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Sertraline ameliorates inflammation in CUMS mice and inhibits TNF-α-induced inflammation in microglia cells

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ABSTRACT

Evidence indicates that inflammation plays a crucial role in depression. Therefore, new antidepressants might be identified by screening drugs for their anti-inflammatory actions. Sertraline hydrochloride (SERT), a widely used antidepressant, has anti-inflammatory effects in clinical studies, but the mechanism involved is unclear. In this study, we used cell and molecular biology to determine the possible anti-inflammatory mechanism of SERT *in vivo* and *in vitro*. Experimental data from the *in vivo* study showed that mice exposed to chronic unpredictable mild stress (CUMS) had significantly higher levels of major inflammatory cytokines (tumor necrosis factor-α [TNF-α], interleukin-1β [IL-1β] and inducible nitric oxide synthase [iNOS]) in peripheral and central tissues compared with the control group. Treatment of CUMS mice with SERT significantly reduced the levels of these inflammatory cytokines and inhibited the phosphorylation of nuclear factor-κB (NF-κB) and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-α). Moreover, SERT reduced serum levels of transaminase in CUMS mice. Our *in vitro* study revealed that SERT suppressed TNF-α-induced NF-κB activation in a dose-dependent manner. SERT also inhibited the TNF-α-induced nuclear translocation of NF-κB by inhibiting IκB-α phosphorylation. Furthermore, SERT inhibited TNF-α-induced inflammatory cytokines in BV2 microglia cells. SERT directly bound to TNF-α and TNF-α receptor 1 (TNFR1) to potently block TNF-α/TNFR1-triggered signaling. These results indicate that SERT might treat depression by inhibiting the activation of microglia via the NF-κB signaling pathway. This study provides a basis for the research and development of antidepressants that act to reduce inflammation and the expression of inflammatory mediators.

1. Introduction

Depression is a common illness that affects > 300 million people worldwide. The most serious consequence of depression is suicide; globally, nearly 800,000 people die by suicide each year [1]. It is a major mental illness with a significant impact on social and occupational functions for individuals suffering from the disease [2]. Recent studies have shown that interactions between inflammation and the brain appear to drive the development of depression; the innate and adaptive immune systems interact with neurotransmitters and neurocircuits to influence the risk of depression [3]. Inflammation is an important biological event that may increase the risk of major depressive episodes, similar to traditional psychosocial factors [4]. Numerous clinical reports have shown that significantly higher concentrations of the proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are present in depressed subjects compared with normal subjects [5,6]. Nuclear factor-κB (NF-κB), a major mediator of inflammatory pathways, regulates inflammatory cytokines such as TNF-α, IL-6 and IL-1 [7–9], as well as anti-apoptotic and tumorigenesis factors [10,11].

Chronic unpredictable mild stress (CUMS) is a classic mouse model of depression first developed by Katz et al. in 1981 [12]. The aim of the CUMS method is to induce the development of a chronically depressive state in response to unpredictable stress stimuli [13]. It was reported that proinflammatory cytokines, including IL-1β, IL-6 and TNF-α, play a crucial role in inflammatory reactions [14]. CUMS increases the expression of proinflammatory cytokines and reduces the expression of anti-inflammatory cytokines in mice [15] and rats [16]. This immuno-

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imbalance affects the regeneration of neurons in CUMS animals [16].

Microglia cells are thought to act as macrophages in the central nervous system [17]. They are critical for the maintenance of normal brain homeostasis, functioning as neuro-immune hubs during brain pathology [18]. Microglia are exquisitely sensitive to perturbation by environmental challenges [17] and they adapt rapidly and respond to changes in the central nervous system (CNS) environment by regulating cytokine production, neuronal plasticity and neurotransmitter synthesis [19]. Furthermore, stress can enhance the release of proinflammatory cytokines from microglia [17]. It was reported that microglia are involved in several neuropsychiatric diseases [20] and that they undergo morphological changes and proliferation in different mouse models of depression [21].

The NF-κB pathway contributes to inflammatory reactions in the prefrontal cortex of CUMS rats [22]. Du et al. reported that fluoxetine inhibited the NF-κB inflammatory pathway and nucleotide oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome activation in CUMS rats. Moreover, it also inhibited NLRP3 inflammasome activation in LPS-induced macrophages and microglia cells [23] suggesting the inflammatory response induced by NF-κB signaling pathways is involved in depression.

Selective serotonin reuptake inhibitors (SSRIs) are a class of antidepressants that are widely used in clinical practice. Sertraline hydrochloride (SERT), fluoxetine and paroxetine are typical SSRIs. As research on SSRIs has expanded, new mechanisms of action have been reported. SSRIs potently inhibit LPS-induced microglial TNF-α and nitrous oxide (NO) production [24]. Clinical research showed that SERT reduced serum inflammatory factors (IL-6, IL-10) in patients with depression [25]. However, whether SERT inhibits inflammation through the NF-κB signaling pathway is still unclear. In this paper, we show the SERT-mediated inhibition of the NF-κB inflammatory pathway in CUMS mice and in TNF-α-induced microglia cells. In this study, we identified SERT as a potent inhibitor of TNF-α induced NF-κB signaling and investigated how this small molecule suppressed NF-κB activation. Moreover, the target of TNF-α/TNF-α receptor 1 (TNFR1) signaling was identified, which provides proof of concept to support the screening of anti-inflammatory drugs for novel antidepressant drug development.

2. Materials and methods

2.1. Reagents

Murine TNF-α was obtained from PeproTech Inc. (Rocky Hill, NJ, USA, 315-01A-20). Recombinant TNFR1 (50496-M02H) and TNFR2 (50128-M08H) were obtained from Sino Biological Inc. (Beijing, China). Sertraline hydrochloride (SERT, HY-B0176A) was obtained from MedChemExpress (Princeton, NJ, USA). Sertraline hydrochloride tablets (SERTT, mainly containing 50 mg SERT/tablet, A1604031) were obtained from Zhejiang Jingxin Pharmaceutical Co., Ltd. (Zhejiang, China). All cell culture reagents were from Gibco (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) was from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies for NF-κB p65 (8242), phospho-NF-κB p65 (pNF-κB p65, 3033 s), TNF-α (3707) and β-actin (4967) were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies for IκB-α (ab49978), phospho-IκBα (pIκB-α, ab133462), IL-1β (ab150777) and induced nitric oxide synthase (iNOS, ab178945) were purchased from Abcam Inc. (Cambridge, MA, USA). Horseradish peroxidase (HRP) conjugated secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA, SA00001–2). Alexa Fluor® 488 donkey anti-rabbit IgG (P/N SA212065) was obtained from Abcam Inc. Enhanced chemiluminescence (ECL, WBKLS0500) was purchased from Millipore Corporation (Billerica, MA, USA). CellTiter 96® AQueous One Solution Reagent (G3582) was purchased from Promega Corporation (Madison, WI, USA). RIPA lysis buffer (R0010) was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). The BCA assay kit was obtained from Thermo Scientific (Waltham, MA, USA). The BCA Protein Assay Kit (P0010S) was obtained from Beyotine Biotechnology (Shanghai, China). Polyvinylidene difluoride (PVDF) membranes were obtained from Merck Millipore Ltd., (Tullagreen Carrigtwohill County Cork, Ireland). DAPI (10236276001) was obtained from Roche Applied Science (Mannheim, Germany).

