Engeletin alleviates depressive-like behaviors by modulating microglial polarization via the LCN2/CXCL10 signaling pathway

Jie Zhang
Binzhou Medical University Hospital

Zheng Song
Binzhou Medical University Hospital

Yanchao Huo
Binzhou Medical University Hospital

Guangqiang Li
Binzhou Medical University Hospital

Liming Lu
Binzhou Medical University Hospital

Xinfu Gao
Binzhou Medical University Hospital

Chuanmei Wei
Binzhou Medical University Hospital

Shuping Zhang
Binzhou Medical University

Xingyue Jiang
Binzhou Medical University Hospital

Yangyang Xu (Yaoliyangxu@126.com)
Binzhou Medical University Hospital

Research Article

Keywords: engeletin, magnetic resonance imaging, inflammatory, chronic social defeat stress, microglial polarization, LCN2, CXCL10

Posted Date: November 10th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3552120/v1

License: ☀️ This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Additional Declarations: No competing interests reported.
Abstract

Background

Microglial polarization and associated inflammatory activity are key mediators of depression pathogenesis. The natural *Smilax glabra rhizomilax* derivative engeletin has been reported to exhibit robust anti-inflammatory activity, but no studies to date have examined the mechanisms through which it can treat depressive symptoms.

Purpose

This study was designed to assess the therapeutic efficacy of engeletin in a murine chronic stress social defeat stress (CSDS) model system and to clarify the underlying mechanisms, with a particular focus on microglial polarization.

Methods

CSDS model mice were used to test the potential antidepressant effects of engeletin. Following a 21-day engeletin treatment period, a range of assays including the sucrose preference test (SPT), social interaction test (SIT), tail suspension test (TST), forced swim test (FST), and open field test (OFT) were used to measure depressive-like behaviors in these mice. Following the completion of such behavioral testing, 3.0 T multifunctional magnetic resonance imaging brain scans including T1-weighted imaging (T1WI), T2-weighted imaging (T2WI), T2 mapping, and diffusion tensor imaging (DTI) were performed. In addition, quantitative real-time PCR (qRT-PCR), and western blotting were used to measure levels of inflammatory cytokines including interleukin (IL)-6, IL-10, IL-1β, and TNF-α. Microglia activation was further evaluated through western blotting and immunohistochemical staining for markers of M1 (CD86, iNOS) and M2 (Arg1, CD206) polarization. The lipocalin-2 (LCN2)/ C-X-C motif chemokine ligand 10 (CXCL10) signaling pathway was additionally assessed via whole transcriptomic sequencing, qRT-PCR, and western blotting. Adeno-associated virus (AAV) particles encoding LCN2-EGFP were then infused into CSDS model mice to evaluate the effects of LCN2 overexpression and engeletin treatment in greater detail.

Results

Treatment for 21 days with engeletin significantly alleviated depressive-like behaviors in CSDS model mice. T1WI and T2WI imaging revealed no significant differences between groups, but the bilateral prefrontal cortex of CSDS mice exhibited significant increases in apparent diffusion coefficient (ADC) and T2 values relative to normal control mice, with a corresponding reduction in fractional anisotropy (FA), while engeletin reversed all of these changes. CSDS resulted in higher levels of IL-1β, IL-6, and TNF-α.
production, enhanced microglial activation, and greater M1 polarization with a concomitant decrease in M2 polarization in the mPFC, whereas engeletin treatment effectively abrogated these CSDS-related pathological changes. Engeletin was further found to suppress the LCN2/CXCL10 signaling axis such that AAV-induced LCN2 overexpression ablated the antidepressant effects of engeletin and reversed its beneficial effects on the M1/M2 polarization of microglia. These data suggest that the antidepressant effects of engeletin are correlated with the polarization of microglia, highlighting a potential avenue for future design of antidepressant strategies that specifically target the microglia.

Conclusion

Engelletin can alleviate CSDS-induced depressive-like behaviors by regulating the LCN2/CXCL10 pathway and thereby altering the polarization of microglia.

Introduction

Major depressive disorder (MDD) affects approximately one out of every six people over the course of their lifetime, and remains a leading cause of morbidity and disability throughout the world. While the cellular mechanisms, molecular pathways, and neural circuits that are correlated with the incidence of depression are increasingly well understood, the precise biological factors that give rise to this disease remain incompletely understood, hampering efforts to design new, effective antidepressants[1, 2].

