Ciliary neurotrophic factor activation of astrocytes mediates neuronal damage via the IL-6/IL-6R pathway

HONG-TAO WANG[1], SI-TONG LU[1-3], ZHI-HUI XIA[1,2], TAO XU[1,4], WEI-YAN ZOU[1,2] and MEI-QUN SUN[1,2]

[1]Anhui Province Key Laboratory of Immunology in Chronic Diseases, Bengbu Medical University, Bengbu, Anhui 233030, P.R. China; [2]Department of Histology and Embryology, Bengbu Medical University, Bengbu, Anhui 233030, P.R. China; [3]Department of Stomatology, Wuhan College of Arts and Science, Wuhan, Hubei 430101, P.R. China; [4] Department of Clinical Laboratory and Diagnostics, Laboratory Medicine College, Bengbu Medical University, Bengbu, Anhui, 233030, P.R. China

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Abstract. The occurrence of epilepsy is a spontaneous and recurring process due to abnormal neuronal firing in the brain. Epilepsy is understood to be caused by an imbalance between excitatory and inhibitory neurotransmitters in the neural network. The close association between astrocytes and synapses can regulate the excitability of neurons through the clearance of neurotransmitters. Therefore, the abnormal function of astrocytes can lead to the onset and development of epilepsy. The onset of epilepsy can produce a large number of inflammatory mediators, which can aggravate epileptic seizures, leading to a vicious cycle. Neurons and glial cells interact to promote the onset and maintenance of epilepsy, but the specific underlying molecular mechanisms need to be further studied. Ciliary neurotrophic factor (CNTF) belongs to the IL-6 cytokine family and is mainly secreted by astrocytes and Schwann cells. In the normal physiological state, CNTF levels are low, but in an epileptic state, CNTF levels in the serum and tears of patients are elevated. Astrocyte activation plays an important role in epileptic seizures. CNTF activates astrocytes to produce a variety of secreted proteins, which are secreted into the astrocyte culture medium (ACM), thus forming a distinct culture medium (CNTF-ACM) that can be used to study the effect of astrocytes on neurons *in vitro*. CNTF-activated astrocytes increase the secretion of the pro-inflammatory factor IL-6. In the present study, CNTF-ACM was applied to primary cerebral cortical neurons to observe the specific effects of IL-6 in CNTF-ACM on neuronal activity and excitability. The results suggested that CNTF-ACM can reduce neuronal activity via the IL-6/IL-6R pathway, promote neuronal apoptosis, increase Ca^{2+} inflow, activate the large conductance calcium-activated potassium channel and enhance neuronal excitability. The results of the present study further revealed the functional changes of astrocytes after CNTF activated astrocytes and the effects on neuronal activity and excitability, thereby providing new experimental evidence for the role of communication between astrocytes and neurons in the mechanism of epileptic seizures.

Correspondence to: Professor Mei-qun Sun, Anhui Province Key Laboratory of Immunology in Chronic Diseases, Bengbu Medical University, 2600 Dong Hai Avenue, Longzihu, Bengbu, Anhui 233030, P.R. China
E-mail: wxr.0924@163.com

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Introduction

Neuroinflammation is an important pathological feature of epilepsy and other brain diseases, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and stroke (1). It involves two key cell types called microglia and astrocytes in the central nervous system (CNS), which can undergo a myriad of molecular and functional reactive changes contingent upon the pathological context, ultimately contributing to the escalation of the disease (2-4). Inhibition of neuroinflammation is a promising therapeutic strategy for inflammation-related brain diseases, and the exploration of action targets is particularly critical.

Ciliary neurotrophic factor (CNTF) is primarily expressed in astrocytes in the CNS and Schwann cells in the peripheral nervous system (5). In both *in vivo* and *in vitro* co-culture, the interaction of normal neurons and astrocytes greatly inhibited the expression of CNTF in astrocytes (6). In addition, evidence from animal models of epilepsy has indicated that after an epileptic seizure, the expression level of CNTF in brain tissue is increased, implying its potential role in pathogenesis (7). Moreover, elevated CNTF levels in the serum and tears are also considered a biomarker of focal epilepsy (8). Nevertheless, the specific pathological effect of CNTF changes in epilepsy remains obscure. It is hypothesized that as a glia-derived neurotrophic factor, CNTF plays a neuroprotective role (9), but it may promote chronic diseases as it belongs to the IL-6 family of cytokines (10). Each IL-6 family member elicits responses essential to the physiological control of immune homeostasis, haematopoiesis, inflammation, development and metabolism. Accordingly, distortion of these cytokine activities often promotes chronic disease, such as inflammatory arthritis, multiple sclerosis, renal injury and scarring (10).

In addition to contributing to epilepsy onset, neuroinflammation can also trigger the tangible release of inflammatory mediators that worsen seizures, creating a vicious cycle (11). Therefore, the role of neuroinflammation in the development of epilepsy has been actively discussed (12,13). Acute inflammation induced by CNTF activates astrocytes and microglia (14). These two neuroglial cells have both pro- and anti-inflammatory effects (15), therefore, the mechanism by which these cells exhibit beneficial effects is a focus for further exploration. It has been shown that persistent overproduction of CNTF by striatal neurons induces the upregulation of the 18kDa transporter protein (TSPO) (a marker of neuroinflammation) in astrocytes (16), which also confirms the critical role of astrocytes in the process of neuroinflammation affected by CNTF.

IL-6, one of the major pro-inflammatory cytokines, is produced within the CNS primarily by activated astrocytes and microglia and has environmentally dependent pro-inflammatory and anti-inflammatory properties, making it now recognized as a crucial target for clinical intervention (17). Elevated IL-6 expression in cerebrospinal fluid and plasma is strongly associated with seizure severity (18). In addition, numerous studies have shown that during the process of epileptogenesis (19,20), IL-6 evaluates and activates different signaling pathways, while also altering the levels of two major neurotransmitters, glutamate and γ-aminobutyric acid (GABA). This results in increased neuronal excitability, as both neurotransmitters are closely related to epileptic seizures (21). Inflammatory communication between neurons and neuroglial cells is closely associated with promoting epileptogenesis and seizure maintenance (22). However, the specific molecular mechanisms involved warrant further investigation.

In the present study, astrocytes and neurons from the cerebral cortex of Sprague-Dawley (SD) rats were isolated and cultured to determine the effects of CNTF-induced inflammatory activation of astrocytes. In addition, IL-6, IL-6Ra and IL-6a were used to explore the effects of activated astrocytes on neuronal activity and excitability to understand the possible mediating mechanism of IL-6 in this process.

Materials and methods

Primary astrocyte culture. Cerebral cortices from SD rats (within 24 h of being born) were finely minced and trypsinized in 0.125% trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.). The cell suspension was prepared with DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (TransGen Biotech Co., Ltd.), 10% calf serum (TransGen Biotech Co., Ltd.), and L-glutamine (2 mmol/l; Beyotime Institute of Biotechnology). The cells were then seeded in poly-L-lysine-coated culture flasks (MilliporeSigma) and incubated at 37˚C in 5% CO_2. The culture medium was changed every 3 days. After confluence at 7-10 days *in vitro*, the cells were cultivated at 37˚C and 250 rpm on a shaking incubator with a rotational radius of 10 cm for 16-18 h to facilitate the separation of oligodendrocytes and microglia. After astrocytes were sub-cultured for 2-4 generations, immunofluorescence staining for GFAP (PeproTech, Inc.) was performed and an astrocyte yield of >95% was achieved (23) (S1). The specific methods for immunofluorescence were as follows: The cell slides were washed with PBS twice, fixed at room temperature with 4% paraformaldehyde for 20 min, and then permeated with 0.1% TritonX-100 at 4˚C. The cells were blocked with 10% rabbit serum (cat. no. C-0006; BIOSS) for 30 min at room temperature and incubated overnight at 4˚C in rabbit anti-rat GFAP antibody (cat. no. 16825-1-AP; 1:200; PeproTech, Inc.). After washing with PBS, the FITC-labeled goat anti-rabbit IgG fluorescent secondary antibody (cat. no. A0562; 1:200; Beyotime Institute of Biotechnology) was added, incubated at room temperature for 2 h. Nuclei were stained with DAPI (cat. no. C1006; Beyotime Institute of Biotechnology) at room temperature for 5 min and washed with PBS three times for 5 min each. The slides were examined under a ZEISS Axio observation microscope (Carl Zeiss AG) and images were captured. All animal studies was approved by The Animal Ethics Committee of Bengbu Medical University (Bengbu, China; approval no. 2020-094).

Primary neuron culture. SD rats were obtained within 24 h of birth and placed on ice and frozen to a state of shock. After which, 75% ethanol was applied to the head and neck for 5 min. The rats were promptly decapitated and immersed in ice-cold PBS buffer containing 2% penicillin-streptomycin. The skulls were sliced open, the brains were removed and transferred to a new culture dish. The cerebral cortex was stripped and immersed in ice-cold HANKS solution containing 2% penicillin-streptomycin. Cerebral cortices from SD rats were harvested, cut into small pieces (~1 mm^3), treated with a 0.125% trypsin-EDTA and dissociated into single cells by gentle suspension. The cell suspension was centrifuged at 4˚C and 200 x g for 3 min and resuspended in DMEM/F12 with penicillin (100 U/ml), streptomycin (100 μg/ml; Beyotime Institute of Biotechnology) and 10% FBS (Hyclone; Cytiva). Suspended primary neurons were seeded in poly-L-lysine-coated culture plate and incubated at 37˚C in 5% CO_2 for 6 h. After which, the medium was replaced with a neurobasal medium supplemented with B27 (Gibco; Thermo Fisher Scientific, Inc.), 2 mM GlutaMAX (Beyotime Institute of Biotechnology), 12.5 μM L-glutamic acid (Beyotime Institute of Biotechnology) and 1% antibiotic-antimycotic (Beyotime Institute of Biotechnology) and incubated at 37˚C in 5% CO_2. After the neurons were seeded in plates for 48 h, cytarabine (5 μmol/l; MedChemExpress) was added to inhibit the proliferation of glial cells for 24 h at 37˚C in 5% CO_2, and then the culture medium was replaced with full volume of neurobasal medium containing B27 incubated at 37˚C in 5% CO_2. After which, the medium was replaced by half volume every 3 days. The cells were cultured until the 7th-10th day, after which immunofluorescence staining for microtubule-associated protein-2 antibody (PeproTech, Inc.) was performed (the identification method is similar to that of astrocytes); the yield of neurons was >95% (24) (Fig. S1).