2.2. Mice and SERT treatment

Male C57BL/6 mice, weighing 18–22 g, were purchased from Liaoning Changsheng Biotechnology Co. Ltd. (SPF, SCXK (LIAO) Liaoning, China, 2015–001). At least 1 week before initiation of the experiments, all mice were housed in a central animal care facility in a temperature (22 °C), humidity and atmosphere-controlled room with unrestricted access to food and water, and allowed to adapt to their new environment. The experiment was carried out in accordance with the guidelines of the Animal Care Committee of Yanbian University (Resolution number, 201501022). After adapting to their new environment for at least one week, mice were randomly divided into four groups: control (not subjected to CUMS or treatment); vehicle (CUMS + vehicle, treated with vehicle); SERT-10 (CUMS + SERT, treated with SERTT at a dose of 10 mg SERT/kg of body weight/d by oral administration); and SERT-5 (CUMS + SERT, treated with SERTT at a dose of 5 mg SERT/kg of body weight/d by oral administration). SERTT was suspended in sterilized distilled water prior to administration to mice. Normal control mice and CUMS mice received the same volume of solution medium. Drug administrations and CUMS stimulation were started at the initiation of the experiment, which lasted for 5 weeks. One hour after the last drug administration, mice were anesthetized and blood was collected by retro-orbital puncture for serum biochemistry. Mice were then sacrificed and the brain was removed followed by separation of brain tissues. Then the tissues were frozen in liquid nitrogen and stored at −80 °C for use in Western blotting. The detailed *in vivo* experimental design is shown in Fig. 1A.

2.3. CUMS method

CUMS stimulation was given on a random schedule and stimulation included food deprivation for 12 h, water deprivation for 12 h, swimming in cold water (4 °C) for 5 min, hot bath (45 °C) for 5 min, tail clamp for 1 min, cage tilting (45°) for 12 h and damp bedding for 12 h. The mice received stimulation every day and once a stimulation was administered to a mouse, it was not used again for the next 2 days [26–28]. CUMS stimulation was given to the mice daily for five consecutive weeks. The control group was housed in a separate cage without stimulation (Table 1).

2.4. Tail suspension test

The tail suspension test (TST) was performed by suspending mice 20 cm above the floor using a string tied 1 cm from the tip of the tail and tied to a horizontal rod. The duration of the test was 6 min and immobility time, defined as when the mouse was completely immobile, was recorded during the last 4 min of the test [29,30]. The tail suspension test was performed in the last week (week 5) of the experiment.

2.5. Forced swimming test

Twenty-four hours after the TST test was performed on the mice, the forced swimming test (FST) was performed by placing mice in an open glass beaker (20 cm in height and 14 cm in diameter) with 10 cm-deep water (25 ± 1 °C), forcing them to swim. The duration of the test was 6 min and immobility time, defined as the mice floating passively without struggling in the water, was recorded during the last 4 min of the test [31,32]. The test was performed at the end of week 4.

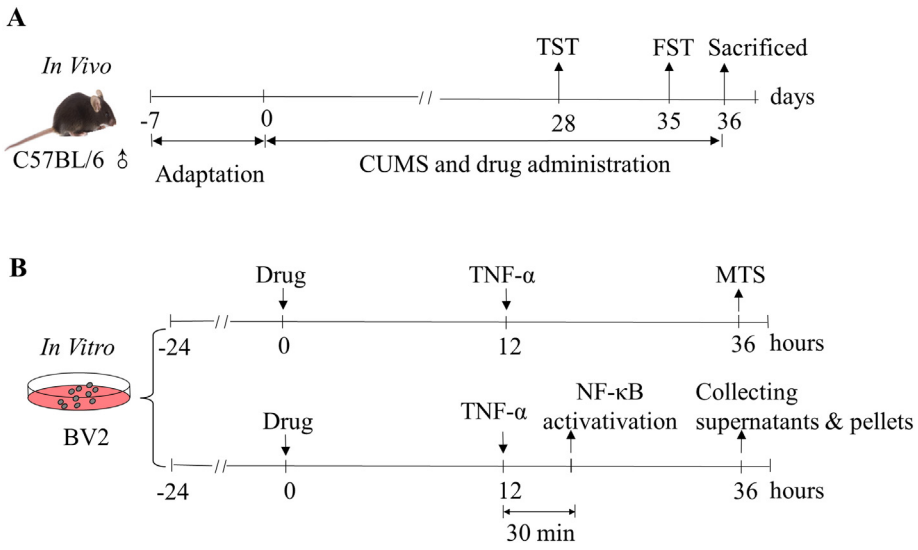


Fig. 1. Schematic representation of the *in vivo* (A) and *in vitro* (B) experimental design. (A) *In vivo* experiment: SERTT was orally administered (daily) to the mice for 35 days, and 30 min after the final dose of SERTT was administered, tests were conducted. SERTT: sertraline hydrochloride tablet; SERT sertraline hydrochloride; CUMS: chronic unpredictable mild stress; FST: forced swimming test; TST: tail suspension test; (B) *In vitro* experiment: MTS assay: detection of cell proliferation; Immunofluorescence staining: detection of nuclear transcription of NF-κB p65.

Table 1
CUMS stimulation.

Stressor	Description	Duration(time)
Food/water deprivation	Deprivation food or water	12 h
Cold water swimming	The mice swim in cold water at 4 °C	5 min
Hot bath	The mice stand on 45 °C hot water	5 min
Tail clamp	Clip the tail with a clip	1 min
Cage tilting	The cages tilted to a 45-degree angle	12 h
Damp bedding	200 mL water in 100 g sawdust bedding	12 h

2.6. The temperature measurement

Following lubrication with glycerol, the temperature probe was inserted 3 cm beyond the rectal opening for approximately 30 s until a stable temperature was reached. The test was performed at the end of week 5, and the mice were not stimulated [33].

2.7. Enzyme-linked immunosorbent assay (ELISA)

At the end of the experiment, blood was collected by retro-orbital puncture and centrifuged at 1000 ×*g* for 10 min at 4 °C to separate the serum. Serum levels of NO, IL-1β, TNF-α, aspartate aminotransferase (AST) and alanine transaminase (ALT) were determined using ELISA kits (MLBIO, China) according to the manufacturer's instructions.