Neuroinflammatory processes are increasingly thought to contribute to the incidence and progression of depressive disorders[3, 4]. Patients suffering from clinical depression exhibit significantly elevated levels of inflammatory cytokines including IL-6, IL-1β, and TNF-α. Consistent with these changes, rodent models of depression established through chronic unpredictable mild stress or lipopolysaccharide (LPS) exposure exhibit significant increases in the levels of these inflammatory mediators[5, 6]. Such neuroinflammation is characterized by the activation of resident immune effector cells within the central nervous system (CNS), including the microglia[7]. Microglia serve as essential regulators of microenvironmental homeostasis through their ability to detect pathogens and eliminate them via phagocytosis, in addition to secreting inflammatory chemokines and cytokines that support tissue repair and nervous system development[8, 9]. The activation of microglia and the consequent induction of neuroinflammation have been tied to the pathogenesis of major depressive disorders. Broadly speaking, activated microglia are classified into proinflammatory M1 cells and anti-inflammatory regenerative M2 cells[10, 11]. Higher levels of M1 microglia polarization and activity are associated with phagocytic and inflammatory activity, and an elevated M1/M2 ratio is related to impaired neurological function and depression in rat[12, 13]. Efforts aimed at suppressing aberrant microglial activation and/or restoring the appropriate homeostatic balance between M1 and M2 microglia thus represent promising new antidepressant strategies.

Engelletin (dihydrokaempferol 3-rhamnoside) is a natural flavonol glycoside derived from Smilax glabra rhizomilax extracts (Fig. 1). Engelletin has previously been shown to exhibit antitumorigenic, antioxidant,
and anti-inflammatory properties[14, 15]. In addition, it has been shown to exert neuroprotective activity, suppressing the production of reactive oxygen species (ROS) and mitigating the severity of ischemia/reperfusion injury and Alzheimer's disease[16, 17]. Preliminary research conducted by our group further revealed that engeletin can alleviate depressive-like behaviors induced by chronic restraint stress (CRS) through its ability to modulate the BDNF/TrkB/mTORC1 signaling axis, thereby enhancing synaptic plasticity[18]. However, no research to date has evaluated the effects of engeletin on the regulation of the M1/M2 polarization of microglia.

To address this gap in knowledge, this study was developed to explore the therapeutic effects of engeletin on depressive-like behaviors and to clarify the underlying antidepressant mechanisms in an effort to establish new approaches to controlling this devastating disease.

Materials and methods

Drugs and animals

Engeletin ($C_{21}H_{22}O_{10}$), was provided by SenBeiJia Biological Technology (Jiangsu, China). Male C57BL/6J mice (weight 20–24g) were purchased from Jinan Pengyue Experimental Animal Center (license number: SCXK20190003). Mice were maintained at a constant room temperature of 22 ± 3°C, with a humidity of 50 ± 15%, and were kept at a 12/12 h light/dark cycle.

Chronic social defeat stress (CSDS) and social interaction (SI) test

Briefly, each adult male C57BL/6 experimental mice was exposed to 5–10 min of physical aggression by a male CD-1 mice. At the completion of the session, C57BL/6J experimental and CD-1 mice were housed overnight in a two-compartment rat cage and separated by a transparent divider to provide sensory, but not physical, contact. The procedure was repeated for a total of 10 consecutive days, in which C57BL/6J experimental mice faced a new aggressor every day.

For the social interaction (SI) test, we measured the time spent in the interaction zone during the first (target absent) and second (target present) trials[19]. The time of mice spent in the interaction zone (IZ) surrounding the plastic box was recorded and analyzed.

Behavioral measurements

Sucrose preference test (SPT)

To observe the anhedonia-like behavior, the SPT was performed as described. The process is as follows: 1) 1% sucrose solution and 2) tap water. SPT was performed for 6 h, and the liquid consumption was assessed by subtracting the bottle weights. The consumption was calculated as follows: The sucrose preference rate = (1% sucrose solution intake)/[(1% sucrose solution intake) + (water intake)]
**Forced swimming test (FST)**

The FST was performed in a clear glass cylinder (height 25 cm, diameter 10 cm) filled with 10 cm of water (25 ± 1°C). All mice were forced to swim for 6 min, and during the last 4 min, the immobility time was recorded.

