doi: 10.4149/gpb_2022041

The ubiquitin-editing enzyme TNFAIP3 exerts neuroprotective roles in epilepsy rats through repressing inflammation

Zhihong Zhuo[1], Huimin Kong[1], Peina Jin[1] and Youhong Ren[2]

[1] *Department of Pediatrics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan Province, China*
[2] *Department of Rehabilitation, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan Province, China*

Abstract. The ubiquitin-editing enzyme TNF alpha-induced protein 3 (TNFAIP3) emerges protective roles in neurological disorder, such as cerebral trauma. However, the molecular mechanisms of TNFAIP3 in epilepsy are not very clear. Hereon, the epileptic mouse models and BV2 microglial cellular models were established by kainic acid (KA) and lipopolysaccharide (LPS) respectively. We found that TNFAIP3 was highly expressed in the hippocampus of epileptic mice. Besides, TNFAIP3 overexpression relieved the spatial learning and memory, reduced the hot plate latency, as well as inhibited neuronal apoptosis in KA-treated mice. *In vivo* and *in vitro* experiments indicated that inflammation, a key characteristic of epilepsy, was inhibited by TNFAIP3 upregulation, as evidenced by the downregulated expression of pro-inflammatory cytokine interleukin (IL)-1β and inducible NO synthase (iNOS), along with the decreased levels of NLRP3 inflammasome, which could activate inflammation. Collectively, we infer that TNFAIP3 relieves neuronal injury in epilepsy by suppressing inflammation.

Key words: Epilepsy — TNFAIP3 — NLRP3 inflammasome — Kainic acid

Introduction

Epilepsy is a chronic neurological disease that affects individuals of all ages and has a worldwide distribution (Neligan et al. 2012). Approximately 65 million people worldwide suffer from epilepsy, and 30% of newly diagnosed epilepsy patients will respond to antiepileptic drugs (Thurman et al. 2011; Keller et al. 2017). Epileptogenesis is affected by various factors, such as heredity and acquired factors (Goldberg and Coulter 2013; Pitkänen et al. 2015). Epilepsy in early childhood is often hard to treat compared with adults (Spencer and Huh 2008), which may due to physiologic immaturities in ion homeostasis and other developmental characteristics (Sheizaf et al. 2007; Usta et al. 2007). Furthermore, epilepsy can be caused by a variety of underlying disease mechanisms, but in nearly half of all cases worldwide, the cause of the seizures is still unclear (Neligan et al. 2012). Therefore, there is an urgent need for researchers to develop appropriate strategies to treat epilepsy.

TNF alpha-induced protein 3 (TNFAIP3), also known as A20, is a ubiquitin-editing enzyme, which negatively regulates nuclear factor-kappa B (NF-κB) by multiple mechanisms (Shembade and Harhaj 2010; Catrysse et al. 2014). It has been reported that the abnormal expression and/or function of TNFAIP3 is related to chronic inflammation and tissue damage, which can contribute to the immunopathology of a variety of human autoimmune and inflammatory diseases. For example, TNFAIP3 is slightly upregulated in the gingival specimens of patients with chronic periodontitis (Crump et al. 2017). In active multiple sclerosis plaques, TNFAIP3 is highly expressed, particularly in macrophages and resident astrocytes (Perga et al. 2021). It is reported that chronic lung damage suppresses TNFAIP3 activity in alveolar macrophages, which promotes the development of pulmonary fibrosis by upregulating many immunosuppres-

Correspondence to: Zhihong Zhuo, Department of Pediatrics, The First Affiliated Hospital of Zhengzhou University, 1 Jianshe East Road, Zhengzhou, Henan Province, China
E-mail: zhuozhihong@126.com

sive and profibrotic proteins (Liu et al. 2019). Furthermore, the inhibition of TNFAIP3 can reduce the growth and tumorigenic potentiality of glioma stem cells (Hjelmeland et al. 2010). Accumulating evidence indicates that the expression of TNFAIP3 needs to be regulated and the aberrant TNFAIP3 levels are associated with numerous conditions, which may lead to different disease outcomes.

Neuroinflammation is present in most pathological conditions of the central nervous system (CNS), either as the main driver of these conditions or as a response to neurodegeneration or homeostasis after disease progression (Corps et al. 2015; Waisman et al. 2015). The dysregulated neuroinflammatory signals can contribute to epilepsy. It is well known that the primary neuropathological feature in human and most animal epilepsy models is neuronal dysfunction (Farrell et al. 2017). In some epilepsy models, neuronal injury occurs within a few hours after status epilepticus and causes changes in the function and structure of the neuronal network (Pitkänen et al. 2002). TNFAIP3 expression is upregulated in the brain tissue of the hereditary epilepsy model, and *Paeonia lactiflora* extract can alleviate neuronal injury (Sunaga et al. 2004). Moreover, TNFAIP3 is revealed to inhibit the activity of inflammasome nucleotide-binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) (Duong et al. 2015), which can alleviate the symptoms of epilepsy in kainic acid (KA)-induced epileptic rats (Mohseni-Moghaddam et al. 2019). Therefore, according to these studies, we proposed that TNFAIP3 might play a protective role in the process of epilepsy. In the present study, we explored the possible role of TNFAIP3 in epilepsy by performing neuropathological investigations, such as behavioral analysis, neuronal apoptosis and inflammation.

Materials and Methods

Animals

C57BL/6 mice aged four weeks were housed in a 12 h/12 h light-dark cycle with free access to food and water at a temperature of 22 ± 1°C and relative humidity of 45–55%. All the animal experiments were approved by the Institutional Animal Care and the animal ethics committee of the First Affiliated Hospital of Zhengzhou University.

Cell culture and lentiviral infection

The microglial BV2 cells were ordered from iCell Bioscience (China) and cultured in a Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS). BV2 cells were cultured in an incubator at 37°C and 5% CO_2.

Lentivirus overexpressing TNFAIP3 (Lv-TNFAIP3) or negative control (Lv-NC) were used to infect BV2 cells. After 48-h infection, cells were treated with 1 μg/ml lipopolysaccharide (LPS) for 24 h.