Bioinformatics analysis. The publicly available gene expression datasets were obtained from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/). The human dataset GSE32534 (epileptic patients, n=5; controls, n=5) was originally reported by Niesen *et al* (25). The mouse dataset GSE157689 (epileptic, n=5; controls, n=4) was

originally reported by Joseph *et al* (26). Differential expression analysis was conducted using the limma package in R (version 4.2.1). This analysis aimed to identify key factors potentially playing significant roles in epileptic conditions. The significance thresholds were set at log fold change (logFC) >|0.5| and adjusted P-value (adj-P) <0.05. The results of the differential expression analysis were visualized using the ggplot2 package in R (version 4.2.1).

ELISA and collection of conditioned culture medium. The cultured astrocytes were randomly divided into the control group (astrocytes were cultured with DMEM/F12 medium containing 10% fetal bovine serum and 10% calf serum) and the CNTF treatment group (50 ng/ml; PeproTech, Inc.) at 37˚C in 5% CO_2 for 12, 24 and 48 h. The levels of IL-6, TNF-α and IL-1β in the culture supernatants of the corresponding groups were detected in accordance with the instructions of the ELISA kits (IL-6, cat. no. EK0412; TNF-α, cat. no. EK0526; IL-1β, cat. no. EK0393; Wuhan Boster Biological Technology, Ltd.). Subsequent to rinsing with PBS thrice, the cells were incubated in fresh, serum-free DMEM for 48 h. Following this, the CNTF-astrocyte culture medium (ACM) was collected, centrifuged at 4˚C and 1,200 x g for 10 min and filtered through a 0.2 μm filter. The untreated ACM served as the control. Finally, the samples were stored at -80˚C until further analyses.

The cultured neurons were randomly divided into the following groups: i) Normal control group (neurobasal medium containing B27); ii) CNTF-treated group (CNTF, 50 ng); iii) CNTF-ACM-treated group (astrocytes were treated with 50 ng/ml CNTF for 48 h, and then replaced with fresh serum-free medium and continued to culture for 48 h to obtain the culture supernatant, and the neuronal cells were cultured with this supernatant); iv) CNTF-ACM combined with IL-6a (BIOSS) treated group (CNTF-ACM + IL-6a, 60 ng/ml) (27); and v) IL-6a-treated group (neurobasal medium containing B27 + IL-6a, 60 ng/ml). All groups were incubated at 37˚C in 5% CO_2 for 48 h, the levels of GABA in the culture supernatants corresponding to each group were detected according to the instructions of the Rat GABA ELISA kit (cat. no. JN352241; Jining Biotech).

Cell counting kit 8 (CCK-8) assay. To test the effect of CNTF-ACM on neuronal activity and the mediating role of IL-6 in this process, neurons were inoculated in 96-well plates at 15,000 cells/well, and neurons were treated according to the same groupings used to detect GABA on the 7th day of culture. The previous culture medium of each well was replaced by the neurobasal medium containing B27 (100 μl) and CCK-8 (Biosharp Life Sciences) solution (10 μl) was added and cultured at 37˚C for 2 h on the 9th day of culture. Subsequently, the optical density at 450 nm was detected by a microplate reader.

Microwell plate methods for glutamate content in cell culture supernatants of each group. The cultured astrocytes were randomly divided into the following groups: i) control group (normal culture medium); ii) IL-6-treated group (IL-6, 30 ng/ml; 24 h); and iii) IL-6-combined with IL-6Ra-treated group (IL-6Ra pre-treatment, 60 ng/ml, 1 h; followed by IL-6, 30 ng/ml, 24 h). The Glutamate Content Assay Kit (cat. no. JN365241; 4˚C) was purchased from Jining Biotech.

The cultured neurons were grouped according to the same groupings used to detect GABA. The culture supernatants of the aforementioned groups were collected into 1.5 ml microfuge tube and centrifuged at 8,000 x g for 10 min at 4˚C. After which, the supernatant samples were aspirated. The enzyme counter was warmed up for 30 min and the wavelength was adjusted to 340 nm. Briefly, 50 μl of the sample, 120 μl reagent I and 20 μl reagent II were added to each well of a 96-well plate and incubated for 2 min, after which the A1 value was read at 340 nm. After which, 10 μl reagent III was added to each well and the 96-well plate was allowed to stand for 20-30 min, and the A2 value was read at 340 nm. The glutamate content was calculated as follows: Glutamate content (μg/ml)=[ΔA ÷ (ε x d) x V2 x Mr x 106] ÷ V1=186.84 x ΔA, ΔA=A2-A1.

Detection of $[Ca^{2+}]i$ and ROS by flow cytometry. Fluo-3 AM (Beyotime Institute of Biotechnology) was diluted in DMEM/F12 medium to a final concentration of 5 μM. Neurons were incubated with diluted Fluo-3 AM for 1 h at 37˚C and then washed twice with PBS. Subsequently, neurons were again incubated with DMEM/f12 solution for 0.5 h at 37˚C and washed with PBS. After which, the cells were digested with 0.25% trypsin without EDTA for 30 s. After digestion was terminated, cells were gently blown until they were suspended in solution. The cell suspension was centrifuged at 200 x g for 5 min at 4˚C, and the supernatant was discarded. The collected cells were washed with PBS and resuspended in 300 μl PBS, followed by flow cytometric analysis (FlowJo V10; BD Biosciences) using the LSRFortessa Cell Analyzer (BD Biosciences). Fluo-3 AM, as a fluorescent probe, can penetrate the cell membrane and enter the cell, where it is cleaved by esterases to form Fluo-3. After binding with Ca^{2+}, its fluorescence intensity significantly increases, making it suitable for flow cytometry to detect changes in intracellular Ca^{2+} concentration.

Neurons were incubated with the 2',7'-dichlorofluorescein diacetate (fluorescent probe of reactive oxygen species; 10 μmol/l; Beyotime Institute of Biotechnology) for 1 h at 37˚C in the dark. The cells were washed twice with PBS and then were incubated with DMEM/f12 solution for 30 min at 37˚C. Subsequently, the cells were washed thrice with PBS and digested with trypsin for 30 s, followed by rewashing and resuspension in PBS. Flow cytometric analysis was carried out using LSRFortessa Cell Analyzer (Cytek Biosciences).

RT-qPCR assay. Total RNA (cultured astrocytes treated with IL-6 in combination with IL-6Ra or alone, and cultured neurons treated with CNTF-ACM in combination with IL-6a or alone, and control groups) was extracted by TRIzol® (Ambion; Thermo Fisher Scientific, Inc.) reagent, and cDNA was synthesized by reverse transcription using the Revertaid First Strand cDNA Synthesis Kit (cat. no. K1622; Thermo Fisher Scientific, Inc.) at 25˚C for 5 min, 42˚C for 1 h, and 70˚C for 5 min.The cDNA chain was amplified by Fast SYBR Green Master Mix kit (TransGen Biotech Co., Ltd.), and quantitative PCR was performed. The composition of the PCR reaction system is as shown: cDNA 2 μl, 2X PerfectStart

Table I. Primers sequences.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
Cx43	CCACTCTCGCCTATGTCTCC	TAGTTCGCCCAGTTTTGCTC
GLT-1	ATTGGTGCAGCCAGTATTCC	CCAAAAGAATCGCCCACTAC
BKCa	CCGTCCACAGCAAATCGGCCA	CCATGTGGGTACTCATGGGCTTGG
Bcl-2	GACTGAGTACCTGAACCGGCATC	CTAGACAGCGTCTTCAGAGACA
BAX	TGTTTGCTGATGGCAACTTC	GATCAGCTCGGGCACTTTAG
GAPDH	GGGTGTGAACCACGAGAAAT	ACTGTGGTCATGAGCCCTTC

Green qPCR SuperMix (including SYBR Green I fluorescent dye, Taq enzyme, dTNP mixture) 10 μl, with 0.4 μl each of the upstream primer (10 μM) and downstream primer (10 μM) and 7.2 μl ddH_2O. The thermocycling program (45 cycles) was set at 94˚C pre-denaturation for 30 s; denaturation at 94˚C for 5 s; annealing at 55-60˚C for 15 s and a final extension at 72˚C for 10 s. $RQ=2^{-\Delta\Delta Cq}$ formula (28) was used to calculate the relative expression of the target gene with GAPDH used as the reference gene. Each experiment was repeated at least thrice and the sequences of each gene primer are shown in Table I.