**Tail suspension test (TST)**

TST is a test to evaluate despair or depressive-like behavior. Briefly, adhesive tape was stuck to the tail’s tip about 1 cm below to suspend mice for 6 min, 50 cm above the floor. The time during which mice remained immobile over a period of 4 min was recorded.

**Open field test (OFT)**

OFT was used to assess the motor abilities of mice. Mice were placed in a plexiglass arena (50 cm × 50 cm × 50 cm) and were allowed to explore the open field freely for 10 min. The total traveled distance was scored.

**Magnetic resonance imaging (MRI)**

The MRI images were captured using a 3.0 T horizontal magnet (Skyra, Siemens, Munich, Germany) with a 25-mm-diameter gradient coil (4 channels for mice, Chen Guang Medical Co .Ltd, Shanghai, China). Axial T1-weighted imaging (T1WI), axial T2-weighted imaging (T2WI), T2-mapping, and diffusion tensor imaging (DTI) were acquired. Imaging parameters are as follows: T1-mprage: repetition time (TR) = 2200 ms, echo time (TE) = 3.86 ms, matrix size (MTX) = 224 × 224, slice thickness (ST) = 0.5 mm, field-of-view (FOV) = 60 mm × 52 mm; T2WI: repetition time (TR) = 4800 ms, echo time (TE) = 102 ms, matrix size (MTX) = 3320 × 320, slice thickness (ST) = 1.5 mm, field-of-view (FOV) = 63 mm × 63 mm; DTI: repetition time (TR) = 3000 ms, echo time (TE) = 67 ms, matrix size (MTX) = 142 × 142, slice thickness (ST) = 1.5 mm, field-of-view (FOV) = 108 mm × 82 mm; T2-mapping: repetition time (TR) = 1360 ms, echo time (TE) = 16.1–69 ms, matrix size (MTX) = 384 × 384, slice thickness (ST) = 2.0 mm, field-of-view (FOV) = 103 mm × 103 mm.

**RNA-Seq and Data Analysis**

The high-throughput RNA sequencing analysis for this study was provided by a commercial service (Biotech Biotechnology Inc, Shanghai, China). First, total RNA was extracted from the mPFC tissue of three groups of mice (three samples per group): CON group, CSDS group and engeletin group, respectively. The RNA quality and quantity were then analyzed using the Quantum Bit RNA Detection Kit and the Quantum Bit 2.0 Fluorometer (Life Technologies, CA, United States).

**Viral constructs and mPFC viral infusion**

For local overexpression of LCN2 in mPFC, a virus packed with a non-fusion protein expression vector, adeno-associated virus (AAV)-CMV-MCS-3flag-T2A-ZsGreen made by Hanbio Biotechnology Co. Ltd. (1.6 × 10^12 vg/mL, Shanghai, China) was injection in C57BL/6J mice. AAV-CMV-ZsGreen (1.4 × 10^12
vg/mL, Shanghai, China) was used as the control vector. Mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) before surgery. 2 ul of virus was stereotaxically microinjected into the bilateral mPFC (2.2 mm anteroposterior, ± 0.3 mm mediolateral, and −2.4 mm dorsoventral relative to bregma) at the rate of 0.1 ul /min for 1 min.

**Immunohistochemical Staining**

Immunohistochemistry was performed as previously described. The primary antibodies included anti-IBA1 (Rabbit, 1:200). The sections were observed using a confocal laser scanning microscope (model FV1000, Olympus).

**RNA extraction and Real-Time PCR**

Gene expression levels were measured by RT-qPCR as described in our previous publication using GAPDH as the internal control. Primer sequences in detail are shown in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>GGCAGAGAACCATGGCCAGAA</td>
<td>AATCGA TGACAGCGCTCAGGC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AAATGCCACCTTTTGACAGTG</td>
<td>GAGTGATAGCTGCCTGCCTGA</td>
</tr>
<tr>
<td>IL-6</td>
<td>GACAAAGCCAGAGTCCTTCAGA</td>
<td>GAGCATTGAAATTGGGGGTAGG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TGCCTCAGCTCTTTCTCATT</td>
<td>GGGCTTGTCACTCGAGTTTT</td>
</tr>
<tr>
<td>LCN2</td>
<td>CCCCATCTCTGCTCACTGTC</td>
<td>TTTTCTGGACCAGCATTG</td>
</tr>
<tr>
<td>CXCL10</td>
<td>TCTGAGTGGGACTCAAGGGA</td>
<td>TTGTGGCAATGATCTCAACATG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCAGTGGCAAAGTGGAGATTG</td>
<td>TGCAGGATGCATTGCTGACA</td>
</tr>
</tbody>
</table>