Intrahippocampal injection of lentivirus (Lv)

Lv-TNFAIP3 or Lv-NC (2 μl) was infused at a speed of 0.2 μl/min bilaterally in the hippocampus region (anterior/posterior: −2.0 mm, medial/lateral: ±1.5 mm, and dorsal/ventral: −1.5 mm). To minimize back flux, the pipette stayed in place for at least 5 min after injection. The mice were allowed to recover for 2 weeks after the injection of Lv. Guidelines for the care and use of laboratory animals were followed for all mouse experiments.

Animal model in epilepsy

The mice were injected with 25 mg/kg KA (Abcam, China) *intraperitoneally* (*i.p.*) 2 weeks after injecting Lv *intrahippocampally*. After that, the mice were observed for 2 h and seizure intensity was scored as follows: stage 0, normal behavior; stage 1, facial twitches including nose, lips, and eyes; stage 2, chewing and head nodding; stage 3, forelimb clonus; stage 4, rearing and falling on forelimbs; stage 5, imbalance and falling on the side or back. Mice with fully induced seizures (stages 4 and 5) were used to determine the number of seizures and duration of the first seizure. After 2 h, the mice were *i.p.* injected with diazepam (10 mg/kg) to suppress epilepsy. Three days after modeling, mice in each group were sacrificed, and the hippocampal tissues were taken for subsequent experiments.

Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted using TRIpure lysate (Bioteke, China) and then reverse-transcribed to cDNA using BeyoRT II M-MLV reverse-transcriptase (Beyotime, China). SYBR Green (Solarbio, China) was used to conduct the qRT-PCR, which used GAPDH as a reference control. The data obtained from the system was calculated according to the $2^{-\triangle\triangle Ct}$ method. The sequences of primers for qRT-PCR were as follows (5'-3'): TNFAIP3-F, GCTCCAGCCTCACTTCC; TNFAIP3-R, TGCACTTGCTTGTCCCT; IL-1β-F, CTCAACTGTGAAATGCCACC; IL-1β-R, GAGTGATACTGCCTGCCTGA; iNOS-F, CACCACCCTCCTCGTTC; iNOS-R, CAATCCACAACTCGCTCC.

Protein extraction and Western blot

RIPA lysate (Solarbio, China) was used for total protein extraction. The protein concentration was measured by a BCA Protein Assay Kit (Solarbio, China). The protein

samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore, USA). The membranes were blocked in 5% milk in Tris-buffered saline with Tween-20, and then incubated with primary antibody against TNFAIP3 (Affinity, China), NLRP3 (Abclonal, China), ASC (Abclonal, China), caspase 1 P20 (Affinity, China), and GAPDH (Proteintech, China) at 4°C overnight and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Solarbio, China) at the room temperature for 1 h. The band signals were visualized and quantified by a gel imaging system (Beijing Liuyi Biotechnology, China).

Hot plate test

In this test, the front paws of mice were placed on a heated plate, which was maintained at 55°C. When the mice were licked or jumped after touching the hot plate, the reaction time was recorded as hot plate latency. To prevent tissue damage, mice that did not respond to pain within 60 s were removed from the hot plate.

Morris water maze

Morris water maze test was performed by video tracking to assess the spatial learning and memory in mice. An opaque morris water maze with a diameter of 120 cm and a height of 50 cm was filled with water, which was controlled at 21–23°C. The maze is divided into four quadrants, northeast (I), northwest (II), southwest (III), and southeast (IV), and the escape platform with a diameter of 10 cm and a height of 30 cm was immersed 1 cm below the surface of the water at the quadrant I. In all experiments, the mice were released from the quadrant III of the maze, and mice were allowed to remain for 60 s. Then the time for the mice to find the hidden platform, percentage of time spent in the target quadrant, and the number of times crossing the platform were recorded by video tracking system. Within 60 s, mice that could not find a target platform were gently guided to the platform. Each mouse was trained twice a day at a regular time for 5 days.

Immunofluorescence staining

Paraffin-embedded hippocampus sections (5 μm) were incubated at 60°C in an oven for 4 h, followed by dewaxing and dehydration. After blocking by 1% BSA, the sections were incubated with TNFAIP3 primary antibody (Affinity, China) at 4°C overnight. The proteins were visualized by incubating with the secondary antibody conjugated to Cy3-labelled goat anti-rabbit IgG (Invitrogen, USA) for 1 h at room temperature, and the nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, Aladdin, China) for another 10 min. After rinsing the sections in PBS, the anti-fluorescence mounting media (Solarbio, China) was applied, and the cells were examined under a microscope (Olympus, Japan). For immunofluorescence double staining, except for TNFAIP3 primary antibody, the sections were also incubated with the Iba1 primary antibody (Santa Cruz, USA) and followed by fluorescein isothiocyanate-labelled goat anti-mouse IgG (Abcam, UK).

Fluoro-Jade B (FJB) staining

Specific labeling of degenerating neurons and cell nuclei was performed in the hippocampus by a FJB staining kit (Merckmillipore, USA). The embedded sections were dewaxed to water and immersed in 80% ethanol solution containing 1% NaOH for 5 min and transferred into 70% ethanol for 2 min. Afterward, the sections were placed in 0.06% potassium permanganate solution for 10 min and followed by staining with FJB staining at room temperature for 20 min. Fluorescent images were exhibited using a microscope (Olympus, Japan).

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-end labeling (TUNEL)/NeuN staining

In Situ Cell Death Detection Kit (Roche, Switzerland) was used to detect neuronal apoptosis. After dewaxing and rehydration, the sections were incubated in 0.1% Triton X-100 for 8 min at ambient temperature. The slices were then incubated for 1 h at 37°C with the TUNEL reaction mixture. After blocking, the samples were incubated with primary antibodies against NeuN (Abcam, UK) at 4°C overnight. Then the corresponding secondary antibody Cy3-labelled goat anti-mouse IgG (Invitrogen, USA) was used to incubate the samples for 1 h at room temperature. Nuclei were counterstained with DAPI (Aladdin, China). The anti-fade mounting medium (Solarbio, China) was used to seal tissue sections between glass slides and coverslips. Finally, the sections were observed under a microscope (Olympus, Japan).