2.8. Cell culture and treatment with SERT and TNF-α

BV2 cells (American Type Culture Collection, Manassas, VA, USA), a mouse microglial cell line, were cultured in 100 mm dishes (untreated) with Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and penicillin (100 U/mL)-streptomycin (100 U/mL), then placed in a humidified incubator at 37 °C with 5% CO_2. The cultures were passaged by trypsinization every third day and cells were plated in 100 mm culture dishes. For the experiments, cells were seeded onto 24- or 96-well plates and 60 mm dishes (all untreated) overnight and culture medium was changed to DMEM with 5% FBS. The cells were treated with SERT in the absence or presence of TNF-α (10 ng/mL) for 24 h, after which cell viability or proteins were assayed. The detailed *in vitro* experimental design is shown in Fig. 1B.

2.9. Measurement of cell viability by MTS assay

BV2 cells were seeded in 96-well plates (untreated) at 1 × 10^5 cells/mL and incubated overnight. Then the cells were pretreated with different concentrations of SERT (0.5–10 μM) for 12 h, and induced with or without TNF-α (10 ng/mL) for 24 h. Finally, 20 μL MTS solution (CellTiter 96®AQueous One Solution Reagent, Promega, Madison, WI, USA) was added into each well (volume of well: 100 μL medium) and the cells were cultured for an additional 3 h. All incubations were performed in a humidified incubator at 37 °C, 5% CO_2. Absorbance was measured at 490 nm using a Multiskan GO (Thermo Electron Corp., Marietta, OH, USA).

2.10. Western blotting

BV2 cells were cultured in 6 cm dishes (untreated) at 5 × 10^5 cells/well and allowed to adhere overnight. The culture medium was removed and replaced with fresh medium containing SERT at the indicated concentrations and then the cells were treated with TNF-α (10 ng/mL). Cells (after treatment) or mouse brain tissue (from the *in vivo* study) were collected and lysed with RIPA lysis buffer. Protein concentrations were determined using a BCA protein assay kit. Equal amounts of protein were separated using 8–10% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with 5% skim milk for 1 h at room temperature then incubated with primary antibodies for 4 h at room temperature or overnight at 4 °C. Primary antibodies for Iba-1, iNOS, TNF-α, IL-1β, NF-κB p65, pNF-κB p65, IκB-α, pIκB-α and β-actin were used for Western blotting. After incubation with primary antibodies, the membranes were washed then HRP conjugated secondary antibodies were added. Antibody labeled protein on the membranes was detected using ECL Western blotting detection reagents, and then developed and fixed. Blots were scanned and bands were quantified with Quantity One software (Bio-Rad, USA). Variations in the density were expressed as fold changes (n = 3), compared with the normal control in each blot.

2.11. LSPR assay

Localized Surface plasmon resonance (LSPR) assays were carried out on an OpenSPRTM (Nicoya Lifesciences, Waterloo, Canada). For the analysis of TNF-α (5 μg) and TNFR1 (5 μg), protein was fixed on the COOH sensor chip by NH2 and COOH, then SERT at concentrations of 2.5–20 μM were injected sequentially into the chamber in PBS running buffer. The interaction of TNF-α and TNFR1 with the fixed small molecules was detected by OpenSPRTM at RT. The binding time was 240 s, the disassociation time was 150 s, the flowrate was 20 μL/min, and the chip was regenerated with 0.25% SDS. The data were retrieved and

analyzed with TraceDrawer software (Ridgeview Instruments AB, Sweden). Briefly, the integrated rate equation describing a 1:1 Langmuir interaction was fit simultaneously to the entire concentration range for each compound. This fit yielded the association rate (Ka), the dissociation rate (Kd), and the dissociation constant (assuming the relationship KD = Kd / Ka) [34].

2.12. Immunofluorescence detection of NF-κB p65

BV2 cells were seeded into 24-well plates (untreated) at 2×10^4 cells/well. Twelve h later, cells were pretreated with 1 μM SERT for 12 h, followed by treatment with TNF-α (10 ng/mL) for 30 min. Cells pretreated with DMSO or treated with TNF-α (10 ng/mL) alone were used as negative and positive controls, respectively. After treatment, cells were rinsed three times in room temperature PBS, fixed in methanol (pre-cooled at −20 °C) for 20 min at 4 °C, then permeabilized with 0.2% Triton X-100 for 15 min on ice. Cells were blocked with 5% bovine serum albumin (BSA) in PBS for 30 min and incubated overnight with primary NF-κB p65 antibody at 4 °C, followed by incubation with Alexa Fluor® 488 donkey anti-rabbit IgG for 30 min at room temperature. Then the cells were stained with DAPI for 5 min before observation using an Olympus IX83 (Tokyo, Japan) inverted fluorescence microscope. The NF-κB p65 protein appeared green under the fluorescence microscope and the nuclei appeared blue. The green and blue images were merged using ImageJ software (NIH, Bethesda, MD, USA) to produce cyan fluorescence in areas of co-localization.

2.13. Statistical analysis

All values are expressed as the mean ± S.D. A comparison of the results was performed by one-way ANOVA and Dunnett multiple comparison tests (Graphpad Software, Inc., San Diego, CA, USA). Statistically significant differences between groups were defined as $P < 0.05$.

3. Results

3.1. SERT improves the behavior of CUMS mice

To evaluate mice for behaviors associated with depression, we performed FST at the end of the week 4 experimental period and TST at the end of week 5. For the FST, exposure to CUMS increased immobility time ($P < 0.05$); treatment with SERT-10 and SERT-5 decreased the immobility time in CUMS mice in a dose-dependent manner ($P < 0.001$, $P < 0.01$, respectively) (Fig. 2A). For the TST, immobility time in the vehicle group was significantly increased compared with the control group ($P < 0.01$). In the same test, compared with the vehicle group, the immobility time of the SERT-10 and SERT-5 groups were significantly reduced in a dose-dependent manner ($P < 0.001$, $P < 0.05$, respectively) (Fig. 2A). We measured mouse body temperature at the end of week 5 and found it was decreased in the vehicle group compared with the control group ($P < 0.05$). Hypothermia was inhibited in the CUMS + SERT groups ($P < 0.001$) (Fig. 2B).

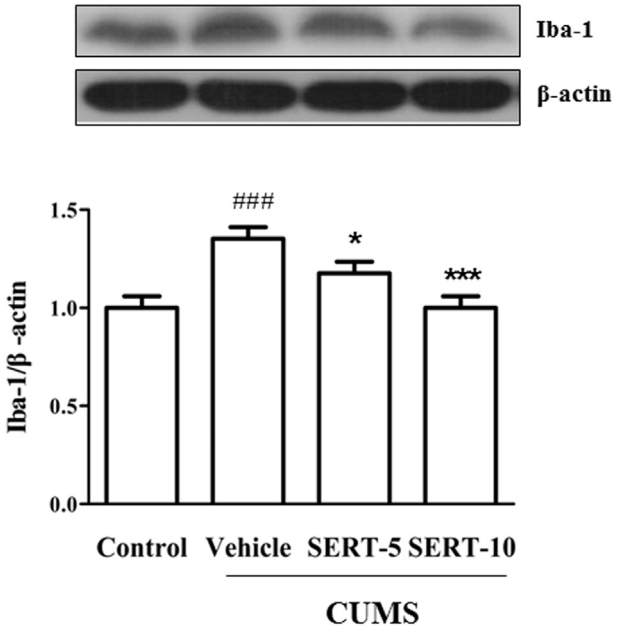


Fig. 3. SERT inhibits the expression of Iba-1 in brain tissues of CUMS mice. Whole brain extracts were analyzed by Western blotting using the indicated antibodies for Iba-1 and β-actin. Data represent the mean ± SD. $^*P < 0.05$, $^{***}P < 0.001$ vs vehicle; $^{\#}$ $^{\#}$ $^{\#}P < 0.001$ vs control. The data are representative of 3 independent experiments.