**Western blottings**

The primary antibodies included rabbit anti- IBA1 (ab178846, 1:1000), rabbit anti-BDNF (ab108319, 1:1000), rabbit anti-iNOS (ab178945, 1:1000), rabbit anti-CD86 (ab239075, 1:1000), and rabbit anti-CD206 (#24595, 1:1000), rabbit anti-Arg1 (PA5-29645, 1:5000), mice anti-LCN2 (ab125075, 1:1000), rabbit anti-CXCL10 (ab306587, 1:1000) and GAPDH (AF0006, 1:1000). Western blotting images were captured using Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.). The acquired data were normalized with the relative GAPDH density.

**Statistical Analysis**

Data were expressed as the mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism 9.4.1. Statistical analysis was performed using one-way or two-way ANOVA test followed by Bonferroni post hoc testing. Differences were considered statistically significant when P < 0.05.
Results

Engeletin treatment alleviates depressive-like behaviors in CSDS model mice

As they exhibit robust validity as predictors of antidepressant-like activity, the FST and TST are behavioral tests that are widely used when assessing possible approaches to combatting depressive symptoms[3]. Engeletin has previously been reported to induce a dose-dependent (5, 10, 20 mg/kg) reduction in immobility time in these two testing paradigms. Whether engeletin exhibits robust antidepressant activity in the CSDS model of depression, however, has yet to be established. To test this possibility, engeletin was intragastrically administered (5, 10, 20 mg/kg) for 21 days following CSDS modeling (Fig. 2A). Under these conditions, engeletin was associated with a significant improvement in percentage reduction in sucrose consumption (Fig. 2B), whereas it had a relatively minimal impact on water drinking (Fig. 2C). The repeated administration of engeletin also restored CSDS-associated reductions in social interactions (Fig. 2D), while also fully reversing the CSDS-related increases in murine immobility observed in the TST and FST (Fig. 2E, F). Neither stress-related modeling nor engeletin administration, however, had any impact on the locomotor behavior of mice in the OFT (Fig. 2G).

The impact of engeletin on MRI scan results in CSDS model mice

Neuroimaging strategies can detect MDD-related functional and structural changes in many neurological circuits[20]. While no differences between the control, CSDS, and engeletin-treated groups were detected via T1WI or T2WI (Fig. 3A), CSDS modeling was associated with a significant increase in the T2 value of the bilateral prefrontal cortex (Fig. 3B). This suggests the presence of inflammation and edema in the prefrontal lobe in this model of depression, while engeletin was able to reverse these changes (Fig. 3C). Fractional anisotropy (FA) values were also significantly decreased in the bilateral prefrontal cortex of these CSDS model mice, with a marked increase in the apparent diffusion coefficient (ADC), with engeletin administration reversing both of these pathological changes (Fig. 3D-F).

Engeletin suppresses CSDS-induced inflammatory cytokine production within the mPFC

To gain additional insight into the immunomodulatory potential of engeletin, qRT-PCR was next used to assess changes in inflammatory cytokine expression. This approach revealed that engeletin treatment (20 mg/kg) was associated with pronounced reductions in pro-inflammatory IL-1β, IL-6, and TNF-α content within the mPFC, with a concomitant rise in anti-inflammatory IL-10 levels (Fig. 4A-D). Western blotting similarly confirmed that engeletin was sufficient to reverse CSDS-induced increases in pro-inflammatory cytokine levels and reductions in IL-10 levels (Fig. 4E-I).
Engeletin modules the polarization of microglia in CSDS model mice

To better understand how engeletin impacts microglial polarization in the context of CSDS-driver neuroinflammatory activity, immunofluorescent staining and western blotting were next employed to evaluate the degree of M1 and M2 polarization within the mPFC of mice in these different experimental groups. Significant increases in IBA1 staining and protein levels were detected in the mPFC samples from the CSDS model group relative to the control group, while engeletin administration alleviated this change (Fig. 5A-C). Engeletin additionally suppressed polarization towards the pro-inflammatory M1 microglial phenotype, as evidenced by reductions in the expression of CD86 and iNOS, while promoting the upregulation of CD206 and Arg-1 consistent with the induction of M2 polarization (Fig. 5D-E). Overall, these data thus suggest that engeletin is capable of serving as an anti-inflammatory mediator through its ability to modulate microgliosis polarization.

