-10 in each group, *P < 0.05 ** P < 0.01, ***P < 0.001.

Figure 10

Schematic mechanism of the changes of microglial GPR55 in neuroinflammation and neurogenesis in CSDS. CSDS caused significant depression- and anxiety-like manifestations and decreased the protein level of GPR55, which was mainly expressed on microglia in hippocampus. The activation of GPR55 was able to attenuate some depression- and anxiety-like behaviors, simultaneously reducing neuroinflammation and protecting neurogenesis in defeated mice.

Supplementary Files

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