Enzyme-linked immunosorbent assay (ELISA)

The content of interleukin (IL)-1β was measured using a mouse IL-1β ELISA kit (Lianke Biotech, China) according to the manufacturer's instructions. The corresponding concentration was calculated according to the standard curve.

Immunohistochemistry (IHC) assay

Immunohistochemical studies were performed on paraffin-embedded tissues. After deparaffinization, endogenous peroxidase quenching was performed by the addition of 3% H_2O_2 and the antigen-antibody reaction was incubated overnight at 4°C. The samples were incubated with primary antibodies Iba1 (Santa Cruz, USA) overnight at 4°C. After

incubation with HRP-conjugated secondary antibody (ThermoFisher, USA), the sections were stained using Diaminobenzidine reagents (Maixin Biotechnology, China) and counterstained with hematoxylin (Solarbio, China). Images were captured using a BX53 microscope (Olympus, Japan).

Statistical analysis

Statistical significance was calculated using Graphpad Prism software. The unpaired *t*-test was performed to compare data between the two groups, and one-way analysis of variance was utilized to examine the differences between multiple groups. All data were presented as the mean ± standard deviation (SD). Statistical significance was indicated by a p value of less than 0.05.

Results

Characterization of TNFAIP3 expression in KA-induced epileptic mice

To analyze the function of TNFAIP3 on epilepsy, we first overexpressed TNFAIP3 by lentiviral infection. Immunofluorescence staining for TNFAIP3 and Iba1 in the CA3 region of the mouse hippocampus verified the efficiency of lentivirus infection (Fig. 1A). The protein levels of TNFAIP3 in the Lv-NC and Lv-TNFAIP3 groups were also verified by Western blot assay (Fig. 1B). The results of immunofluorescence showed that weak red staining for TNFAIP3 in CA1, CA3, and dentate gyrus (DG) regions was detected in the sham group, while the strong staining for TNFAIP3 was presented in epileptic mice models (Fig. 1C). As indicated in Figure 1D and E, TNFAIP3 was highly expressed in KA-kindled epileptic mice.

Characterization of TNFAIP3 upregulation on behavioral disabilities in epileptic mice

Considering the protective effects of TNFAIP3 in epilepsy, we detected the behavioral disabilities in mice after KA administration. The number of epileptic seizure and the time of duration of first seizure in mice with the score of 4 and 5 was recorded, and we found that TNFAIP3 overexpression mitigated the state of epilepsy (Fig. 2A, B). Hot plate test was performed to assess sensory abilities in mice, and the results showed that the reaction time to pain was declined in KA-induced epileptic mice, while the latency time of feeling pain was prolonged after TNFAIP3 overexpression (Fig. 2C). To determine the role of TNFAIP3 in cognitive disorder, morris water maze was performed. Through 4-day records, we found that the escape delay in KA+LV-TNFAIp3 group was lower than that in KA+LV-NC group on the fourth day (Fig. 2D). Moreover, KA-administered mice made fewer passes through the targeted platform, whereas TNFAIP3 overexpression resulted in an increase in the number of crossing the platform in mice (Fig. 2E). As shown in Figure 2F, the path trajectory in the quadrants was recorded by the tracking system. We found that the overexpression of TNFAIP3 increased the percentage of time in epileptic mice spent in the targeted quadrant (Fig. 2G).

Characterization of TNFAIP3 upregulation on neuronal apoptosis in epileptic mice

Apoptosis is well known as an important feature of epilepsy. On this basis, the effects of TNFAIP3 on neuronal degeneration were assessed in hippocampal neurons (CA3).

The images of FJB staining showed that KA injection resulted in neurodegeneration in the hippocampus of mice, but TNFAIP3 overexpression improved the state (Fig. 3A). As shown in Figure 3B, the TUNEL-positive cells were increased in the KA group compared with the sham group, whereas the number of apoptotic cells was reduced in KA-induced mice after TNFAIP3 overexpression.

Characterization of TNFAIP3 upregulation on inflammation in epileptic mice

By investigating the physiological role of TNFAIP3 in the regulation of inflammatory activity, we found that the overexpression of TNFAIP3 significantly decreased the mRNA levels of IL-1β and inducible NO synthase (iNOS) compared with the negative control in KA-injected mice (Fig. 4A). Immunohistochemistry assay showed that TNFAIP3 overexpression reduced the number of Iba1 in the CA3 region of epilepsy-induced mice (Fig. 4B). The protein expression pattern of TNFAIP3 was confirmed by Western blot (Fig. 4C). In addition, Western blot analysis revealed that TNFAIP3 overexpression reduced the protein levels of NLRP3, ASC, and caspase 1 P20 (Fig. 4D).

Characterization of TNFAIP3 upregulation on inflammation in mouse BV2 cells

Next, we performed *in vitro* experiments to verify the function of TNFAIP3 in inflammatory response. The protein levels of TNFAIP3 performed by Western blot suggested that BV2 cells were successfully infected with Lv-TNFAIP3 (Fig. 5A). The results of ELISA showed that IL-1β concentration was notably increased after LPS stimulation compared with the control group and it was inhibited after TNFAIP3 overexpression (Fig. 5B). Subsequently, we verified the efficiency of TNFAIP3 overexpression in response to LPS by Western blot assay (Fig. 5C). Meanwhile, compared with the negative control, TNFAIP3-overexpressed cells induced by