Engeletin suppresses CSDS-induced LCN2/CXCL10 pathway signaling

To clarify the mechanisms whereby engeletin controls microglial polarization, mPFC samples were collected for transcriptomic sequencing from the CSDS and engeletin treatment groups. Analyses of the top 30 most significantly differentially expressed genes revealed pronounced LCN2 and CXCL10 downregulation within the mPFC following engeletin treatment (Fig. 6A, B). In line with these results, significant decreases in LCN2 and CXCL10 expression following engeletin treatment were confirmed via qRT-PCR and western blotting (Fig. 6C-G).

Engeletin suppresses LCN2/CXCL10 signaling to alleviate depressive-like behaviors

LCN2 was next overexpressed in the mPFC using an AAV-LCN2-EGFP vector in order to probe whether the antidepressant effects of engeletin are mechanistically related to LCN2/CXCL10 pathway inhibition (Fig. 7A). AAV-LCN2-EGFP expression remained stable for 14 days following stereotaxic infusion, and overexpression was validated through both immunofluorescent imaging and western blotting (Fig. 7B-C). Behavioral analyses revealed that CSDS model mice in which LCN2 was overexpressed that were treated with engeletin exhibited significant reductions in sucrose preference and social interactions (Fig. 7D-F) relative to CSDS model mice administered the AAV-CON vector and engeletin, together with increased immobility in the TST and FST assays (Fig. 7G-I).

LCN2 overexpression abrogates the impact of engeletin on the polarization of microglia
In order to clarify the potential link between the LCN2/CXCL10 signaling axis and the impact of engeletin on the polarization of microglia within CSDS model mice, changes in microglial marker expression were assessed in the mPFC following LCN2 overexpression. These experiments revealed that overexpressing LCN2 eliminated the ability of engeletin to suppress iNOS and CD86 upregulation following CSDS modeling (Fig. 8A-C), while reversing engeletin-related increases in Arg-1, CD206, and BDNF expression in these mice (Fig. 8D–G). These results suggest that the ability of engeletin to inhibit M1 microglial polarization while favoring polarization towards an M2 phenotype in this mouse CSDS model system is at least partially dependent on LCN2/CXCL10 pathway activation.

**Discussion**

Here, a murine model of CSDS-induced depression was employed to explore the potential antidepressant activity of the *Smilax glabra* Roxb.-derived flavonol glycoside engeletin. Ultimately these analyses revealed that engeletin is able to alleviate CSDS-related depressive-like phenotypes through its ability to suppress LCN2/CXCL10 pathway activation and thereby modulate microglial polarization.

Stress responses entail the engagement of a diverse range of physiological and behavioral responses[21]. Exposure to chronic stress over extended periods can contribute to the incidence of MDD and other adverse outcomes. The precise biological mechanisms that give rise to MDD remain incompletely understood, and therapeutic options remain limited owing to the heterogeneous nature of this disease and the pathogenic and clinical levels[22, 23]. Diagnosing MDD in a clinical setting is also highly dependent on structured interviews and subjective criteria, contributing to a greater risk of patients being misdiagnosed, thus increasing the burden of this disease.

Structural MRIs have led to the identification of several brain abnormalities in MDD patients, with these changes most commonly manifesting in gray matter areas related to decision-making, emotional procession, and reward circuitry within the limbic system and frontal lobe[24, 25]. Multiparameter MRI scans are currently regarded as the most effective approach to noninvasively analyzing the pathological basis of MDD *in vivo*[26]. T1WI and T2WI strategies enable the visualization of brain anatomy, whereas quantitative indices such as DTI measurements and T2 values can provide quantitative sensitivity when attempting to detect injury to the nervous system and gauge the efficacy of neuroprotective interventions[27]. The T2 hyperintensity of the bilateral prefrontal cortex is associated with increases in inflammation, vascular permeabilization, myelin turnover, water content, and the accumulation of byproducts of myelin and axonal breakdown[28, 29]. ADC and FA values are key quantitative DTI parameters that are closely correlated with the pathogenesis of depression. These quantitative DTI and T2 parameters can also be evaluated and compared with the outcomes of behavioral and histological analyses to gain more robust insight into the incidence of depression and to explore therapeutic outcomes[30, 31]. Here, ADC, FA, and T2 values for the bilateral prefrontal cortex were significantly altered in CSDS model mice, while engeletin administration reversed these changes and alleviated depressive-like behavioral phenotypes. Overall these findings conclusively demonstrate that T2 and DTI measures can
offer value as biomarkers of depression that can gauge disease progression and enable the rigorous assessment of interventional strategies.