3.2. SERT inhibits the expression of Iba-1 in brain tissues of CUMS mice

To confirm that SERT inhibits the activation of microglia in CUMS mice, the expression of Iba-1 in brain tissues was detected by Western blot. Compared with the control group, CUMS activated brain

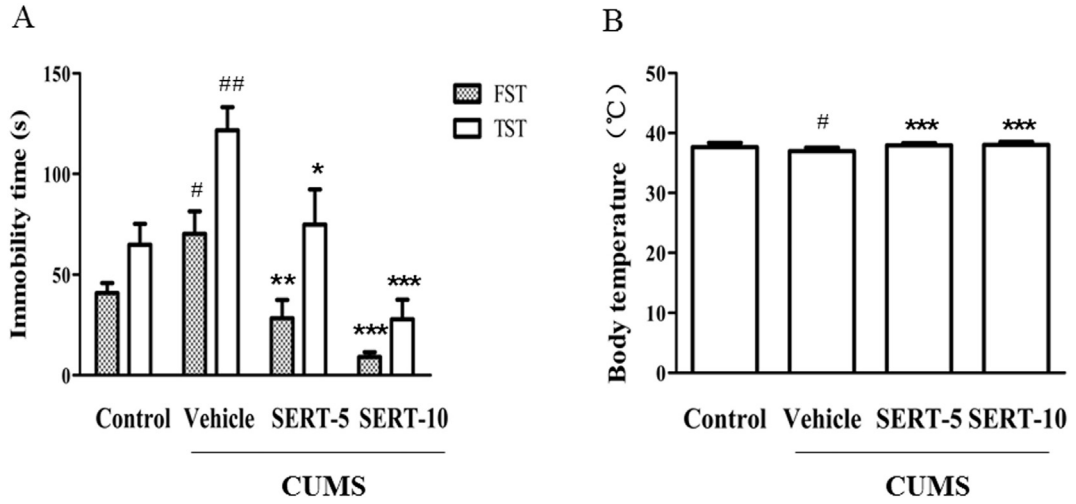


Fig. 2. Effects of treatment on (A) immobility time of FST and TST. Data represent the mean ± SEM. (B) Body temperature (°C). Data represent the mean ± SD. n = 10/group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs vehicle; $^{\#}P < 0.05$, $^{\#}$ $^{\#}P < 0.01$ vs control. Data are representative of 3 independent experiments.

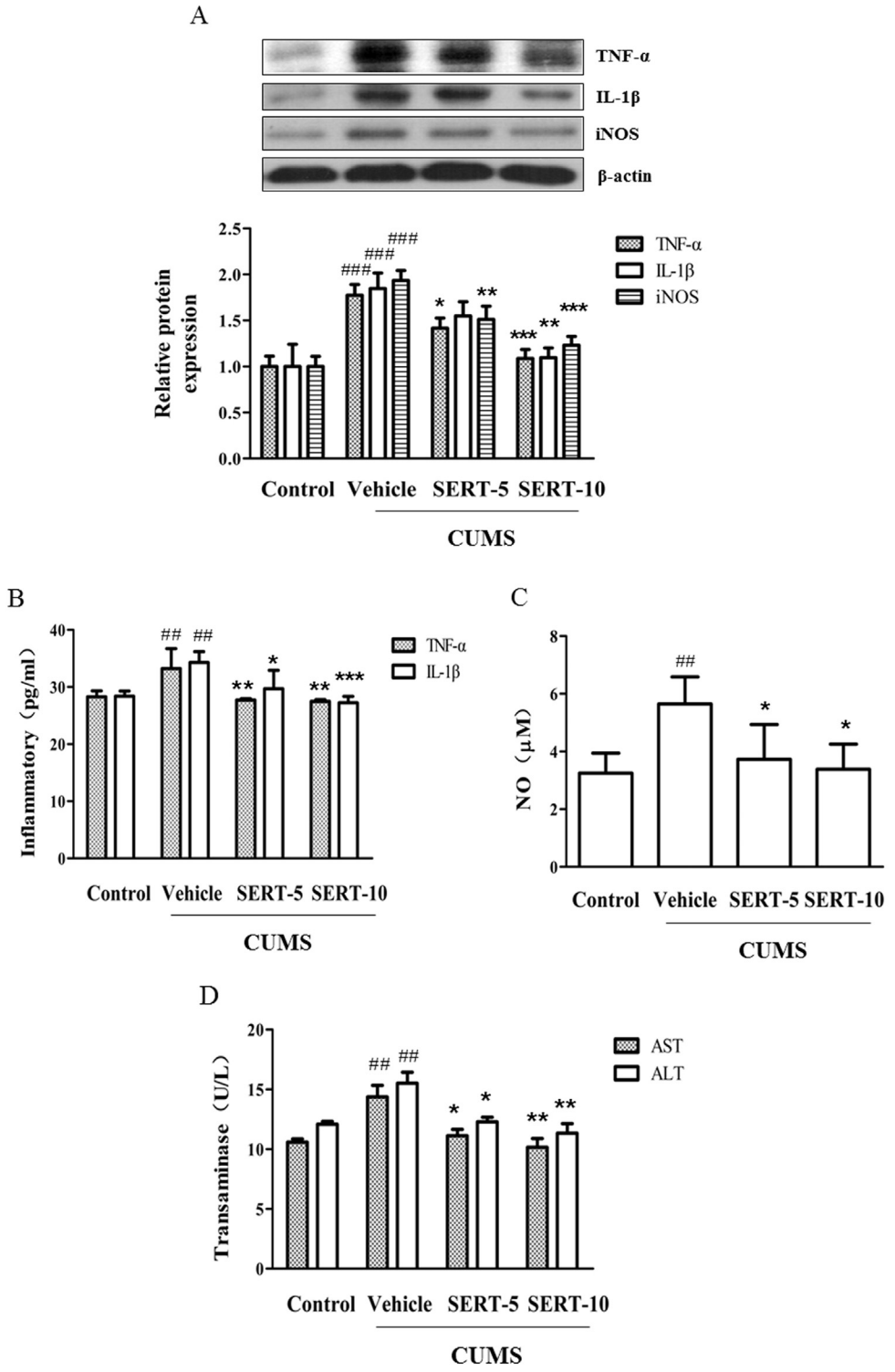


Fig. 4. SERT inhibits inflammatory factors in the brain tissues and serum of mice. Whole brain extracts were analyzed by Western blotting using the indicated antibodies for (A) TNF-α, iNOS, IL-1β and β-actin. Data represent the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs vehicle; $^{\#\#\#}P < 0.001$ vs control. ELISA of (B) TNF-α and IL-1β (n = 4, λ = 450 nm); (C) NO (n = 4, λ = 570 nm) and (D) AST and ALT (n = 4, λ = 505 nm). Data represent the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs vehicle; $^{\#\#}P < 0.01$ vs control. The data are representative of 3 independent experiments.

microglial cells and significantly increased the expression of Iba-1 ($P < 0.001$), whereas SERT-10 and SERT-5 treatment significantly reduced the expression of Iba-1 in a dose-dependent manner ($P < 0.001$, $P < 0.05$, respectively) (Fig. 3).