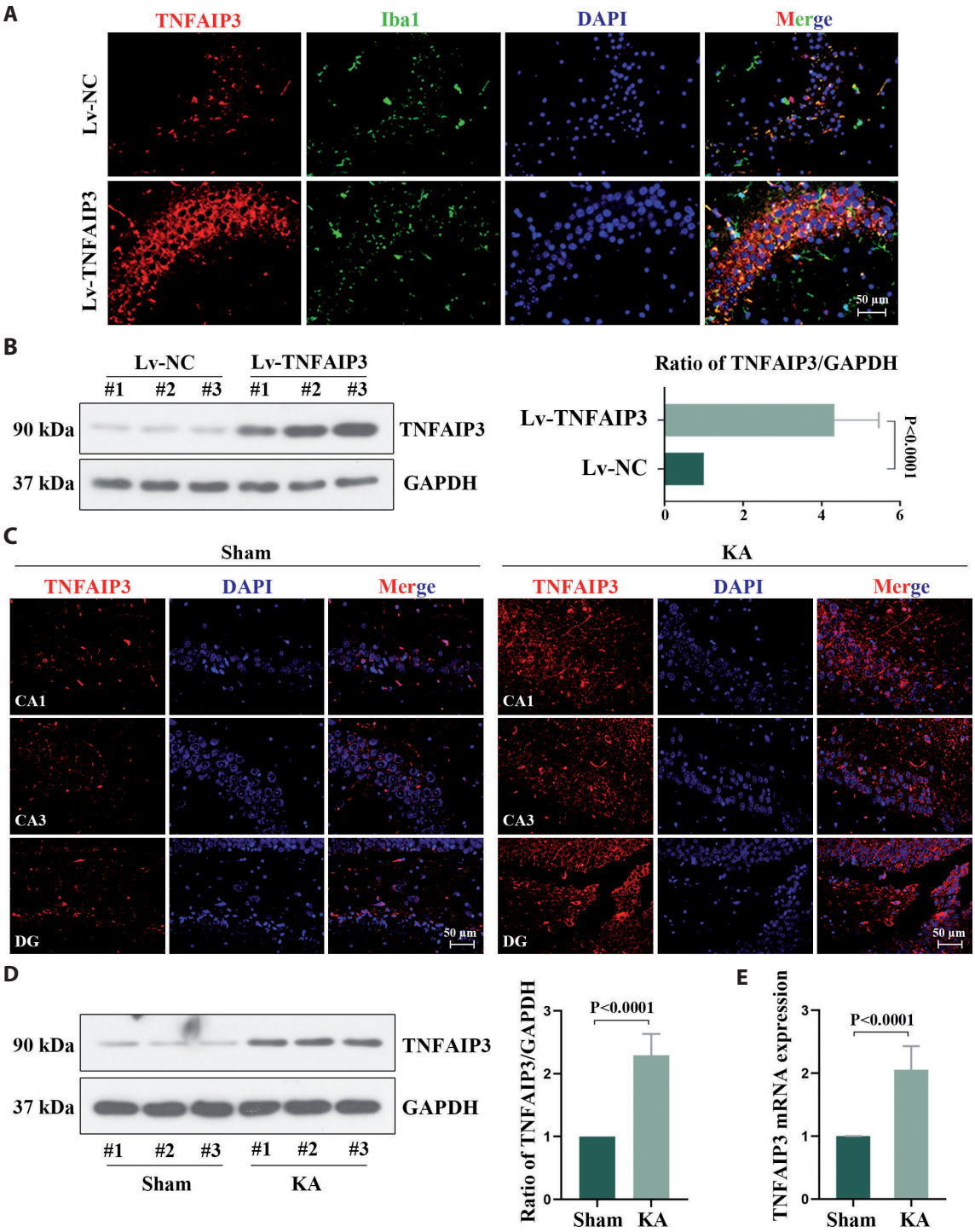

Figure 1. The expression of TNFAIP3 in the hippocampus of KA-induced epilepsy mice. **A.** The expression of TNFAIP3 and Iba1 in the CA3 region of the hippocampus was observed by immunofluorescence staining, and nuclei were fluorescently labeled with DAPI. Scale bars: 50 μm; magnification: ×400. **B.** The protein expression of TNFAIP3 was detected in each group by Western blot, and the level of TNFAIP3 (normalized to GAPDH) was quantified. The 3-day mouse models of epilepsy were used to analyze the expression of TNFAIP3 in the hippocampus for the following experiments. **C.** The expression of TNFAIP3 in CA1, CA3, and DG regions of the hippocampus was detected by immunofluorescence staining. Scale bars: 50 μm; magnification: ×400. Western blot (**D**) and qRT-PCR (**E**) showed the protein and mRNA levels of TNFAIP3 in KA-injection mice. The level of TNFAIP3 (normalized to GAPDH) were quantified. TNFAIP3, TNF alpha-induced protein 3; KA, kainic acid; DG, dentate gyrus.