Western blotting detection of the expression of Cx43, GLT-1, KCa1.1, Bcl-2 and Bax. Cells collected from different groups (cultured astrocytes treated with IL-6 in combination with IL-6Ra or alone, and cultured neurons treated with CNTF-ACM in combination with IL-6a or alone, and control groups) were lysed on ice with RIPA lysate (Biosharp Life Sciences; cat. no. BL504A) to extract total proteins and protein quantification was performed with a BCA Protein Assay Kit (Beyotime Institute of Biotechnology). SDS-PAGE Protein Sampling Buffer (5X) was mixed in a 4:1 ratio, and protein was denatured in a 100˚C incubator for 5 min. Proteins (30 μg/well) were separated by gel electrophoresis (80 V for 0.5 h; and 120 V for 1 h) using 10% (w/v) SDS-PAGE gels, followed by transfer to a PVDF membrane (MilliporeSigma; constant pressure of 100 V for 1 h). The PVDF membranes were then incubated with 5% skim milk for 2 h at room temperature. After which, the following antibodies were added: Rabbit anti-rat antibody KCa1.1 (Abbexa, Ltd.; cat. no. APC-151;1:200), connexin 43 (Cx43; cat. no. 26980-1-AP; 1:4,000; PeproTech, Inc.), glutamate transporter-1 (GLT-1; cat. no. 21829-1-AP; 1:4,000; PeproTech, Inc.), Bax (cat. no. 50599-2-Ig; 1:8,000; PeproTech, Inc.), Bcl-2 (cat. no. 26593-1-AP; 1:1,500; PeproTech, Inc.) and GAPDH (Biosharp Life Sciences; cat. no. BL006B; 1:2,000) and incubated at 4˚C for ~12 h. The samples were washed with TBST three times for 10 min. After which, the HRP-labelled goat anti-rabbit IgG antibody (Biosharp Life Sciences; cat. no. BL003A; 1:15,000) was added and incubated at 25˚C for 2 h. The samples were rinsed with TBST (0.05% Tween-20) three times for 10 min for the final ECL (BeyoECL Plus; Beyotime Institute of Biotechnology; cat. no. P0018S) chemiluminescence reaction. Blotting signaling was detected with ChemiDoc XRS+ gel imaging system (Bio-Rad Laboratories, Inc.).

Statistical Analysis. Experiments were performed in triplicate, data are expressed as means ± SD and were analyzed using SPSS software (v16; SPSS, Inc.). The differences between groups were tested using one-way analysis of variance, followed Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

CNTF induces astrocytes to release pro-inflammatory factors IL-6, TNF-α and IL-1β. ELISA results showed that the levels of IL-6, TNF-α and IL-1β in cell culture supernatants were time-dependently increased after CNTF (50 ng/ml) treatment of astrocytes at different time points (12, 24 and 48 h). A significant increase in IL-6 (Fig. 1A), TNF-α (Fig. 1B) and IL-1β (Fig. 1C) between the 48 h group and the control group was noted (P<0.05; Fig. 1). The results of the differential analysis presented the data, highlighting IL-6 as the most significantly upregulated gene in both the human (GSE32534) and mouse (GSE157689) datasets. (Fig. S2). Therefore, it was decided to focus on IL-6 in the present study.

IL-6 downregulates Cx43 expression and upregulates GLT-1 expression and glutamate release in astrocytes. To clarify the potential mechanism of CNTF-induced astrocyte activation in neuroinflammation, the effects of IL-6 on the expression of Cx43 and GLT-1 in astrocytes were first examined by RT-qPCR and western blotting. The results showed that 30 ng/ml IL-6 significantly decreased the mRNA and protein expression of Cx43 in astrocytes (P<0.05; Fig. 2A, C and D) and significantly increased the mRNA and protein expression of GLT-1 in astrocytes (P<0.01; Fig. 2B-D). After pretreatment with IL-6Ra, the mRNA and protein expression of Cx43 and GLT-1 showed significant reversal (P<0.05; Fig. 2). The effect of IL-6 on glutamate levels in astrocyte culture supernatants was also examined using the microplate assay, and the results showed that IL-6 significantly promoted the release of glutamate from astrocytes (P<0.05; Fig. 2E), whereas IL-6Ra pretreatment significantly down-regulated the level of glutamate release (P<0.05; Fig. 2E).

CNTF-ACM enhances oxidative stress, activates BKCa channels, increases neuronal excitability, decreases neuronal activity and promotes neuronal apoptosis via the IL-6/IL-6R pathway. To illustrate the potential mechanism of CNTF-induced astrocyte activation in neuroinflammation, the cultured neurons were treated with CNTF-ACM, and the results of flow cytometry showed that CNTF-ACM significantly elevated neuronal $[Ca^{2+}]i$ (P<0.01; Fig. 3A and B) and ROS

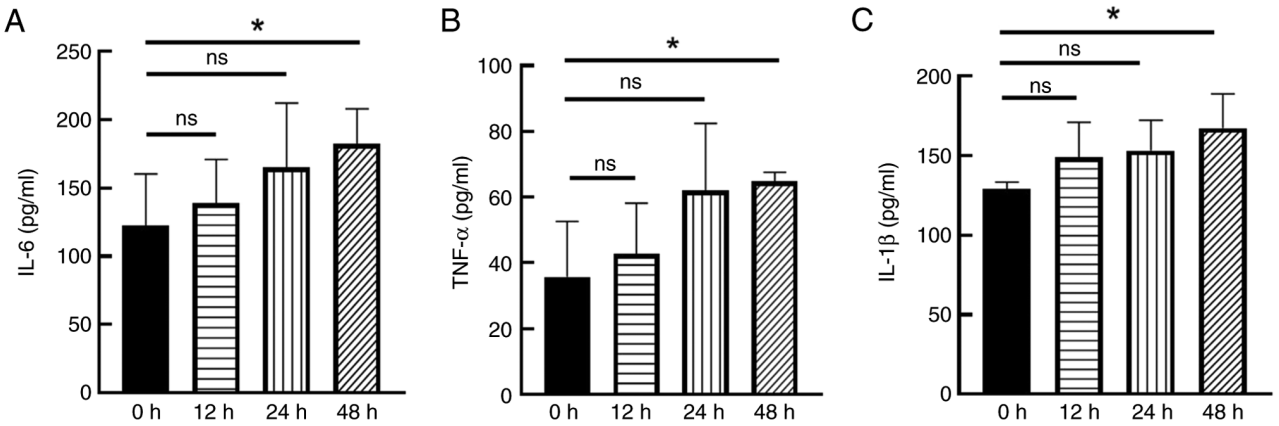

Figure 1. CNTF induces astrocytes to release pro-inflammatory factors. The levels of (A) IL-6, (B) TNF-α and (C) IL-1β in cell culture supernatants were detected by ELISA after CNTF (50 ng/ml) treatment of astrocytes for 12, 24 and 48 h. All data were generated from three independent experiments. *P<0.05. ns, not significant.

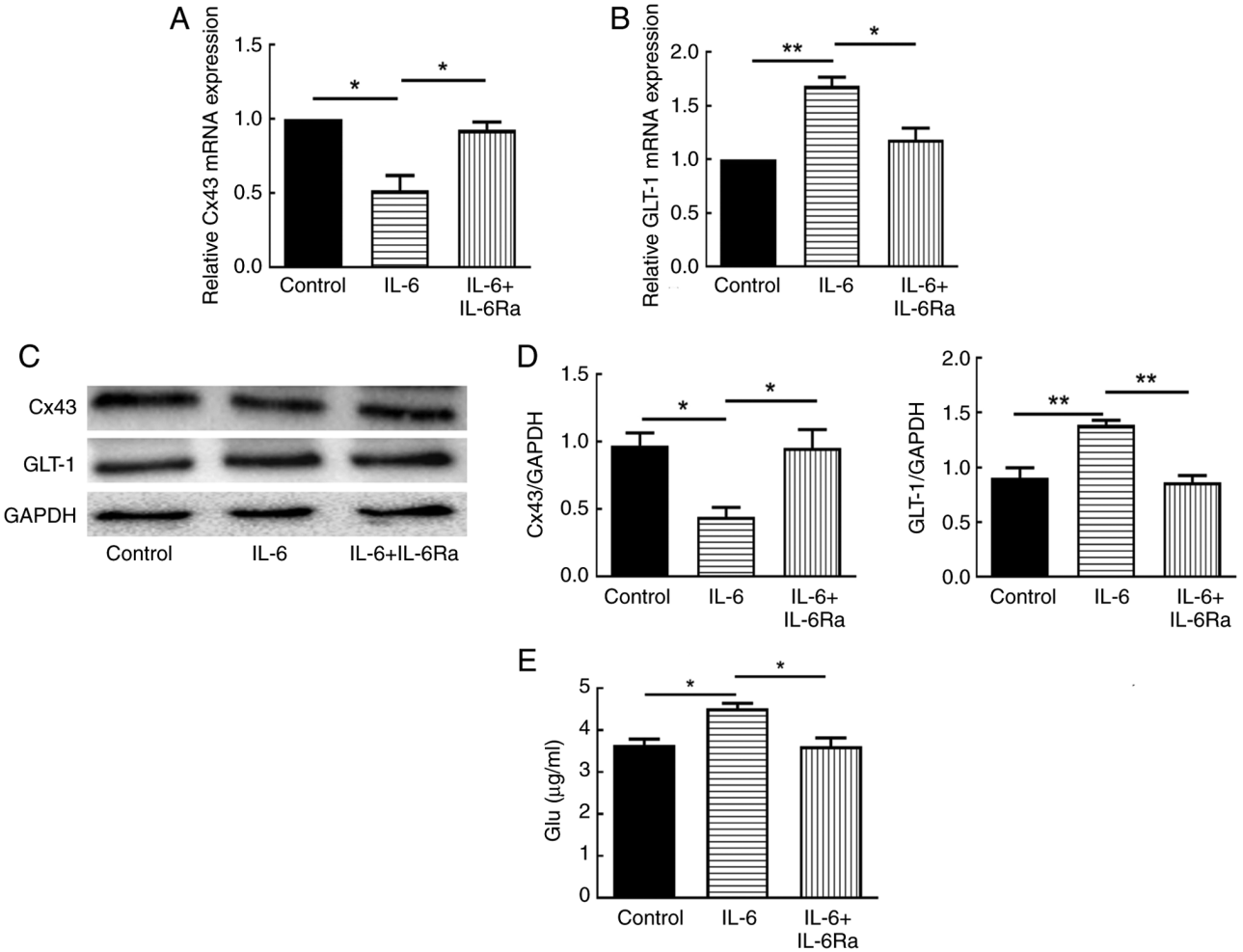

Figure 2. IL-6 downregulates the expression of Cx43, upregulates the expression of GLT-1, promotes glutamate release from astrocytes in astrocytes and IL-6Ra can reverse those outcomes. The effects of IL-6 on the expression of (A) Cx43 and (B) GLT-1 in astrocytes were detected by RT-qPCR and (C) the protein levels were detected by Western blotting. (D) Relative protein expression (normalized to GAPDH) was measured by densitometry. (E) The content of glutamic acid in the cell culture supernatant was determined by the microplate method. *P<0.05; **P<0.01. GLT-1, glutamate transporter-1; Cx43, connexin 43.