3.3. SERT inhibits inflammatory factors in brain tissues and serum

To demonstrate the anti-inflammatory effects of SERT, we measured inflammatory factors (TNF-α, IL-1β and iNOS) in mouse brain tissues by Western blotting and in mouse serum by ELISA. TNF-α, IL-1β and iNOS levels in the brain tissues of mice were significantly increased in the vehicle group compared with the control group ($P < 0.001$) whereas

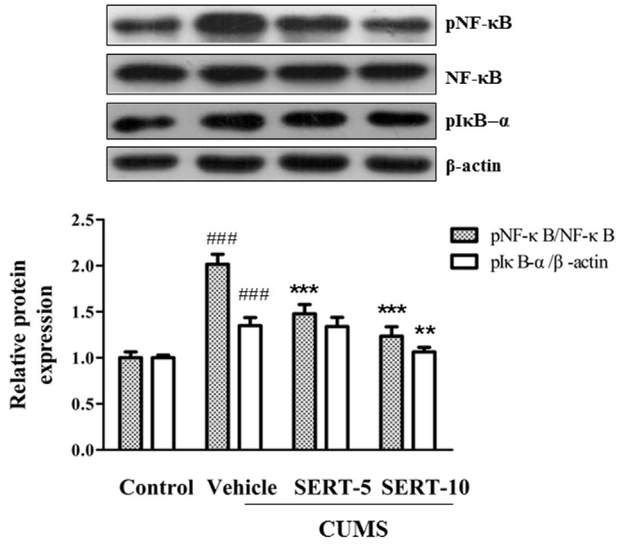


Fig. 5. SERT inhibits CUMS-induced NF-κB inflammasome activation in brain tissues of mice. Whole brain extracts were analyzed by Western blotting using the indicated antibodies for pNF-κB p65, NF-κB p65, pIκB-α and β-actin. Data represent the mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ vs vehicle; $^{###}P < 0.001$ vs control. The data are representative of 3 independent experiments.

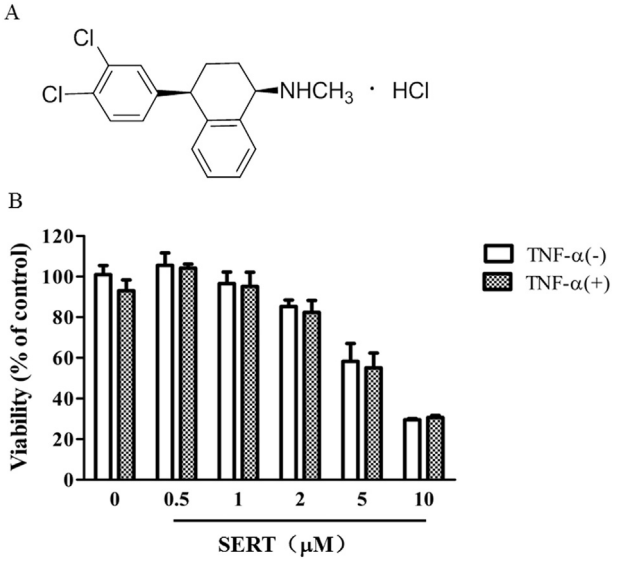


Fig. 6. Viability of BV2 cells simulated by TNF-α for 24 h. (A) Chemical structure of SERT. (B) BV2 cells were treated with the indicated concentrations of SERT for 12 h, then incubated with TNF-α (10 ng/mL) for 24 h. After incubation, cell viability was determined by MTS assay. Data represent the mean ± standard deviation. The data are representative of three independent experiments.

the levels of each of these proinflammatory mediators were reduced in the brain tissues of CUMS mice treated with SERT (Fig. 4A). These inhibitory effects were dose-dependent. As shown in Fig. 4B–D, CUMS significantly increased the levels of inflammatory proteins in the serum. TNF-α, IL-1β, NO, AST and ALT levels were higher in the vehicle group compared with the control group ($P < 0.01$), and this effect was reversed by SERT treatment in a dose-dependent manner.

3.4. SERT inhibits the NF-κB signaling pathway in brain tissues of CUMS mice

To confirm that CUMS activates the NF-κB signaling pathway and to investigate the inhibitory effect of SERT, the expressions of related proteins in brain tissues were detected by Western blotting. The levels of pNF-κB p65and pIκB-α were increased in CUMS mice. However, after treatment with SERT, the levels of pNF-κB p65 and pIκB-α were significantly decreased ($P < 0.001$) (Fig. 5) in a dose-dependent manner.

3.5. SERT inhibits TNF-α-induced NF-κB activation

To evaluate the effect of SERT and TNF-α on BV2 cell viability, cells were incubated with or without TNF-α (10 ng/mL) in the presence of various concentrations of SERT (0.5–10 μM). MTS assay showed that SERT did not cause significant cellular toxicity in BV2 cells treated with SERT with or without TNF-α at doses up to 2 μM (Fig. 6B). We chose concentrations of SERT (0.5 and 1 μM) that resulted in cell viability of > 90% for the follow up experiments.

3.6. SERT inhibits TNF-α-induced microglial activation

To confirm that SERT inhibits the activation of TNF-α-induced microglia, the expression of Iba-1 in BV2 cells was detected by Western blotting (Fig. 7). Compared with the normal group, TNF-α significantly increased the expression of Iba-1 in BV2 cells ($P < 0.001$), whereas treatment with SERT-1 and SERT-0.5 significantly reduced the expression of Iba-1 in a dose-dependent manner ($P < 0.001$, $P < 0.05$, respectively).