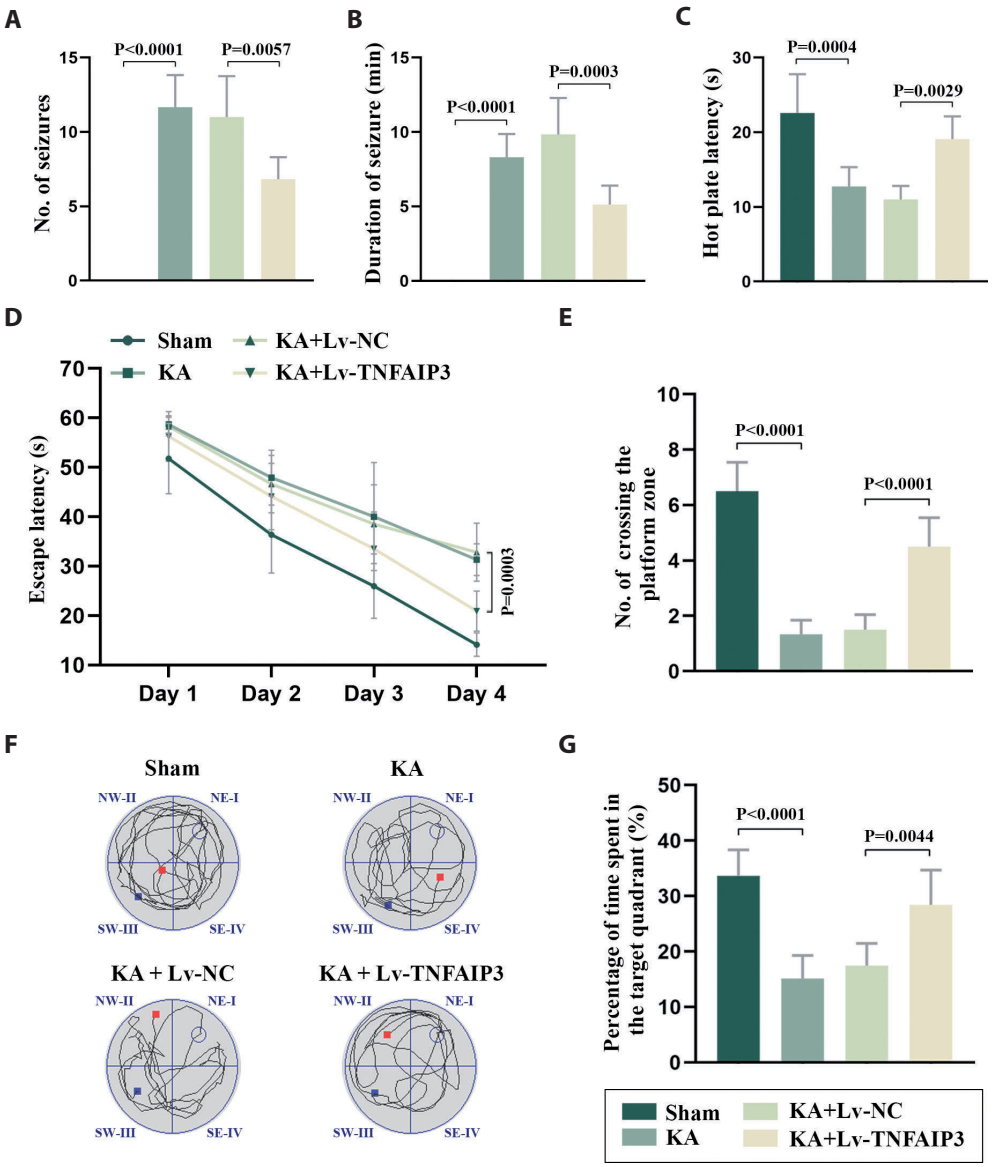

Figure 2. The effect of TNFAIP3 upregulation on behavioral barriers in KA-induced epilepsy mice. **A.** The total number of epileptic seizures. **B.** The duration of the first seizure in mice with a score of 4 or 5. **C.** The hot plate latency of each mouse to react to pain. Morris water maze test was performed to record the escape latency (**D**), the number of entries in the platform zone (**E**), track of probe test (**F**), and the time spent in the hidden quadrant (**G**). The blue circle represents the location of the hidden platform, and the rectangle filled with blue and red are the location of the mice to enter and leave quadrants respectively. NE-I, northeast; NW-II, northwest; SW-III, southwest; SE-IV, southeast. (See online version for color figure.)

LPS markedly decreased the protein levels of NLRP3, ASC, and caspase 1 P20 (Fig. 5D).

Discussion

In the present study, we found that the expression of TNFAIP3 was up-regulated in KA-induced epileptic models. Behavioral analysis showed that TNFAIP3 overexpression mitigated sensory and cognitive impairments after epilepsy. Further investigation revealed that TNFAIP3 overexpression blocked the apoptosis of hippocampal neurons and inhibited the neuroinflammation *via* NLRP3 inflammasome inactivation in KA-induced mice. The effects of TNFAIP3 on inflammatory response were confirmed through *in vitro* experiments. Hence, these findings indicated that TNFAIP3 might exert a neuroprotective role in epileptogenesis.

Status epilepticus can cause short-term and enduring damage to the CNS, particularly to neurons in the hippocampus and other peripheral systems (Wilder et al. 1977), and the epileptic patients undergo cognitive impairment, including learning and memory damage (Elger et al. 2004). Any brain damage, including the hippocampus and cerebral cortex, can cause spatial learning and memory deficits, the highly prevalent comorbidity of epilepsy (Veng et al. 2003). At present, we found that TNFAIP3 overexpression significantly declined the duration of the first seizure, along with ameliorated learning and memory disturbances in

KA-induced epileptic mice, demonstrating that TNFAIP3 exerted protective roles in behavioral disorder. Neuronal injury occurs within a few hours of status epilepticus in some epilepsy models, causing changes in the function and structure of the neuronal network (Pitkänen et al. 2002). Moreover, it is reported that neuronal apoptosis of hippocampus is a major cause of decline in learning and memory abilities in epileptic patients (Zhao et al. 2019). Consistently, we found that TNFAIP3 blocked the apoptosis of hippocampal neurons in KA-induced epileptic mice, suggesting that TNFAIP3 presented an anti-apoptotic function. Previous studies found that the apoptosis rate of neurons overexpressing TNFAIP3 (28.46 ± 3.87%) was lower than in sham neurons (53.06 ± 5.36%) (Miao et al. 2005). A similar conclusion was reached by our work in this study that TNFAIP3 overexpression led to hippocampal neuronal loss in the CA3 area. It has been reported that TNFAIP3 protects neurons from damage by inhibiting neuronal apoptosis (Chen et al. 2019). In addition, studies have shown that the deficiency of hepatocyte-specific TNFAIP3 can make mice prone to spontaneous liver inflammation and cell apoptosis (Catrysse et al. 2016).

Accumulating evidence indicates that NF-κB, as a well-known proinflammatory mediator, plays a key role in self-regulation and protective immune response (Sun et al. 2013). The activation of NF-κB leads to the induction of various in-