levels (P<0.05; Fig. 3C and D). The results of the CCK8 assay showed that CNTF-ACM significantly decreased neuronal viability (P<0.01; Fig. 4A); RT-qPCR and western blotting assays showed that CNTF-ACM significantly upregulated the expression of KCa1.1 (P<0.05; Figs. 4D and 5D), promoted the expression of apoptosis-related factor Bax (P<0.05; Figs. 4C and 5C) and decreased the expression of Bcl-2 (P<0.05; Figs. 4B, 5A and B). The results of the microplate assay

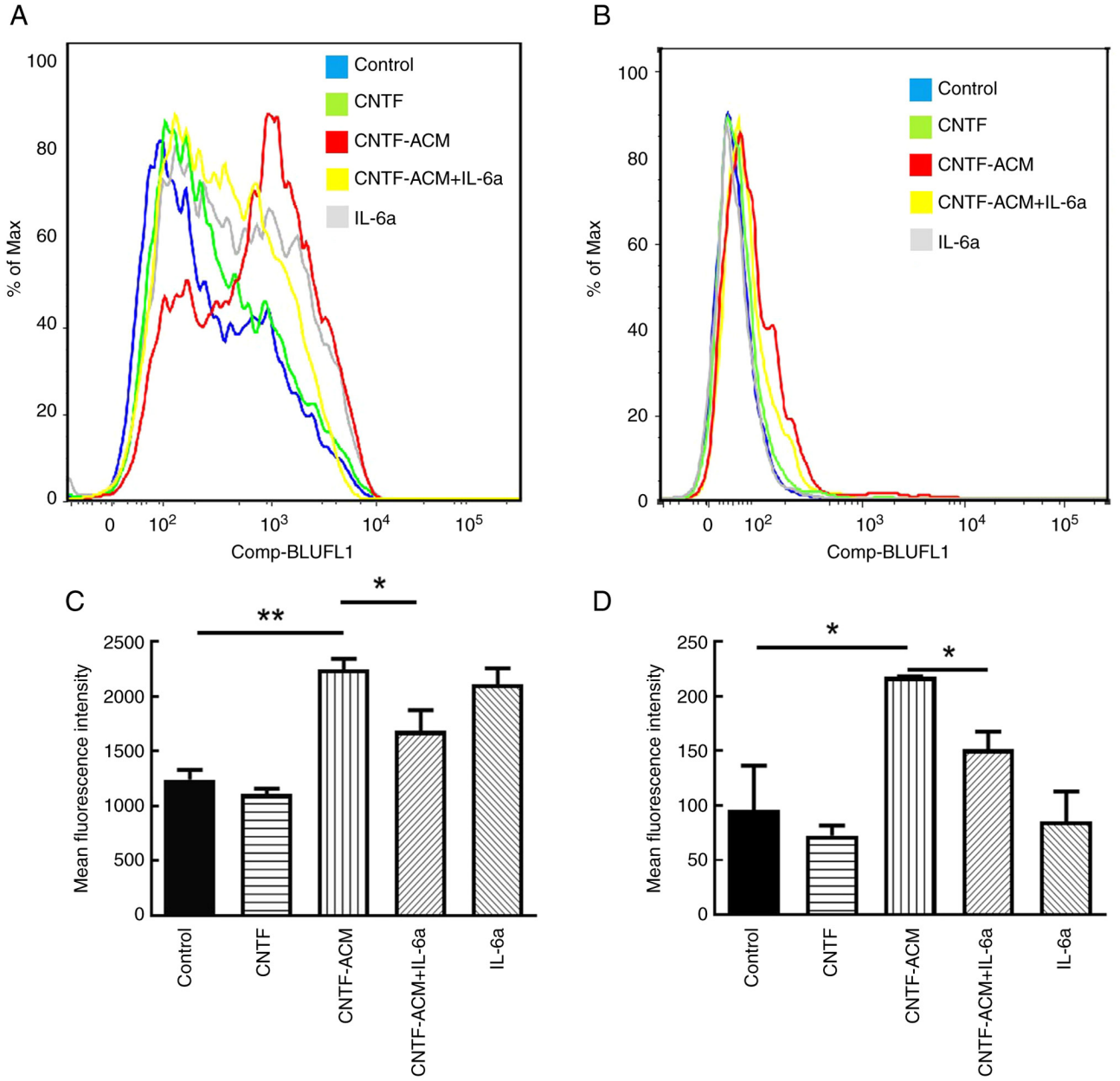

Figure 3. CNTF-ACM promotes the increase of $[Ca^{2+}]i$ and ROS production in neurons. The neurons were labeled with (A) Fluo-3 AM fluorescent probe or (B) DCFH-DA detected by flow cytometry. Analysis of the mean fluorescence intensity of (C) $[Ca^{2+}]i$ and (D) ROS. $^{*}P<0.05$; $^{**}P<0.01$. CNTF, ciliary neurotrophic factor; ACM, astrocyte culture medium.

showed that CNTF-ACM significantly increased the level of glutamate in the supernatants of neuronal cultures (P<0.05; Fig. 6A). The ELISA results showed that CNTF-ACM significantly decreased the level of GABA in the supernatants of neuronal cultures (P<0.05; Fig. 6B). However, pretreatment of CNTF-ACM with IL-6a for 1 h significantly reversed all the aforementioned changes (P<0.05, Figs. 3-6).

Discussion

There is a positive feedback loop between neuroinflammation and epileptogenesis; hence, neuroinflammation is closely related to the pathogenic process associated with seizures, especially in refractory epilepsy (29). The results of the present study showed that CNTF-induced astrocyte-mediated inflammation leads to glutamate excitotoxicity and oxidative stress, followed by neuronal damage, and the IL-6/IL-6R pathway plays an important regulatory role in this process, which reveals its significance as a therapeutic target in epilepsy.

It has been shown that astrocytes are the main cell type in which CNTF affects neuroinflammatory processes (16), while immunoinflammatory dysfunction of neuroglia is a major factor that induces or promotes seizures (30,31). Therefore, to delve into the possible role of CNTF-induced neuroinflammation in the pathogenesis of epilepsy, the pro-inflammatory effects of CNTF on astrocytes were first explored, and the results showed that 50 ng/ml CNTF promoted the release of several pro-inflammatory cytokines, namely IL-6, IL-1β and TNF-α, from astrocytes in a time-dependent manner. The 48 h time groups with significant differences compared with

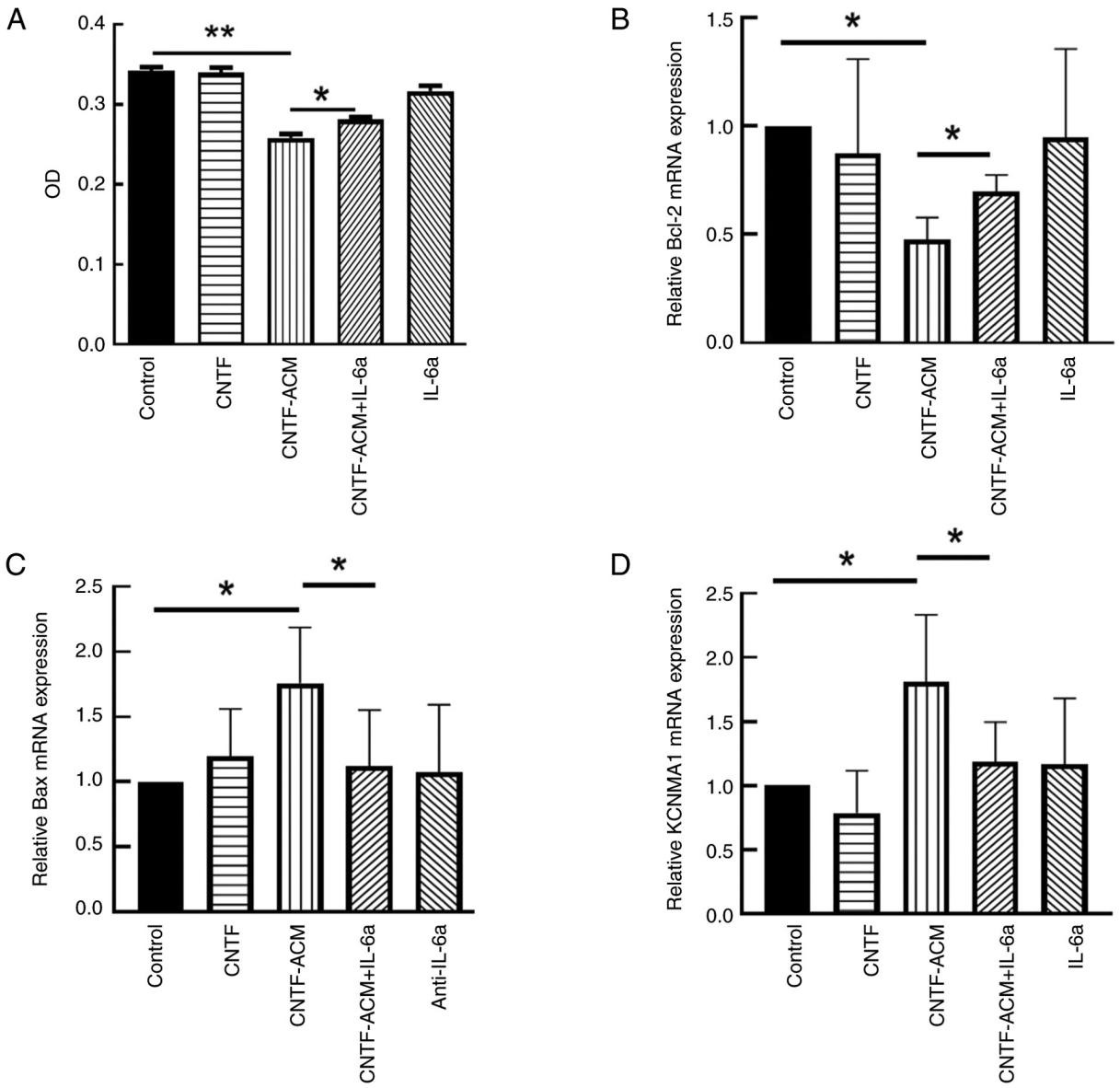

Figure 4. IL-6 in CNTF-ACM inhibits neuronal activity and activates BKCa channels, increases neuronal excitability and promotes neuronal apoptosis. On day 9, each well was replaced with a new medium (100 μl) and CCK-8 solution (10 μl). (A) The OD at 450 nm was measured with a microplate reader instrument. (B) The relative expression of Bcl-2, (C) Bax and (D) KCNMA1 (KCa1.1) detected by RT-qPCR. GAPDH was used as the internal reference. *P<0.05; **P<0.01. CNTF, ciliary neurotrophic factor; ACM, astrocyte culture medium; OD, optical density; BKCa, large conductance calcium-activated potassium channels.

the control group were selected to obtain the CNTF-activated astrocyte conditioned cultures (24,32,33) (*in vitro* model of CNTF-mediated astrocytes activation). Based on the differential expression of the publicly available gene expression datasets, GSE32534 and GSE157689, to identify key factors that may play an important role in epileptic conditions, IL-6 showed the most significant upregulation in both datasets. Therefore, it was decided to focus on IL-6 in the present study.