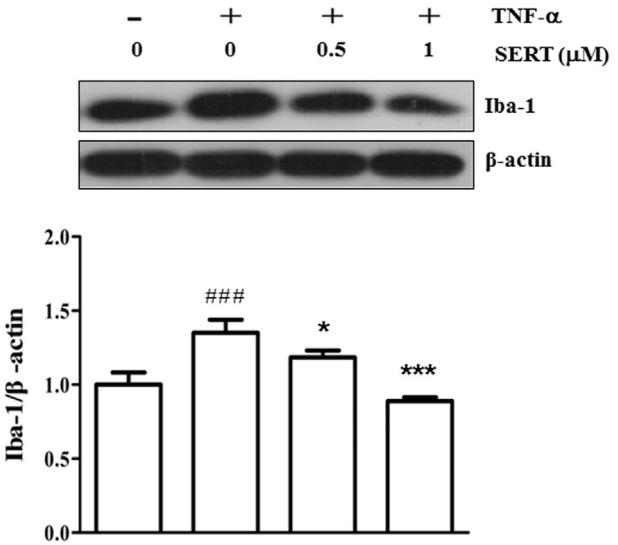


Fig. 7. SERT inhibits TNF-α-induced microglial activation. BV2 cells were treated with 0.5 μM or 1 μM of SERT, then incubated with TNF-α (10 ng/mL) for 24 h. Whole cell extracts were analyzed by Western blotting using the indicated antibodies for Iba-1 and β-actin. Data represent the mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$ vs TNF-α; $^{###}P < 0.001$ vs control. The data are representative of 3 independent experiments.

3.7. SERT inhibits TNF-α-induced inflammatory factors and directly binds to TNF-α and TNFR1

To confirm the optimum effective concentration of SERT necessary to inhibit TNF-α-induced inflammatory factors, we exposed BV2 cells to various concentrations of SERT for 12 h and then treated them with TNF-α for 24 h. The levels of TNF-α and iNOS were examined by Western blotting. SERT inhibited TNF-α-induced inflammatory factors downstream of the NF-κB signaling pathway by a dose-dependent mechanism. The optimum effective concentration of SERT was 1 μM (Fig. 8A).

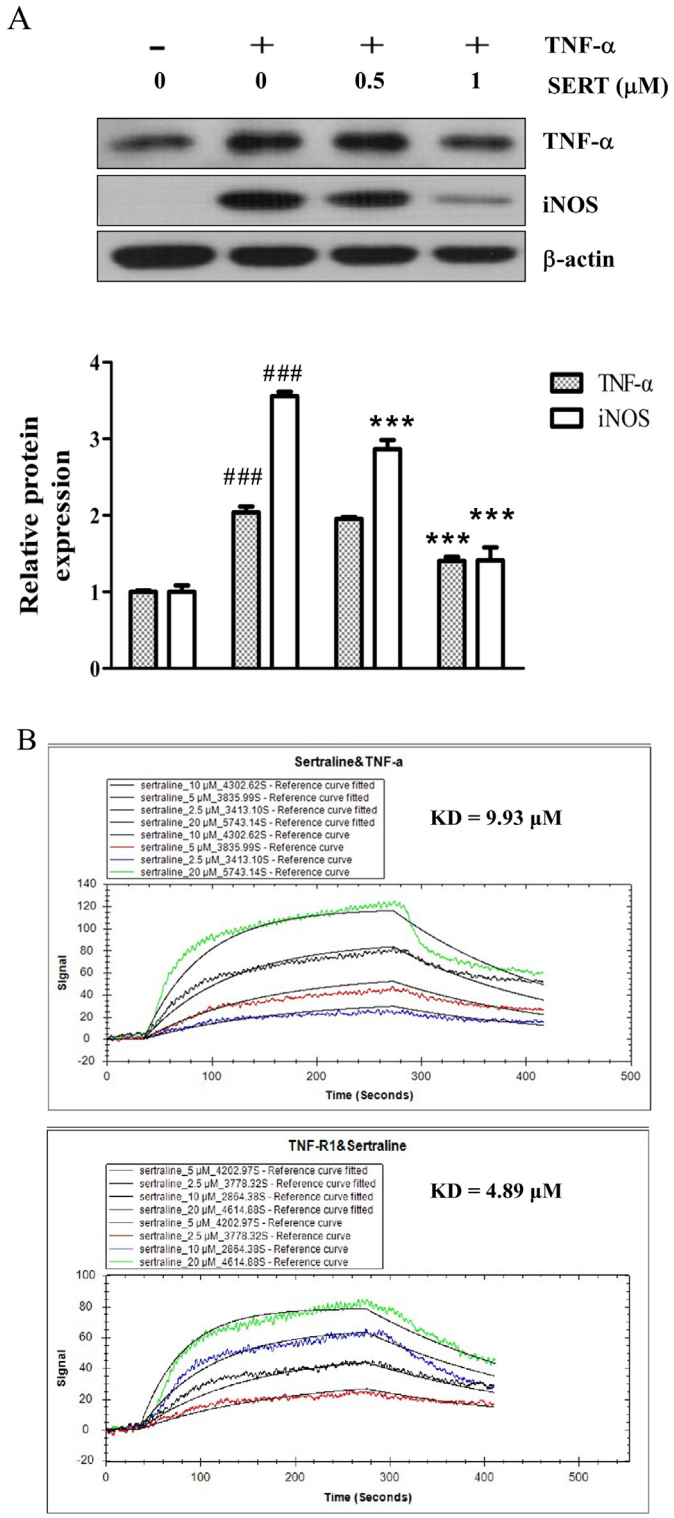


Fig. 8. The effect of SERT treatment on the expression of inflammatory factors (A) and the mechanism of TNF-α or TNFR1 antagonistic activity of SERT (B). (A) BV2 cells were treated with 0.5 μM or 1 μM of SERT, then incubated with TNF-α (10 ng/mL) for 24 h. Whole cell extracts were analyzed by Western blotting using the indicated antibodies for TNF-α, iNOS and β-actin. Data represent the mean ± SD. $^{***}P < 0.001$ vs TNF-α; $^{###}P < 0.001$ vs control. (B) A LSPR binding kinetic curve was determined using different concentrations of SERT. Mouse TNF (i) and TNFR1 (ii) were respectively coupled to COOH sensor chips, and SERT was applied from 2.5 to 20 μM.

LSPR has been widely used to measure binding between mobile analytes and immobilized biomolecules without the use of labels. The signals can be acquired qualitatively and quantitatively. Using the LSPR assay, we examined whether SERT directly acted on extrinsic TNF-α or blocked TNF-α receptors (TNFR1). We detected that the KDs of SERT binding to TNF-α and TNFR1 were 9.93 μM and 4.89 μM, respectively (Fig. 8B). Thus, SERT binds directly to and has a high affinity for TNF-α and TNFR1. TNFR1 had a higher specific binding affinity than TNF-α. This demonstrated that SERT directly acts on extrinsic TNF-α, but also blocks the function of its receptors (TNFR1) to inhibit TNF-α production.