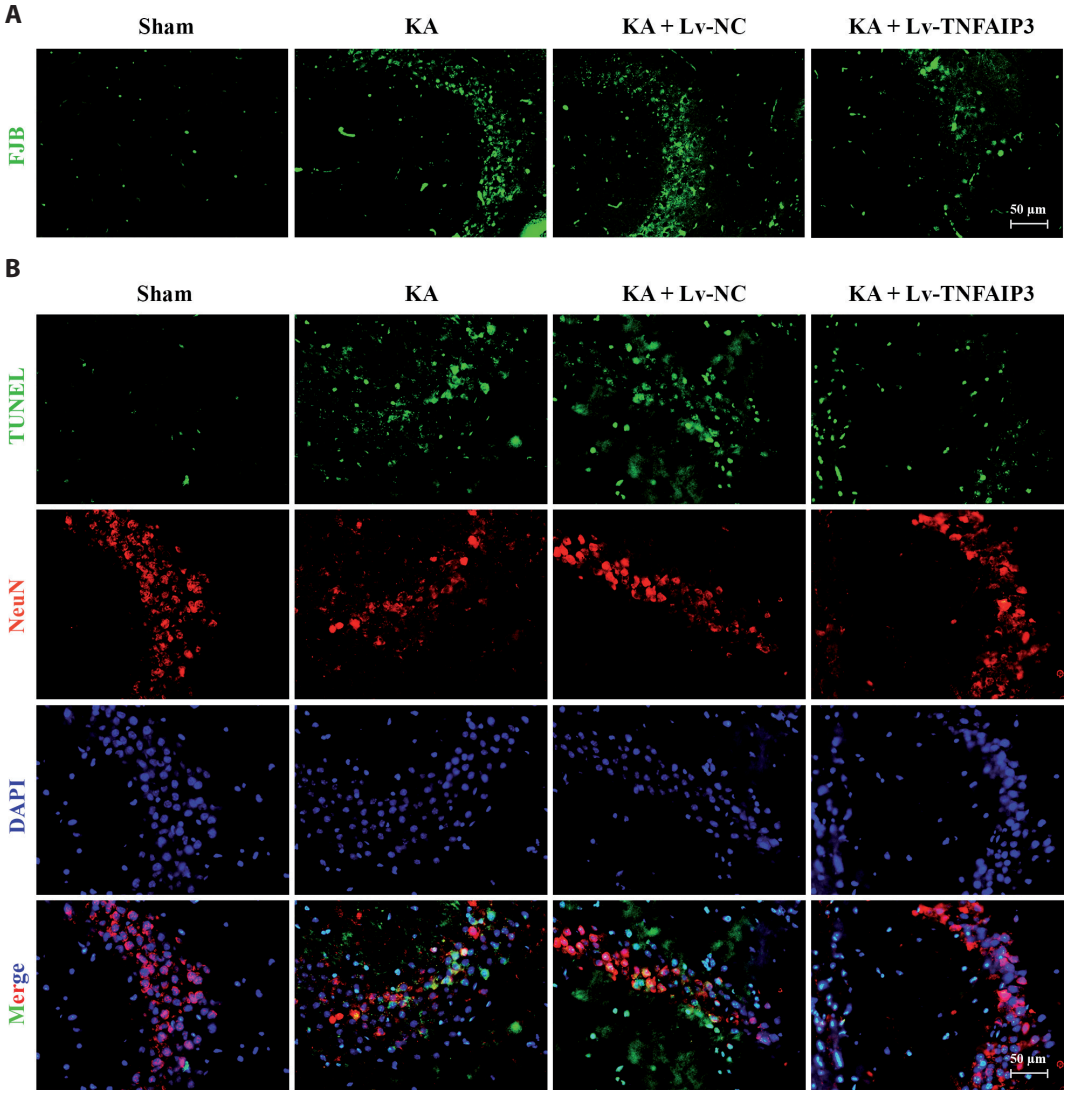

Figure 3. The effect of TNFAIP3 upregulation on neuronal apoptosis in KA-induced epilepsy mice. **A.** The degeneration of hippocampal neurons (CA3) was detected by FJB staining. **B.** Double immunofluorescence analysis was performed with TUNEL (green) and a neuronal marker (NeuN, red), and nuclei were fluorescently labeled with DAPI (blue). Scale bars 50 μm; magnification × 400. FJB, Fluoro-Jade-B; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling.

flammatory mediators and the activation of ubiquitin editing enzyme TNFAIP3. In turn, TNFAIP3 negatively regulates the NF-κB signaling pathway and maintains the steady-state of the CNS specificity (Abbasi et al. 2015). It has been reported that proinflammatory cytokine IL-1β can initiate and exacerbate epileptic activity (Dubé et al. 2005), and the inhibition of IL-1β biosynthesis regulates acute seizures (Maroso et al. 2011). Besides, iNOS is the major NOS isoform in the brain, and is closely related to the pathophysiological process of the nervous system (Stoyanova and Lazarov 2005). Here, we found that TNFAIP3 overexpression downregulated the expression of pro-inflammatory cytokine IL-1β and iNOS in epileptic mice, indicating that TNFAIP3 might profile a key role in the process of inflammation. NLRP3 inflammasome is a multiprotein complex that mediates the activation of the enzyme caspase-1. This process by NLRP3 can induce the maturation and secretion of the pro-inflammatory factor IL-1β, so it is deemed to be a key mediator of IL-1β function (Haneklaus et al. 2013). Previous reports proved that the inhibition of NLRP3 inflammasome played neuroprotection in status epilepticus-induced rats (Meng et al. 2014). Because of the connection between IL-1β and NLRP3 inflammasome, we further investigated the effect of TNFAIP3 on the NLRP3 inflammasome. The protein levels of NLRP3, ASC, and caspase 1 P20 were found to decrease after TNFAIP3 upregulation, illustrating that TNFAIP3 might suppress the NLRP3 inflammasome pathway to reduce the inflammation in epilepsy. The indirect role of NLRP3-ASC-caspase-1 inflammasome in the positive feedback amplification of the IL-1β signal needs further study. In addition to the canonical inflammasome (e.g., NLRP3) pathway, the non-canonical inflammasome pathway has been described (Man and Kan-