IL-6 is an important pro-inflammatory cytokine in the IL-6 family and is elevated in a variety of neurological disorders including epilepsy (19,20). In CNS, IL-6 is mainly produced by activated astrocytes and microglia, and excess IL-6 continues to activate astrocytes in an autocrine manner, leading to a vicious cycle (23), which not only contributes to epilepsy onset but also increases the susceptibility (34). The role of astrocyte gap junction dysfunction as well as impaired extracellular potassium ion and glutamate buffering in the pathogenesis of epilepsy has been widely validated (35-37). Astrocytes take up excess extracellular potassium ions via Kir4.1 and then transfer them from high to low concentrations via gap junctions coupled between themselves, termed spatial buffering, which subsequently maintains or influences neuronal excitability (38). The expression of Kir4.1 may be affected by inflammatory environments (39), and recent studies have shown that IL-1β and IL-6 promote astrocyte activation and downregulate Kir4.1 expression (23,40,41). It is also hypothesized that IL-1β and TNF-α may play important roles in the pathological process of epilepsy and deserve further exploration. In future studies, the research scope will be further expanded based on the results of the present study, including an in-depth exploration of the role of these two cytokines.

Excitotoxicity due to extracellular glutamate is a pathogenic mechanism in numerous CNS disorders including

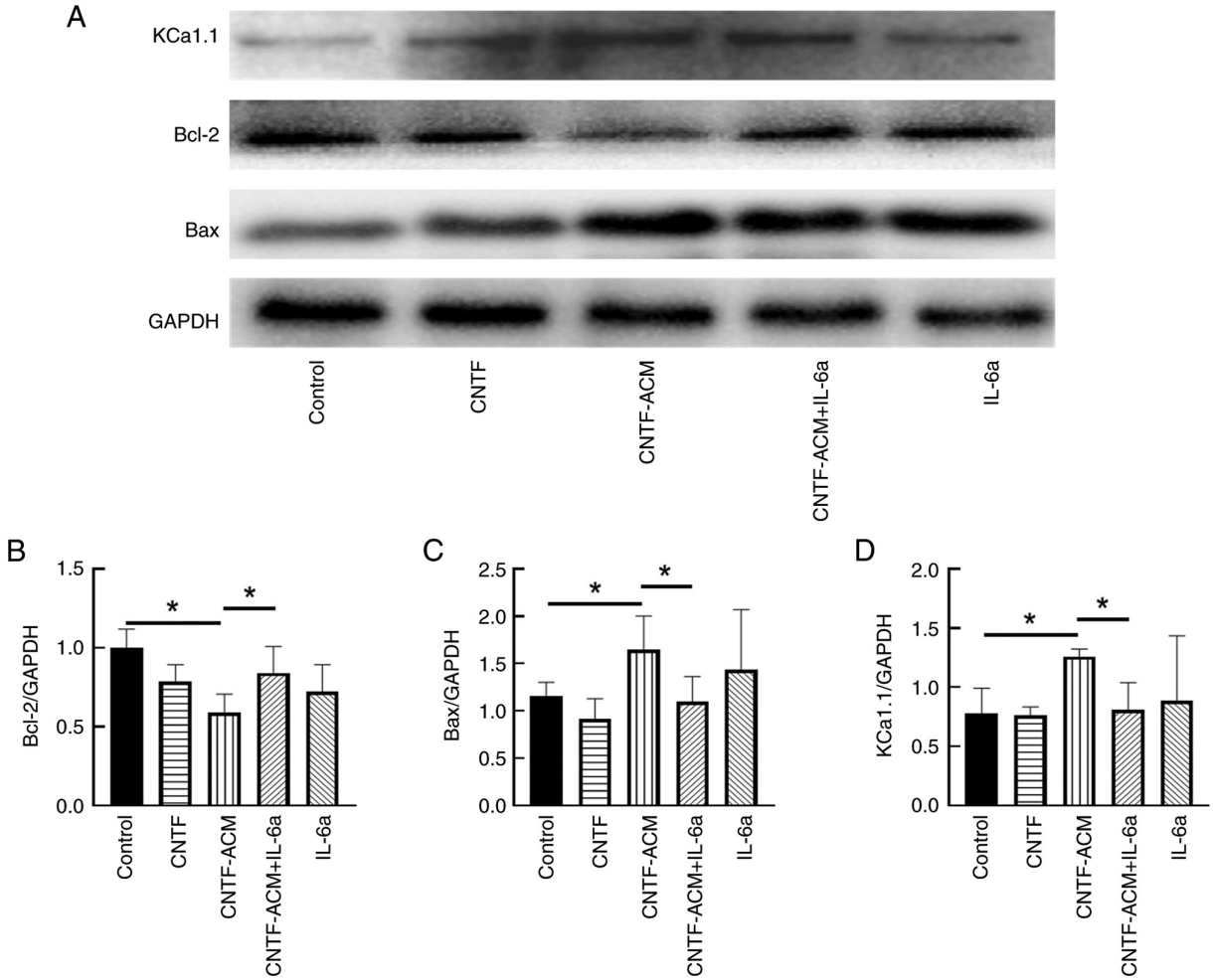

Figure 5. CNTF-ACM mediates neuronal damage through the IL-6/IL-6R pathway. (A) Bcl-2, Bax and KCa1.1 expression were analyzed by immunoblotting with GAPDH as the loading control. (B) Bcl-2, (C) Bax and (D KCa1.1. Relative protein expression (normalized to GAPDH) was measured by densitometry. A total of three independent experiments were performed. Results are expressed as the mean ± standard deviation. *P<0.05 vs. CNTF-ACM group. CNTF, ciliary neurotrophic factor; ACM, astrocyte culture medium.

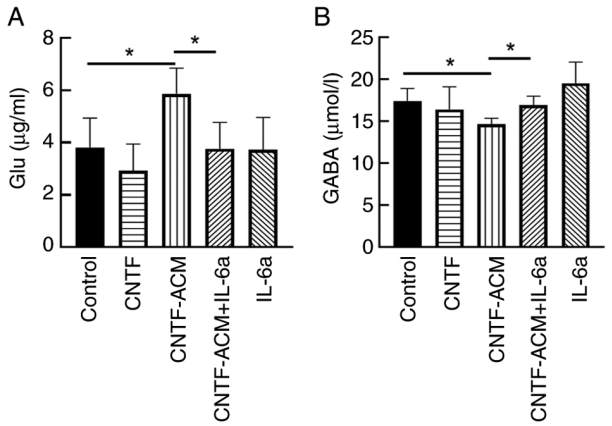

Figure 6. IL-6 in CNTF-ACM increases glutamate release and inhibits GABA level in neurons. Microwell plate method for (A) Glu and (B) GABA content in cell culture supernatants of each group. *P<0.05 vs. CNTF-ACM group. CNTF, ciliary neurotrophic factor; ACM, astrocyte culture medium; Glu, glutamate.

epilepsy (42). Regulation of extracellular glutamate is primarily through clearance by appropriate transporters, and GLT-1, predominantly found in astrocytes, is responsible for ~90% clearance (43). However, dysfunction of GLT-1 in astrocytes is common in both patients with epilepsy (44) and animal models of epilepsy (45,46). In addition to extracellular glutamate uptake, astrocytes could also regulate glutamate homeostasis by releasing glutamate into the synaptic gap, which is involved in the step to excitotoxicity in neurological disorders (47,48). Under these circumstances, impaired uptake and excessive release of glutamate can lead to elevated glutamate concentrations in the synaptic gap, which subsequently brings about neuronal hyperexcitability and excitotoxicity (49,50). To further explore the possible effects of the inflammatory environment on the glutamate buffering function of astrocytes, astrocytes were treated with 30 ng/ml IL-6 for 24 h. The results showed that the expression of GLT-1 and the level of glutamate in the culture supernatant were significantly elevated, suggesting that IL-6 promotes the release of glutamate from astrocytes, which may be a pathway for astrocytes to participate in the modulation of neuronal excitability in the local inflammatory environment. While the majority of previous studies have shown decreased GLT-1 expression in epilepsy models (51,52), the results of the present study found markedly elevated GLT-1 expression in activated

astrocytes in the inflammatory environment, suggesting that the ability of astrocytes to uptake extracellular glutamate may be enhanced. Recently conducted research has reported that in a pentylenetetrazole-induced epilepsy model, the level of extracellular glutamate was noticeably increased, and the expression of GLT-1 was significantly upregulated, but the time for reuptake of glutamate was prolonged (53). This was consistent with the findings of the present study, and may confirm an adaptive mechanism for high levels of glutamate under certain conditions; specifically that high levels of glutamate increase the expression of glutamate transporter proteins, but it is not sufficient to remove excess glutamate.