3.8. SERT inhibits TNF-α-induced IκB-α phosphorylation and degradation, and NF-κB p65 nuclear translocation

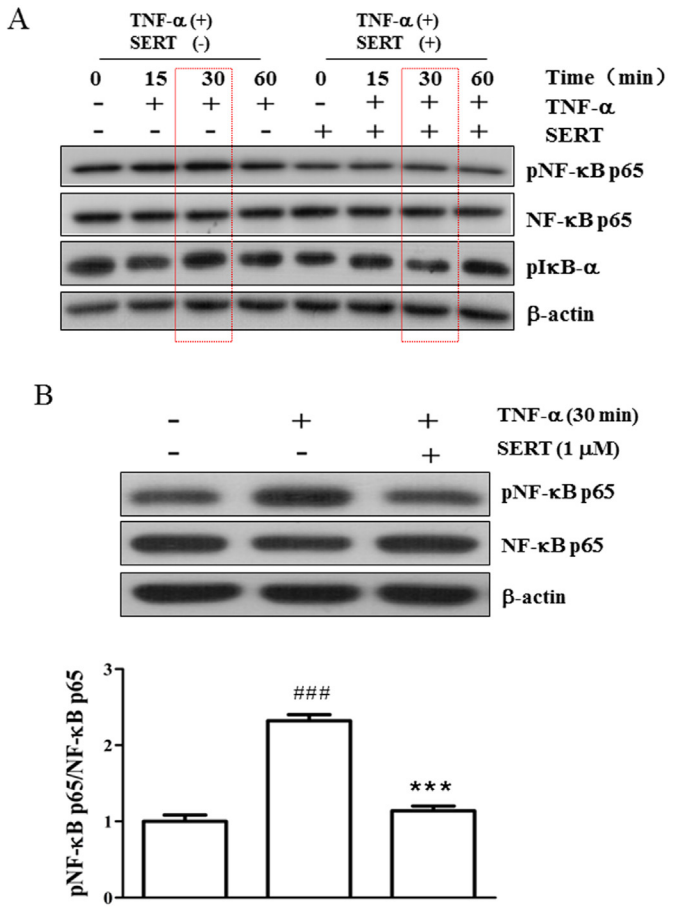
To determine whether the inhibition of TNF-α-induced NF-κB p65 activation was caused by the inhibition of IκB-α phosphorylation, we exposed BV2 cells to SERT for 12 h and then treated them with TNF-α for different durations. The results showed that TNF-α induced the phosphorylation and degradation of IκB-α as quickly as 30 min (Fig. 9A). To show this effect more clearly, we examined pNF-κB p65 and NF-κB p65 after 30 min exposure to TNF-α in a single experiment. This result was similar to that observed previously (Fig. 9B). To confirm these results, we performed immunofluorescence cell staining to observe NF-κB p65 translocation in BV2 cells. The results indicated that in untreated cells, as well as those pretreated with SERT (1 μM) followed by TNF-α treatment, NF-κB p65 was localized in the cytoplasm, whereas in cells treated with TNF-α alone, NF-κB p65 was translocated to the nucleus (Fig. 9C).

4. Discussion

Microglia, a major glial component of the CNS, are also known to play a critical role as resident immunocompetent and phagocytic cells in the CNS. Microglia cells were suggested to be involved in several neuropsychiatric diseases, such as depression [20], Alzheimer's disease [35] and diabetic encephalopathy [36]. These diseases are all related in that morphological changes in the microglia are observed [37]. It was reported that activated microglia may be a potential biomarker for mood states and a measure of pharmacological response in bipolar disorder therapy [19]. Iba-1, a microglia/macrophage specific protein, was reported to be increased after CUMS [38] or LPS [39] challenge. However, CNS inflammatory diseases associated with microglial activation show a marked reduction in neuroinflammation with the administration of minocycline [40].

SERT, a SSRI, is the newest generation of antidepressants and are the primary drugs of choice for the treatment of depressive disorders. SERT was reported to improve memory [41], attention [42], and decrease the expression of TNF-α and IL-6 in patients with depression [43] CUMS rats [44]. However, few studies of SERT have reported an association between antidepressant effects and microglial activation. Previous *in vitro* studies reported SERT significantly inhibited LPS [24] or IFN-γ [45] -induced microglia cell inflammatory protein production (TNF-α, NO). However, whether SERT blocks the TNF-α-induced NF-κB signaling pathway in microglial cells has not been reported. In this study, the anti-inflammatory mechanism of SERT was systematically studied using the CUMS model *in vivo* and TNF-α-induced BV2 microglia cells *in vitro*.

NF-κB family transcription factors are master regulators of immune and inflammatory processes in response to injury and infection [46]. In the latent state, NF-κB is sequestered in the cytosol by IκB (inhibitor of NF-κB) protein [47]. With stimulation from TNF-α or other stimulants, the phosphorylation of IκB results in IκB proteasomal degradation and the release of NF-κB for nuclear translocation and the activation of gene transcription [48]. NF-κB regulates the transcription of various inflammatory cytokines, such as IL-8, IL-1β, TNF-α and iNOS [49]. When



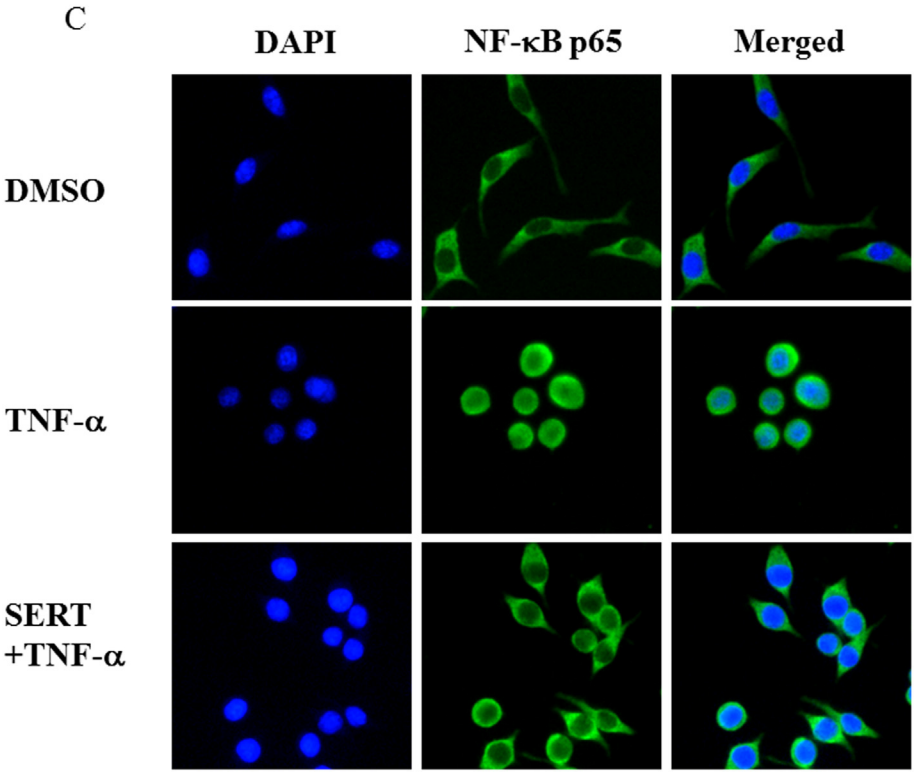


Fig. 9. Effect of SERT on TNF-α-induced NF-κB p65 nuclear translocation. (A) BV2 cells were incubated with 1 μM SERT for 12 h and then incubated with TNF-α (10 ng/mL) for the indicated times. Whole cell extracts were analyzed by Western blotting using the indicated antibodies for pNF-κB p65, NF-κB p65, pIκB-α and β-actin. (B) BV2 cells were incubated with 1 μM SERT for 12 h and then incubated with TNF-α (10 ng/mL) for 30 min. Whole cell extracts were analyzed by Western blotting using the indicated antibodies for pNF-κB p65, NF-κB p65 and β-actin. Data represent the mean ± SD. $^{***}P < 0.001$ vs TNF-α; $^{###}P < 0.001$ vs control. (C) BV2 cells were incubated with 1 μM SERT for 12 h followed by TNF-α (10 ng/mL) stimulation for 30 min. After fixation, cells were stained with anti-NF-κB p65 antibody followed by Alexa Fluor® 488 secondary antibody (green), the nucleus was counterstained with DAPI (blue) and the cells were examined by fluorescence microscopy. Scale bars: 20 mm. Images were acquired for each fluorescence channel, using suitable filters with the 40× objective. Green and blue images were merged using ImageJ software. The data are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