Neuroinflammation is increasingly understood to play an important role in the pathogenesis of depression[13, 32]. The use of anti-inflammatory drugs to suppress microglia-mediated inflammation has been shown to alleviate depressive symptoms, and a growing body of evidence suggests that depressive-like phenotypes can arise owing to the imbalance between pro- and anti-inflammatory cytokine production. Here, engeletin was found to promote the upregulation of anti-inflammatory IL-10 within the mPFC in CSDS model mice while suppressing the expression of pro-inflammatory IL-1β, IL-6, and TNF-α. As peripheral cytokines are generally limited in their ability to cross the blood-brain barrier, microglia serve as a main source of these inflammatory mediators within the CNS, promoting neuroinflammation that is central to the pathogenesis of CSDS-associated depressive-like behaviors.

Microglia are mesoderm-derived cells present within neurological tissues that are central to depression-related pathways and to the incidence of neuroinflammation[9]. While microglial polarization has been found to correlate with depressive behavior, the specific mechanisms underlying this correlation and their therapeutic relevance remain to be fully clarified. Microglia that exhibit M1 and M2 polarization profiles secrete differing levels of pro- and anti-inflammatory cytokines, in addition to expressing different functional and morphological markers[33]. M1 microglia, which generally express iNOS and CD86, produce high concentrations of inflammatory cytokines such as IL-6, TNF-α, IFN-γ, and IL-1β, whereas M2 microglia express Arg1 and CD206, and secrete anti-inflammatory factors including IL-10, IL-4, and TGF-β[8]. M2 microglial polarization has been demonstrated to have beneficial effects following chronic stress in the context of neurological disease[34]. Here, treatment with engeletin resulted in M2 microglia activation and the suppression of M1 polarization, in turn increasing IL-10 and TGF-β secretion while suppressing IL-1β, TNF-α, and IL-6 production within the mPFC. These results thus suggested that engeletin is capable of treating CSDS through the tuning of the microglial balance of M1/M2 polarization and the alleviation of neuroinflammation, potentially providing a novel avenue for the treatment of neurological disease.

LCN2 has increasingly been shown to play a central role in the incidence of neuroinflammatory pathology in the CNS[35]. Also referred to as NGAL or 24p3, LCN2 is a lipocalin family member that binds to a range of hydrophobic molecules, interacts with particular cell surface receptors, and controls concentrations of iron within cells, thereby influencing an array of pathways[36, 37]. While the expression of LCN2 at baseline is relatively limited, it can be rapidly upregulated whereupon it serves as a regulator of viability, migratory activity, innate immunity, and tissue morphology. Multiple studies have identified links between LCN2, depression, and behavior[38]. For example, mice exposed to a 6 h restraint model of stress exhibited a 7-fold increase in hippocampal LCN2 expression. Restraint stress also reportedly induces an increase in amygdalar LCN2 expression, with such upregulation primarily taking place within neurons and being functionally linked to increases in immature neuroplastic spines consistent with fear memory formation[39, 40]. Stress-naïve Lcn2-knockout rodents also resent with an increase in spine density in the amygdalar basolateral complex as compared to wild-type control animals[41]. Naude et al.[42] found that
plasma LCN2 levels in patients with depression were significantly increased relative to non-depressed controls, and plasma LCN2 levels were also reportedly higher in patients suffering from recurrent depression as compared to first-episode depression.