Astrocytes can redistribute elevated K^+ and neurotransmitters from the sites of increased neuronal activity through a large number of gap junction couplings (54), or they can release gliotransmitters through hemichannels, which affect neuronal excitability (55,56). Cx43 is a prime connectivity protein expressed in astrocytes and acts as a major component in the construction of astrocyte gap junctions and hemichannels (57,58). However, the expression of Cx43 in both excised human epileptic tissues and animal models of epilepsy showed different observations of decreased levels (59,60), increased levels (61) or unchanged levels (62). Epilepsy of different etiologies or refractory epilepsy may have different effects on protein expression in tissue samples (58-62). However, *in vivo* and *in vitro* studies have shown that the pro-inflammatory cytokines, IL-1β and TNFα, released by microglia, inhibit gap junction coupling between astrocytes (59,63,64), but activate hemichannels (65). The results of the present study showed that IL-6 downregulated Cx43 expression, indicating that IL-6 may cause decreased buffering capacity of astrocytes for extracellular K^+ and neurotransmitters through the reduction of gap junction coupling and aberrant opening of hemichannels, which ultimately leads to excitotoxicity of neurons. This hypothesis needs further experimental verification.

Taken together, IL-6 may continuously activate astrocytes through autocrine secretion and subsequently downregulate the expression of Kir4.1 and Cx43 as well as promote glutamate release, which subsequently affects neuronal excitability. It is worth noting in the present study that all the aforementioned processes were reversed by pretreatment with IL-6a. These results suggest that IL-6/IL-6R may be a non-neuronal anti-epileptic target, and provide the experimental basis for clinical treatment with IL-6 receptor blockers.

Activated astrocytes can be involved in the process of epileptic activity and release a variety of neuroactive factors that can act on neurons not only indirectly by autocrine activation of astrocytes but also directly by affecting the structure and function of neurons by paracrine secretion (66,67). A previous study treated primary cultured neurons with CNTF-ACM and showed that fibroblast growth factor-2 contained in CNTF-ACM was involved in the upregulation of $[Ca^{2+}]i$, as well as in the enhanced activity of large conductance calcium-activated potassium channels that affect neuronal excitability (24). Based on previous studies and to further investigate the direct effects of astrocyte-mediated neuroinflammation on neuronal structure and function, cultured neurons in the present study were treated with a combination of CNTF-ACM and IL-6a. The results showed that CNTF-ACM significantly elevated $[Ca^{2+}]i$ and the levels of ROS, decreased neuronal viability and promoted neuronal apoptosis; whereas, IL-6a partially reversed the aforementioned changes, suggesting that IL-6 secreted by CNTF-activated astrocytes is involved in the neuronal damage process previously described.

Abnormalities in intracellular Ca^{2+} homeostasis in neurons may be associated with epilepsy (31). Ca^{2+}, a second messenger in cellular signaling, is predominantly stored in the endoplasmic reticulum and mitochondria in neurons and is involved in virtually all neurophysiological functions including the regulation of membrane excitability and mitochondrial function, synaptic transmission, intracellular and paracellular signaling in neurons, the formation of ROS and apoptosis/necrosis (68,69). Mitochondria are responsible for maintaining intracellular Ca^{2+} homeostasis through Ca^{2+} uptake and release (70), and are also the main site of ROS production (71). However, mitochondrial dysfunction can lead to oxidative stress triggered by excessive ROS production, leading to neuronal death (72). Sustained high levels of $[Ca^{2+}]i$ have been shown to cause mitochondrial calcium overload (73,74) and increased ROS production (75). During epileptogenesis, oxidative stress can induce neuronal death via pro-inflammatory and pro-apoptotic factors released from activated glial cells (30,76). Therefore, mitochondria play a key role in epilepsy-induced cellular damage. In addition, ROS can cause mitochondrial dysfunction by initiating toxic signaling cascades against mitochondria or by damaging mitochondrial DNA (77). In the present study, it was demonstrated that CNTF-ACM significantly elevated the levels of $[Ca^{2+}]i$ and ROS, as well as upregulated the mRNA and protein expression of Bax, downregulated the mRNA and protein expression of Bcl-2 and decreased neuronal viability. It is suggested that neuronal apoptosis and reduced neuronal activity resulting from astrocyte-mediated neuroinflammation may be associated with intracellular calcium overload and oxidative stress.

The large conductance calcium-activated potassium channel is a potassium channel whose activity is dependent on intracellular calcium ions (78). It is not only involved in the maintenance of neuronal resting potential and the onset and development of action potentials, but is also coupled to intracellular calcium ions (78), regulating the synthesis and secretion of a variety of neurotransmitters, such as Glu and GABA, that in turn modulate the excitability of neurons (79). Numerous studies have confirmed that hyperactivity of large conductance calcium-activated potassium channels (BKCa) increases neuronal excitability (80-82), and it has also been demonstrated that epileptic seizures promote BKCa function (83), suggesting that activation of BKCa may be the cause of epilepsy or the result of epileptic development. In addition, loss of function due to mutations in the BKCa gene can cause seizures (84). This suggests that the functional abnormalities of BKCa are closely related to epileptogenesis and have a complex pathologic role. It has been shown that LPS enhances the expression and function of BKCa channels in mesenchymal stem cells (MSCs), and the use of blockers of BKCa channels significantly reduces the level of IL-6 secreted by LPS-stimulated MSCs (85). However, IL-6 at a concentration of 10 ng/ml inhibited BKCa channel activity in smooth muscle cells (86). At present, little is known about the association between neuroinflammation and BKCa activity. The results of the present study showed that CNTF-ACM upregulated

neuronal BKCa expression, promoted neuronal glutamate release and inhibited neuronal GABA release, while IL-6a partially reversed these changes. The results of the present experiment suggest that CNTF-induced astrocyte-derived IL-6 upregulates neuronal BKCa expression and increases neuronal excitability. Although the association between BKCa expression and glutamate release was not investigated in the present experiment, a possible inflammatory pathway in the development of epilepsy was elucidated.

In summary, CNTF-induced astrocyte-mediated neuroinflammation further leads to neuronal intracellular calcium overload through the released pro-inflammatory factor IL-6. Subsequently, the overloaded intracellular calcium ions may promote the overproduction of ROS, leading to oxidative damage in neurons. They may also promote the expression of BKCa and the release of glutamate, leading to excitotoxic damage in neurons.

In conclusion, neuroinflammation is a crucial mechanism in numerous neurological disorders. In the present study, the possible mechanisms by which CNTF induces astrocyte-mediated inflammatory responses that are involved in neuronal damage through autocrine and paracrine modes were explored, and the significance of IL-6 as a target for the treatment of inflammatory evidence-associated neurological diseases was elucidated. It was found that CNTF mediates the neuroinflammatory response by inducing astrocytes to secrete IL-6. The role of CNTF in triggering neuronal immune signaling was demonstrated and evidence that the CNTF-IL-6/IL-6R axis mediated the immune cascade across the AST-Neuron-glutamate/GABA network was shown. These findings revealed an as yet unknown pathway in the inflammation of the nervous system, with implications for the mechanisms behind epileptic seizures. However, the causal relationship between inflammation-induced intracellular calcium overload and ROS overproduction, as well as the direct relationship between BKCa and the release of glutamate need to be further investigated.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HTW wrote the original draft, conceptualized the study, curated the data, and was involved in the formal analysis, investigation and methodology, software visualization and validation. STL curated the data, and performed the formal analysis, investigation and methodology. ZHX curated the data and performed the study methodology. TX curated, analyzed and interpreted the data, and wrote the original draft. WYZ performed the study methodology, obtained the resources, performed study supervision and validation, and reviewed and edited the manuscript. MQS conceptualized the study, acquired the funding, performed the investigation and methodology, obtained the resources, performed study supervision, and reviewed and edited the manuscript. HTW and MQS confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal studies were approved by The Animal Ethics Committee of Bengbu Medical University (Bengbu, China; approval No. 2020-094).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Vezzani A, French J, Bartfai T and Baram TZ: The role of inflammation in epilepsy. Nat Rev Neurol 7: 31-40, 2011.
2. Herrera G, Silvero C MJ, Becerra MC, Lasaga M and Scimonelli T: Modulatory role of α-MSH in hippocampal-dependent memory impairment, synaptic plasticity changes, oxidative stress, and astrocyte reactivity induced by short-term high-fat diet intake. Neuropharmacology 239: 109688, 2023.
3. Zhang T, Liang W, Ou W, Zhang M, Cui S and Zhang S: Daphnetin alleviates neuropathic pain in chronic constrictive injury rats via regulating the NF-κB dependent CXCL1/CXCR2 signaling pathway. Pharm Biol 61: 746-754, 2023.
4. Lovotti M, Mangan MSJ, McManus RM, Shkarina K, Vasconcelos MB and Latz E: Monitoring of inflammasome activation of macrophages and microglia in vitro, part 2: Assessing inflammasome activation. Methods Mol Biol 2713: 431-451, 2024.
5. Richardson PM: Ciliary neurotrophic factor: A review. Pharmacol Ther 63: 187-198, 1994.
6. Kang SS, Keasey MP, Cai J and Hagg T: Loss of neuron-astroglial interaction rapidly induces protective CNTF expression after stroke in mice. J Neurosci 32: 9277-9287, 2012.
7. Moradi P, Ganjkhani M, Anarkooli IJ and Abdanipour A: Neuroprotective effects of lovastatin in the pilocarpine rat model of epilepsy according to the expression of neurotrophic factors. Metab Brain Dis 34: 1061-1069, 2019.
8. Shpak A, Guekht A, Druzhkova T, Rider F, Gudkova A and Gulyaeva N: Increased ciliary neurotrophic factor in blood serum and lacrimal fluid as a potential biomarkers of focal epilepsy. Neurol Sci 43: 493-498, 2022.
9. Bechstein M, Häussler U, Neef M, Hofmann HD, Kirsch M and Haas CA: CNTF-mediated preactivation of astrocytes attenuates neuronal damage and epileptiform activity in experimental epilepsy. Exp Neurol 236: 141-150, 2012.
10. Jones SA and Jenkins BJ: Recent insights into targeting the IL-6 cytokine family in inflammatory diseases and cancer. Nat Rev Immunol 18: 773-789, 2018.
11. Liang W, Wang J, Sui J, Yun F, Shen Y, Zhou J, Wu Y, Shen D and Zhang Q: Inflammation as a target for the treatment of fever-associated epilepsy in zebrafish larvae. Int Immunopharmacol 116: 109802, 2023.