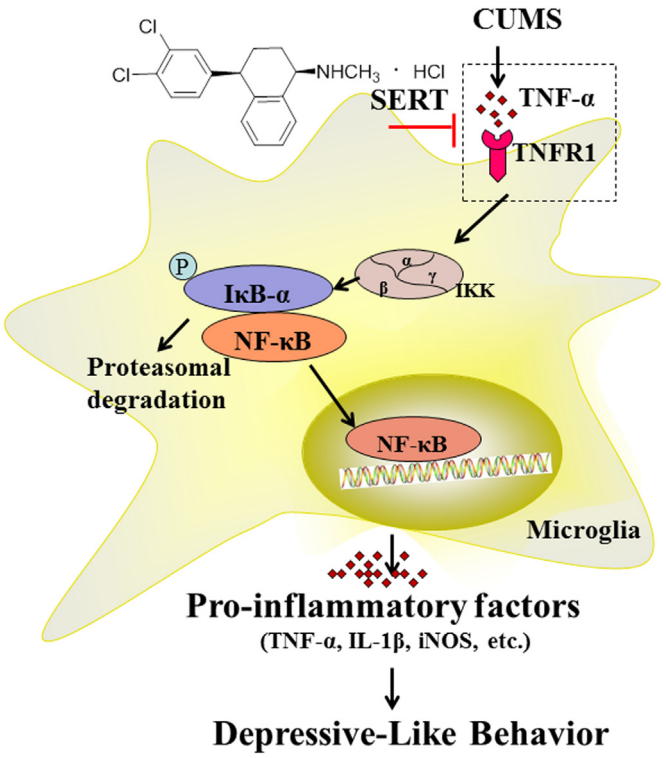


Fig. 10. Proposed anti-inflammatory effects of SERT mediated through TNF-α-induced NF-κB signaling. SERT ameliorates inflammation in CUMS mice and TNF-α-induced inflammation in BV2 cells by inhibiting NF-κB activation.

NF-κB is activated, its inflammatory factors are activated simultaneously. Exposure of mice to CUMS activated the NF-κB pathway and NLRP3 inflammasomes with the overexpression of inflammatory mediators; these changes were reversed by chronic (daily) treatment with senegenin [50] and atractylenolide I [51]. Furthermore, a previous report showed that fluoxetine inhibited LPS-induced NF-κB activation in microglia [52]. Recent studies confirmed a relationship between depression and inflammation. These studies showed that levels of TNF-α [53] and IL-1β [54] in the serum of patients with depression were significantly higher compared with the normal group. Similarly, TNF-α, IL-1β and IL-6 protein levels were maintained at significantly higher levels in the CUMS stressed mice compared with the normal control group [55]. Interestingly, functional allelic variants of genes encoding IL-1β and TNF-α, as well as genetic variations affecting T-cell function, may increase the risk for depression [56]. Of note, the secretion of TNF-α was revealed to elevate the levels of adrenocorticotropic hormone, cortisol and adrenocorticotropic hormone, which have a direct effect on the hypothalamic-pituitary-adrenal (HPA)-axis. Furthermore, upregulation of the HPA-axis can cause depression [57]. Increasing evidence has shown that soluble TNF-α inhibitors improved cognitive impairment in mice [58], and have antidepressant effects or improve antidepressant responses [57]. Here, we established a CUMS *in vivo* model of depression and TNF-α-induced inflammation *in vitro* to investigate the anti-inflammatory mechanism of SERT. The results demonstrated that SERT reduced the levels of pNF-κB p65 and pIκB-α and the levels of the downstream inflammatory factors iNOS, TNF-α and IL-1β in the central tissues and microglia cells. This finding suggests that SERT exerts anti-inflammatory and antidepressant-like activities by inhibiting the NF-κB and downstream signaling pathways.

Animal models and clinical observations reported that chronic liver inflammation was associated with changes in the CNS [40]. In this study CUMS significantly increased the levels of AST and ALT and treatment with SERT significantly decreased the levels of AST and ALT. This indicated that SERT might reduce liver inflammation. Furthermore, serum transaminase level is an important gold indicator for liver inflammation and elevated serum levels of TNF-α caused hepatitis [34]. A previous study found that CUMS induced hepatic injury in rats [59,60]. More importantly, SERT attenuated proinflammatory cytokines (TNF-α, IL-1β, NO) in peripheral tissues. We speculated that alleviation of the liver-brain inflammation axis is a novel mechanism mediating the improvement of SERT on CUMS stressed depressive-like behavior.

The anti-depression mechanism of SERT is to inhibit the re-uptake of serotonin into pre-synaptic cells, thereby increasing extracellular levels of the neurotransmitter that are available to bind post-synaptic neurons [61]. It was reported that only SERT at clinical dosages increased extracellular dopamine (DA) levels in the nucleus accumbens and striatum, indicating the SERT effect on DA is a unique property among SSRIs [62]. Recently in another experiment, we found that SERT significantly increased the levels of DA and 5-hydroxytryptamine (5-HT) in the CUMS model mice (data not shown).

TNF-α signaling through TNFR1, which is expressed in the CNS, mainly results in activation of the transcription factors NF-κB and AP-1 and induces pro-inflammatory effects that exacerbate neuroinflammation and secondary neuronal damage [63]. Thus, anti-inflammatory strategies that block TNF-α/TNFR1 signaling might be useful new antidepressants. In this study, we detected the target of SERT in the TNF-α-induced signaling pathway. The results showed that SERT directly bound to TNF-α and TNFR1 to potently inhibit TNF-α-induced inflammation and effectively block TNFR1-triggered NF-κB signaling activities and other downstream pathways.

In summary, SERT ameliorated inflammation caused by the activation of NF-κB signaling in CUMS mice and in TNF-α-stimulated BV2 microglia cells (Fig. 10). Furthermore, a novel anti-inflammatory strategy blocking TNF-α/TNFR1 signaling may be used to develop new antidepressants for the treatment of depression.

Declarations of interest

None.

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