Chemokines play an essential role in guiding the movement of particular cell populations to specific physiological sites. In addition to their importance in the maintenance of systemic homeostasis, these chemotactic cytokines can also contribute to pathological changes within the CNS, particularly in the context of development, injury, synaptic transmission, and disease-related neuroinflammatory activity[43]. LCN2 was recently identified as a promoter of chemokine expression within the CNS, with neurons, endothelial cells, astrocytes, and microglia all serving as potential cellular producers of chemokines[38]. CXCL10 secretion induced by LCN2 has been reported to induce microglial, astrocytic, and neuronal migration through mechanisms at least partially regulated by JAK2/STAT3 and IKK/NF-κB pathways[44]. Here, chronic stress was associated with significant LCN2/CXCL10 axis activation, whereas the administration of engeletin markedly suppressed LCN2 and CXCL10 expression within the mPFC in a manner that was reversed by LCN2 overexpression in the mPFC of these engeletin-treated CSDS model mice.

LCN2 was significantly downregulated in apoptosis-resistant microglia, and subsequent research in which LCN2 was knocked down or overexpressed revealed that it serves as a vital mediator of apoptotic sensitization and the amoeboid transformation of activated microglia[45, 46]. The precise physiological importance of LCN2 as a regulator of the M1/M2 polarization of microglia, however, remains poorly understood. Here, the antidepressant-like effects of engeletin were primarily found to be attributable to its ability to modulate LCN2/CXCL10 pathway signaling and microglial polarization. The overexpression of LCN2 was sufficient to reverse the antidepressant-like benefits of engeletin in CSDS model mice while reducing the expression of M2-associated proteins (Fig. 9). These data provide further support for the utility of LCN2 as a diagnostic biomarker of depression that can be assessed in combination with a range of other inflammatory markers, growth factors, and metabolic or endocrine changes when assessing the incidence of depressive symptoms.

Together, these data demonstrate that chronic stress is associated with LCN2 upregulation whereas engeletin treatment is capable of mitigating inflammation-associated damage and promoting the M2 polarization of M1 microglia through the suppression of LCN2/CXCL10 signaling activity.

**Declarations**

**Funding**

The National Natural Science Foundation of China (Grant No: 31570352 and 31170321), Project of Shandong Medical and Health Science and Technology Development Plan (Grant No: 2017WS153 and 202209010908), Project of Shandong Traditional Chinese Medicine Technology (Grant No: Q-2023011), and Binzhou Medical University Science and Technology Program (Grant No: BY2016KJ11 and
BY2020KJ43). We would like to thank all the reviewers who participated in the review and MJ Editor (www.mjeditor.com) for its linguistic assistance during the preparation of this manuscript.

**Availability of data and materials**

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no competing financial interests.

**Ethics approval and consent to participate**

All experimental procedures in this study were conducted in accordance with the National Institutes of Health Guidelines for Care and Use of Laboratory Animals and all animal protocols were approved by the Laboratory Animals Care and Use Committee of Binzhou Medical University Hospital (20230206-61).

**References**


Figures
Figure 1

The chemical structure of engeletin.

A

<table>
<thead>
<tr>
<th></th>
<th>CSDS</th>
<th>SI</th>
<th>Engeletin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>11</td>
<td>31</td>
</tr>
<tr>
<td>32</td>
<td>33</td>
<td>34</td>
<td>35</td>
</tr>
</tbody>
</table>

Figure 2

Engeletin alleviates CSDS-induced depressive-like behavior. (A) An overview of the relative timing of CSDS modeling, engeletin treatment, and behavioral testing (SPT, SIT, TST, FST, OFT), which was performed at the end of the study for all mice. (B-G) The effects of engeletin on SPT (B, C), SIT (D), TST (E), FST (F), and OPT (G) test performance. Results are means ± SEM. (n = 12). *P < 0.05, **P < 0.01, ****P < 0.0001, one- or two-way ANOVAs with Bonferroni post hoc testing.
Figure 3

The impact of engeletin on multimodel MRI results in CSDS model mice. (A) Representative T1WI and T2WI images from the CON, CSDS, and engeletin groups. (B-C) Representative T2 mapping images and corresponding quantification. (C) (D) Representative DTI images. (E-F) DTI index quantification (FA, ADC) in the bilateral prefrontal cortex. Results are means ± SEM (n = 12). *P < 0.05, **P < 0.01, one-way ANOVAs with Bonferroni post hoc testing.
Figure 4

Engeletin suppresses inflammatory cytokine production within the mPFC in CSDS model mice. (A-D) qRT-PCR analyses of IL-1β, IL-6, TNF-α, and IL-10 expression. (E-I) Western immunoblotting analyses of IL-1β, IL-6, TNF-α, and IL-10 expression. Results are means ± SEM (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way ANOVAs with Bonferroni post hoc testing.
Figure 5