12. Hou X, Xiao S, Xu X, Qin M, Cheng X and Xu X: Glycoprotein non-metastatic melanoma protein B (GPNMB) protects against neuroinflammation and neuronal loss in pilocarpine-induced epilepsy via the regulation of microglial polarization. Neuroscience 551: 166-176, 2024.
13. Ahmad SR, Zeyaullah M, AlShahrani AM, Dawria A, Ali H, Mohieldin A, Altijani AA, Razi U, Mehdi M, Akram S and Hussain ER: Deciphering the enigma of neuron-glial interactions in neurological disorders. Front Biosci (Landmark Ed) 29: 142, 2024.
14. Kahn MA, Ellison JA, Speight GJ and de Vellis J: CNTF regulation of astrogliosis and the activation of microglia in the developing rat central nervous system. Brain Res 685: 55-67, 1995.
15. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, Bennett ML, Münch AE, Chung WS, Peterson TC, *et al*: Neurotoxic reactive astrocytes are induced by activated microglia. Nature 541: 481-487, 2017.
16. Ceyzériat K, Nicolaides A, Amossé Q, Fossey C, Cailly T, Fabis F, Garibotto V, Escartin C, Tournier BB and Millet P: Reactive astrocytes mediate TSPO overexpression in response to sustained CNTF exposure in the rat striatum. Mol Brain 16: 57, 2023.
17. Rohani R, Aliaghaei A, Abdollahifar MA, Sadeghi Y, Zare L, Dehghan S and Heidari MH: Long-Term effects of hippocampal low-frequency stimulation on pro-inflammatory factors and astrocytes activity in kindled rats. Cell J 23: 85-92, 2021.
18. Leo A, Nesci V, Tallarico M, Amodio N, Gallo Cantafio EM, De Sarro G, Constanti A, Russo E and Citraro R: IL-6 Receptor Blockade by Tocilizumab Has Anti-absence and Anti-epileptogenic Effects in the WAG/Rij Rat Model of Absence Epilepsy. Neurotherapeutics 17: 2004-2014, 2020.
19. Uludag IF, Duksal T, Tiftikcioglu BI, Zorlu Y, Ozkaya F and Kirkali G: IL-1β, IL-6 and IL1Ra levels in temporal lobe epilepsy. Seizure 26: 22-25, 2015.
20. Uludag IF, Bilgin S, Zorlu Y, Tuna G and Kirkali G: Interleukin-6, interleukin-1 beta and interleukin-1 receptor antagonist levels in epileptic seizures. Seizure 22: 457-461, 2013.
21. Xiaoqin Z, Zhengli L, Changgeng Z, Xiaojing W and Li L: Changes in behavior and amino acid neurotransmitters in the brain of rats with seizure induced by IL-1beta or IL-6. J Huazhong Univ Sci Technolog Med Sci 25: 236-239, 2005.
22. Vezzani A, Balosso S and Ravizza T: The role of cytokines in the pathophysiology of epilepsy. Brain Behav Immun 22: 797-803, 2008.
23. Lu S, Wang J, Sun T, Yan H, Zou W, Li H, Qi Q and Sun M: IL-6 promotes the activation of rat astrocytes and down-regulation of the expression of Kir4.1 channel. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 38: 316-320, 2022 (In Chinese).
24. Sun M, Liu H, Xu H, Wang H and Wang X: CNTF-Treated astrocyte conditioned medium enhances large-conductance calcium-activated potassium channel activity in rat cortical neurons. Neurochem Res 41: 1982-1992, 2016.
25. Niesen CE, Xu J, Fan X, Li X, Wheeler CJ, Mamelak AN and Wang C: Transcriptomic profiling of human peritumoral neocortex tissues revealed genes possibly involved in tumor-induced epilepsy. PLoS One 8: e56077, 2013.
26. Joseph DJ, Von Deimling M, Hasegawa Y, Cristancho AG, Ahrens-Nicklas RC, Rogers SL, Risbud R, McCoy AJ and Marsh ED: Postnatal Arx transcriptional activity regulates functional properties of PV interneurons. iScience 24: 101999, 2020.
27. Guo Y, Nemeth J, O'Brien C, Susa M, Liu X, Zhang Z, Choy E, Mankin H, Hornicek F and Duan Z: Effects of siltuximab on the IL-6-induced signaling pathway in ovarian cancer. Clin Cancer Res 16: 5759-5769, 2010.
28. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
29. Zabrodskaya Y, Paramonova N, Litovchenko A, Bazhanova E, Gerasimov A, Sitovskaya D, Nezdorovina V, Kravtsova S, Malyshev S, Skiteva E and Samochernykh K: Neuroinflammatory dysfunction of the blood-brain barrier and basement membrane dysplasia play a role in the development of drug-resistant epilepsy. Int J Mol Sci 24: 12689, 2023.
30. Tan TH, Perucca P, O'Brien TJ, Kwan P and Monif M: Inflammation, ictogenesis, and epileptogenesis: An exploration through human disease. Epilepsia 62: 303-324, 2021.
31. Purnell BS, Alves M and Boison D: Astrocyte-neuron circuits in epilepsy. Neurobiol Dis 179: 106058, 2023.
32. Sun M, Liu H, Xu H, Wang H and Wang X: CNTF-ACM promotes mitochondrial respiration and oxidative stress in cortical neurons through upregulating L-type calcium channel activity. Mol Cell Biochem 420: 195-206, 2016.
33. Escartin C, Brouillet E, Gubellini P, Trioulier Y, Jacquard C, Smadja C, Knott GW, Kerkerian-Le Goff L, Déglon N, Hantraye P and Bonvento G: Ciliary neurotrophic factor activates astrocytes, redistributes their glutamate transporters GLAST and GLT-1 to raft microdomains, and improves glutamate handling in vivo. J Neurosci 26: 5978-5989, 2006.
34. Foiadelli T, Santangelo A, Costagliola G, Costa E, Scacciati M, Riva A, Volpedo G, Smaldone M, Bonuccelli A, Clemente AM, *et al*: Neuroinflammation and status epilepticus: A narrative review unraveling a complex interplay. Front Pediatr 11: 1251914, 2023.
35. Andrioli A, Fabene PF, Mudò G, Barresi V, Di Liberto V, Frinchi M, Bentivoglio M and Condorelli DF: Downregulation of the astroglial connexin expression and neurodegeneration after pilocarpine-induced status epilepticus. Int J Mol Sci 24: 23, 2022.
36. Çarçak N, Onat F and Sitnikova E: Astrocytes as a target for therapeutic strategies in epilepsy: current insights. Front Mol Neurosci 16: 1183775, 2023.
37. Hotz AL, Jamali A, Rieser NN, Niklaus S, Aydin E, Myren-Svelstad S, Lalla L, Jurisch-Yaksi N, Yaksi E and Neuhauss S: Loss of glutamate transporter eaat2a leads to aberrant neuronal excitability, recurrent epileptic seizures, and basal hypoactivity. Glia 70: 196-214, 2022.
38. Bellot-Saez A, Kékesi O, Morley JW and Buskila Y: Astrocytic modulation of neuronal excitability through K(+) spatial buffering. Neurosci Biobehav Rev 77: 87-97, 2017.
39. Zurolo E, de Groot M, Iyer A, Anink J, van Vliet EA, Heimans JJ, Reijneveld JC, Gorter JA and Aronica E: Regulation of Kir4.1 expression in astrocytes and astrocytic tumors: A role for interleukin-1 β. J Neuroinflammation 9: 280, 2012.
40. Sun M, Wang H, Qi Q, Yan H, Zou W, Dong X, Wang Z, Wang J and Wang X: IL-1β promotes the proliferation of astrocytes and downregulates the expression of Kir4.1. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 33: 446-449, 2017 (In Chinese).
41. Sun M, Yan H, Zou W, Wang Y, Li H and Wang X: Lipopolysaccharide induces astrocyte activation and downregulates the expression of Kir4.1 channel. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 32: 196-200, 2016 (In Chinese).
42. Ma L, Wu Q, You Y, Zhang P, Tan D, Liang M, Huang Y, Gao Y, Ban Y, Chen Y and Yuan J: Neuronal small extracellular vesicles carrying miR-181c-5p contribute to the pathogenesis of epilepsy by regulating the protein kinase C-δ/glutamate transporter-1 axis in astrocytes. Glia 72: 1082-1095, 2024.
43. Hazell AS, Rao KV, Danbolt NC, Pow DV and Butterworth RF: Selective down-regulation of the astrocyte glutamate transporters GLT-1 and GLAST within the medial thalamus in experimental Wernicke's encephalopathy. J Neurochem 78: 560-568, 2001.
44. Rakhade SN and Loeb JA: Focal reduction of neuronal glutamate transporters in human neocortical epilepsy. Epilepsia 49: 226-236, 2008.
45. Peterson AR, Garcia TA, Cullion K, Tiwari-Woodruff SK, Pedapati EV and Binder DK: Targeted overexpression of glutamate transporter-1 reduces seizures and attenuates pathological changes in a mouse model of epilepsy. Neurobiol Dis 157: 105443, 2021.
46. Muñoz-Ballester C, Berthier A, Viana R and Sanz P: Homeostasis of the astrocytic glutamate transporter GLT-1 is altered in mouse models of Lafora disease. Biochim Biophys Acta 1862: 1074-1083, 2016.
47. Mahmoud S, Gharagozloo M, Simard C and Gris D: Astrocytes maintain glutamate homeostasis in the CNS by controlling the balance between glutamate uptake and release. Cells 8: 184, 2019.
48. Yang J, Vitery M, Chen J, Osei-Owusu J, Chu J and Qiu Z: Glutamate-Releasing SWELL1 channel in astrocytes modulates synaptic transmission and promotes brain damage in stroke. Neuron 102: 813-827.e6, 2019.
49. Cao W, Xiong S, Ji W, Wei H, Ma F and Mao L: Neuroprotection Role of Vitamin C by Upregulating Glutamate Transporter-1 in Auditory Cortex of Noise-Induced Tinnitus Animal Model. ACS Chem Neurosci 15: 1197-1205, 2024.
50. Skórkowska A, Krzyżanowska W, Bystrowska B, Torregrossa R, Whiteman M, Pomierny B and Budziszewska B: The Hydrogen Sulfide Donor AP39 reduces glutamate-mediated excitotoxicity in a rat model of brain ischemia. Neuroscience 539: 86-102, 2024.