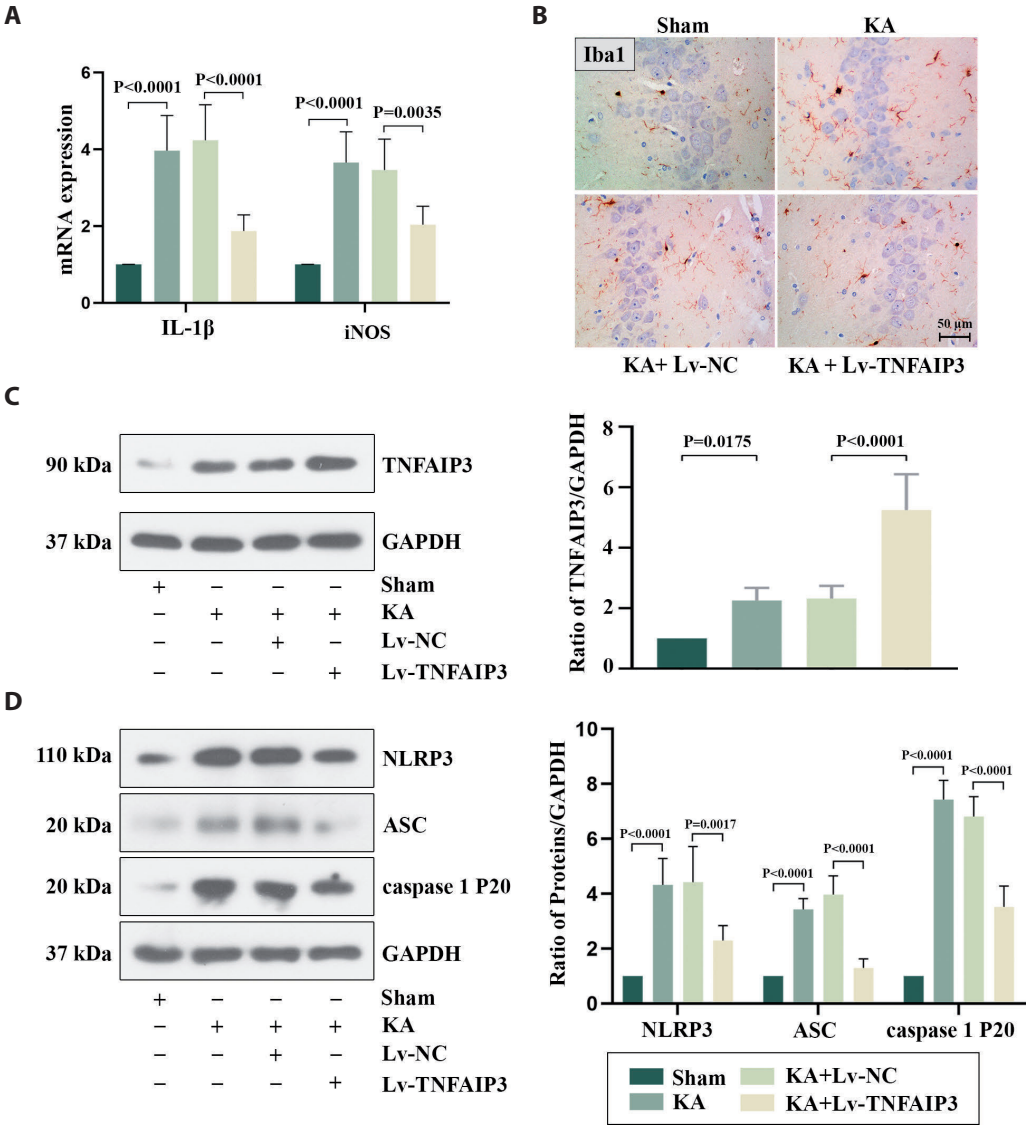

Figure 4. The effect of TNFAIP3 upregulation on inflammation in KA-induced epilepsy mice. **A.** The expression of IL-1β and iNOS in the hippocampus was detected by qRT-PCR. **B.** Iba1 expression in hippocampal tissues (CA3) was detected by immunohistochemistry. Western blot analysis for TNFAIP3 (**C**) and NLRP3, ASC, caspase 1 P20 (**D**) in the hippocampus, and the levels were quantified by normalizing to GAPDH.