Engaletin modulates the activation and polarization of microglia within the mPFC. (A-B) Representative immunofluorescent staining for IBA1 (A) and corresponding quantification. Scale bar: 20 μm. (C-E) Western blotting analyses of IBA1 (C), markers of M1 microglial polarization (CD86, iNOS) (D), and markers of M2 microglial polarization (Arg1, CD206) (E). Results are means ± SEM (n = 5). *P < 0.05, **P < 0.01, ****P < 0.0001, one-way ANOVAs with Bonferroni post hoc testing.
Engeletin suppresses LCN2/CXCL10 signaling activity in the mPFC. (A-B) Partial heatmaps and volcano plots highlighting differences in gene expression between the CSDS and engeletin groups (|log2FC| ≥ 1, difference ≥ 2 fold), with blue and red respectively indicating downregulated and upregulated genes (n = 3). (C-D) qRT-PCR analyses of changes in LCN2 and CXCL10 expression in the CON, CSDS, and engeletin groups (n = 5). (E-G) Representative western blotting (E) and corresponding quantification (F, G) of LCN2
and CXCL10 expression in the mPFC (n = 5). Results are means ± SEM. *P < 0.05, **P < 0.01, ****P < 0.0001, one-way ANOVAs with Bonferroni post hoc testing.

**Figure 7**

**LCN2 overexpression ablates the *in vivo* antidepressant-like activity of engeletin.** (A) Overview of the experimental approach used to assess behavioral responses in mice following AAV-mediated LCN2

---

**Table:**

<table>
<thead>
<tr>
<th>Group</th>
<th>SPT (s)</th>
<th>TST (s)</th>
<th>FST (s)</th>
<th>OFT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>31</td>
<td>32</td>
<td>33</td>
<td>34</td>
</tr>
<tr>
<td>CSDS</td>
<td>10</td>
<td>11</td>
<td>31</td>
<td>32</td>
</tr>
<tr>
<td>SI</td>
<td>31</td>
<td>32</td>
<td>33</td>
<td>34</td>
</tr>
<tr>
<td>Engeletin</td>
<td>31</td>
<td>32</td>
<td>33</td>
<td>34</td>
</tr>
<tr>
<td>AAV-LCN2</td>
<td>31</td>
<td>32</td>
<td>33</td>
<td>34</td>
</tr>
</tbody>
</table>

---

**Graphs:**

1. **C:** LCN2 and GAPDH expression levels in the brain.
2. **D:** Therapeutic drug efficacy in a model of depression.
3. **E:** Total drinking behavior.
4. **F:** Time in interaction zone.
5. **G:** Immobile in TST (s).
6. **H:** Immobility in FST (s).
7. **I:** Total Distance in OFT (mm).
overexpression. (B) Fluorescent images of a fixed brain section expressing AAV-LCN2-EGFP in the mPFC on day 14 following AAV stereotactic infusion. Scale bar: 100 µm. (C) Western blotting confirmed successful AAV-LCN2-EGFP overexpression (n = 5). (D-H) LCN2 overexpression was found to reverse the antidepressant-like effects of engeletin on murine behaviors in the SPT (D, E), SIT (F), TST (G), and FST (H) (n = 12). (I) No significant differences in locomotor function were observed among groups in the OFT (n = 12). Results are means ±SEM. *P < 0.05, ***P < 0.001, ****P < 0.0001, one-way ANOVAs with Bonferroni post hoc testing.

Figure 8
LCN2 overexpression in the mPFC reverses the engeletin-mediated shift in the M1 and M2 polarization of microglia in CSDS model mice. (A-C) Representative western blotting (A) and corresponding quantification (B, C) demonstrating that the overexpression of LCN2 reverses engeletin-related reductions in the M1 polarization of microglia (iNOS, CD86). (D-G) Representative western blotting (D) and corresponding quantification (E-G) demonstrating that the overexpression of LCN2 reverses the engeletin-induced enhancement of M2 microglial polarization (CD206, Arg1, BDNF). Results are means ± SEM. (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way ANOVAs with Bonferroni post hoc testing.

Figure 9

Schematic model of the mechanism by which engeletin suppresses CSDS-induced depressive-like behaviors by modulating microglial polarization via the LCN2/CXCL10 signaling pathway.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
• rawdata.rar