51. Hameed MQ, Hui B, Lin R, MacMullin PC, Pascual-Leone A, Vermudez SAD and Rotenberg A: Depressed glutamate transporter 1 expression in a mouse model of Dravet syndrome. Ann Clin Transl Neurol 10: 1695-1699, 2023.
52. Mohamed AM, Ali DA, Kolieb E and Abdelaziz EZ: Ceftriaxone and selenium mitigate seizures and neuronal injury in pentylenetetrazole-kindled rats: Oxidative stress and inflammatory pathway. Int Immunopharmacol 120: 110304, 2023.
53. Taspinar N, Hacimuftuoglu A, Butuner S, Togar B, Arslan G, Taghizadehghalehjoughi A, Okkay U, Agar E, Stephens R Jr, Turkez H and Abd El-Aty AM: Differential effects of inhibitors of PTZ-induced kindling on glutamate transporters and enzyme expression. Clin Exp Pharmacol Physiol 48: 1662-1673, 2021.
54. Wallraff A, Köhling R, Heinemann U, Theis M, Willecke K and Steinhäuser C: The impact of astrocytic gap junctional coupling on potassium buffering in the hippocampus. J Neurosci 26: 5438-5447, 2006.
55. Moraga-Amaro R, Jerez-Baraona JM, Simon F and Stehberg J: Role of astrocytes in memory and psychiatric disorders. J Physiol Paris 108: 240-251, 2014.
56. Orellana JA and Stehberg J: Hemichannels: New roles in astroglial function. Front Physiol 5: 193, 2014.
57. Bruzzone S, Guida L, Zocchi E, Franco L and De Flora A: Connexin 43 hemi channels mediate Ca2+-regulated transmembrane NAD+ fluxes in intact cells. FASEB J 15: 10-12, 2001.
58. De Bock M, Wang N, Decrock E, Bultynck G and Leybaert L: Intracellular Cleavage of the Cx43 C-Terminal Domain by Matrix-Metalloproteases: A novel contributor to inflammation. Mediators Inflamm 2015: 257471, 2015.
59. Bedner P and Steinhäuser C: Role of impaired astrocyte gap junction coupling in epileptogenesis. Cells 12: 1669, 2023.
60. Vizuete AFK, Leal MB, Moreira AP, Seady M, Taday J and Gonçalves CA: Arundic acid (ONO-2506) downregulates neuroinflammation and astrocyte dysfunction after status epilepticus in young rats induced by Li-pilocarpine. Prog Neuropsychopharmacol Biol Psychiatry 123: 110704, 2023.
61. Liu B, Ran X, Yi Y, Zhang X, Chen H and Hu Y: Anticonvulsant effect of carbenoxolone on chronic epileptic rats and its mechanism related to connexin and high-frequency oscillations. Front Mol Neurosci 15: 870947, 2022.
62. Elisevich K, Rempel SA, Smith BJ and Edvardsen K: Hippocampal connexin 43 expression in human complex partial seizure disorder. Exp Neurol 145: 154-164, 1997.
63. Bedner P, Dupper A, Hüttmann K, Müller J, Herde MK, Dublin P, Deshpande T, Schramm J, Häussler U, Haas CA, *et al*: Astrocyte uncoupling as a cause of human temporal lobe epilepsy. Brain 138(Pt 5): 1208-1222, 2015.
64. Haghikia A, Ladage K, Hinkerohe D, Vollmar P, Heupel K, Dermietzel R and Faustmann PM: Implications of antiinflammatory properties of the anticonvulsant drug levetiracetam in astrocytes. J Neurosci Res 86: 1781-1788, 2008.
65. Retamal MA, Froger N, Palacios-Prado N, Ezan P, Sáez PJ, Sáez JC and Giaume C: Cx43 hemichannels and gap junction channels in astrocytes are regulated oppositely by proinflammatory cytokines released from activated microglia. J Neurosci 27: 13781-13792, 2007.
66. Sano F, Shigetomi E, Shinozaki Y, Tsuzukiyama H, Saito K, Mikoshiba K, Horiuchi H, Cheung DL, Nabekura J, Sugita K, *et al*: Reactive astrocyte-driven epileptogenesis is induced by microglia initially activated following status epilepticus. JCI Insight 6: e135391, 2021.
67. Li T, Lan JQ and Boison D: Uncoupling of astrogliosis from epileptogenesis in adenosine kinase (ADK) transgenic mice. Neuron Glia Biol 4: 91-99, 2008.
68. Griffioen G: Calcium dyshomeostasis drives pathophysiology and neuronal demise in age-related neurodegenerative diseases. Int J Mol Sci 24: 13243, 2023.
69. Gola L, Bierhansl L, Csatári J, Schroeter CB, Korn L, Narayanan V, Cerina M, Abdolahi S, Speicher A, Hermann AM, *et al*: NOX4-derived ROS are neuroprotective by balancing intracellular calcium stores. Cell Mol Life Sci 80: 127, 2023.
70. Cai Q and Jeong YY: Mitophagy in Alzheimer's disease and other age-related neurodegenerative diseases. Cells 9: 150, 2020.
71. Petrosillo G, Ruggiero FM and Paradies G: Role of reactive oxygen species and cardiolipin in the release of cytochrome c from mitochondria. FASEB J 17: 2202-2208, 2003.
72. Esteras N, Kopach O, Maiolino M, Lariccia V, Amoroso S, Qamar S, Wray S, Rusakov DA, Jaganjac M and Abramov AY: Mitochondrial ROS control neuronal excitability and cell fate in frontotemporal dementia. Alzheimers Dement 18: 318-338, 2022.
73. Jung S, Chung Y, Lee Y, Lee Y, Cho JW, Shin EJ, Kim HC and Oh YJ: Buffering of cytosolic calcium plays a neuroprotective role by preserving the autophagy-lysosome pathway during MPP(+)-induced neuronal death. Cell Death Discov 5: 130, 2019.
74. Park J, Jang KM and Park KK: Effects of Apamin on MPP(+)-Induced Calcium Overload and Neurotoxicity by Targeting CaMKII/ERK/p65/STAT3 signaling pathways in dopaminergic neuronal cells. Int J Mol Sci 23: 15255, 2022.
75. Stutzmann GE: The pathogenesis of Alzheimers disease is it a lifelong 'calciumopathy'. Neuroscientist 13: 546-559, 2007.
76. Hwang Y, Kim HC and Shin EJ: Enhanced neurogenesis is involved in neuroprotection provided by rottlerin against trimethyltin-induced delayed apoptotic neuronal damage. Life Sci 262: 118494, 2020.
77. Schulien AJ, Justice JA, Di Maio R, Wills ZP, Shah NH and Aizenman E: Zn(2+)-induced Ca(2+) release via ryanodine receptors triggers calcineurin-dependent redistribution of cortical neuronal Kv2.1 K(+) channels. J Physiol 594: 2647-2659, 2016.
78. Shah KR, Guan X and Yan J: Structural and functional coupling of calcium-activated BK channels and calcium-permeable channels within nanodomain signaling complexes. Front Physiol 12: 796540, 2022.
79. Griguoli M, Sgritta M and Cherubini E: Presynaptic BK channels control transmitter release: Physiological relevance and potential therapeutic implications. J Physiol 594: 3489-3500, 2016.
80. Sun AX, Yuan Q, Fukuda M, Yu W, Yan H, Lim G, Nai MH, D'Agostino GA, Tran HD, Itahana Y, *et al*: Potassium channel dysfunction in human neuronal models of Angelman syndrome. Science 366: 1486-1492, 2019.
81. Du W, Bautista JF, Yang H, Diez-Sampedro A, You SA, Wang L, Kotagal P, Lüders HO, Shi J, Cui J, *et al*: Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. Nat Genet 37: 733-738, 2005.
82. Brenner R, Chen QH, Vilaythong A, Toney GM, Noebels JL and Aldrich RW: BK channel beta4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. Nat Neurosci 8: 1752-1759, 2005.
83. Shruti S, Clem RL and Barth AL: A seizure-induced gain-of-function in BK channels is associated with elevated firing activity in neocortical pyramidal neurons. Neurobiol Dis 30: 323-330, 2008.
84. Benton MD, Lewis AH, Bant JS and Raman IM: Iberiotoxin-sensitive and -insensitive BK currents in Purkinje neuron somata. J Neurophysiol 109: 2528-2541, 2013.
85. Song A, Wang J, Tong Y, Fang J, Zhang Y, Zhang H, Ruan H, Wang K and Liu Y: BKCa channels regulate the immunomodulatory properties of WJ-MSCs by affecting the exosome protein profiles during the inflammatory response. Stem Cell Res Ther 11: 440, 2020.
86. Zhang X, Wang L, Xu L and Zou L: Effects of Atractylodes macrocephala on the cytomembrane Ca2+-activated K+ currents in cells of human pregnant myometrial smooth muscles. J Huazhong Univ Sci Technolog Med Sci 28: 200-203, 2008.