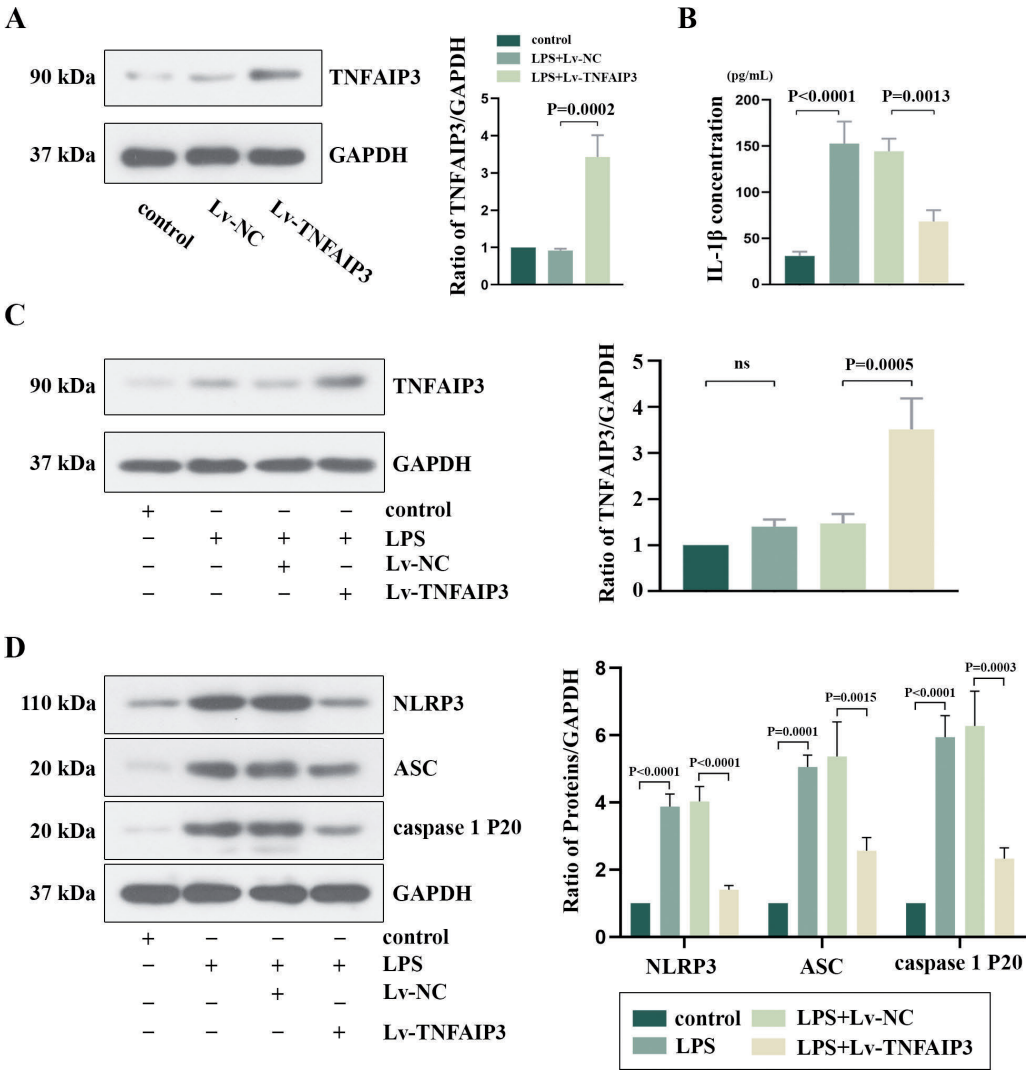

Figure 5. The effect of TNFAIP3 upregulation on inflammation in mouse BV2 cells (Microglia). **A.** Western blot was used to verify the efficiency of BV2 cells after 48 h-lentivirus infection. The level of TNFAIP3 (normalized to GAPDH) was quantified. BV2 cells infected with overexpressed TNFAIP3 lentivirus were treated with lipopolysaccharide (LPS), and the cells were used for the following experiments. **B.** The content of IL-1β was quantified in culture supernatants by ELISA. Western blot analysis for TNFAIP3 (**C**) and NLRP3, ASC, caspase 1 P20 (**D**). The levels were quantified by normalizing to GAPDH ns, non significant.

neganti 2015; Rathinam et al. 2019). In this pathway, no NLRs are required, and the protein caspase11 is activated and cleavaged GSDMD to produce pores to release K^+. The decrease of intracellular K^+ activates NLRP3 to produce IL-1β and IL-18 (Sanz and Garcia-Gimeno 2020). Therefore, future studies are worth exploring the role of TNFAIP3 on different inflammasome pathways.

Epilepsy is related to intense and persistent inflammation in the microenvironment of the CNS, along with subtle neuronal damage, gliosis, and microgliosis (Alyu and Dikmen 2017). After the CNS is injured, two primary cells (microglia and astrocytes) related to neuroinflammation are activated, which can be seen from their morphological and phenotypic changes (Polikov et al. 2005; Eggen et al. 2013; Jensen et al. 2013). Microglia is a mononuclear phagocyte of the CNS, which has significant contributions to maintaining the homeostasis and the pathology of CNS (Voet et al. 2018). The activation of microglia and the neuroinflammation produced by pro-inflammatory cytokines in the brain play a positive role in epilepsy (Ho et al. 2015). Here, *in vitro* experiments in LPS-activated microglial cells demonstrated that TNFAIP3 might negatively regulate NLRP3 inflammasome to alleviate epilepsy. In line with previous studies, Voet et al. (2018) suggested that NLRP3 critically controlled microglia activation and NLRP3 in microglia protected against LPS-induced inflammation. In addition to protecting against epilepsy, TNFAIP3 can also protect against arthritis by negatively regulating the NLRP3 inflammasome (Vande Walle et al. 2014). Previous studies also indicated that mice lacking TNFAIP3 were hypersensitive to both lipopolysaccharide and tumor necrosis factor, and could lead to severe inflammation, cachexia, and die prematurely (Lee et al. 2000). Although the cellular and molecular mechanisms of epilepsy are not yet clear, Musto et al. (2016) speculated

that the unregulated inflammatory process could lead to abnormal neural connections and the overexcited neural networks that mediate epileptogenesis.

In conclusion, our results confirmed that TNFAIP3 was highly expressed in KA-induced mice models. Then we identified that TNFAIP3 improved spatial learning and memory abilities, as well as ameliorated apoptosis and inflammation in epileptic mice, which further confirmed the significance of TNFAIP3 in the maintenance and protection of epileptic CNS through *in vitro* experiments. Therefore, TNFAIP3 as an inhibitor may develop as an important target in the treatment of chronic epilepsy.

Conflict of interest. The authors declare that they have no competing interests.

Funding. This work was supported by the Natural Science Foundation of Henan Province (Grant No. 202300410469).

References

Abbasi A, Forsberg K, Bischof F (2015): The role of the ubiquitin-editing enzyme A20 in diseases of the central nervous system and other pathological processes. Front. Mol. Neurosci. **8,** 21 https://doi.org/10.3389/fnmol.2015.00021

Alyu F, Dikmen M (2017): Inflammatory aspects of epileptogenesis: contribution of molecular inflammatory mechanisms. Acta Neuropsychiatr. **29,** 1-16 https://doi.org/10.1017/neu.2016.47

Catrysse L, Farhang Ghahremani M, Vereecke L, Youssef SA, Mc Guire C, Sze M, Weber A, Heikenwalder M, de Bruin A, Beyaert R, et al. (2016): A20 prevents chronic liver inflammation and cancer by protecting hepatocytes from death. Cell Death Dis. **7,** e2250 https://doi.org/10.1038/cddis.2016.154

Catrysse L, Vereecke L, Beyaert R, van Loo G (2014): A20 in inflammation and autoimmunity. Trends Immunol. **35,** 22-31 https://doi.org/10.1016/j.it.2013.10.005

Chen X, Qian B, Kong X, Hao J, Ye Y, Yang K, Xu T, Zhang F (2019): A20 protects neuronal apoptosis stimulated by lipopolysaccharide-induced microglial exosomes. Neurosci. Lett. **712,** 134480 https://doi.org/10.1016/j.neulet.2019.134480

Corps KN, Roth TL, McGavern DB (2015): Inflammation and neuroprotection in traumatic brain injury. JAMA Neurol. **72,** 355-362 https://doi.org/10.1001/jamaneurol.2014.3558

Crump KE, Oakley JC, Xia-Juan X, Madu TC, Devaki S, Mooney EC, Sahingur SE (2017): Interplay of toll-like receptor **9,** myeloid cells, and deubiquitinase a20 in periodontal inflammation. Infect. Immun. **85,** e00814-816 https://doi.org/10.1128/IAI.00814-16

Dubé C, Vezzani A, Behrens M, Bartfai T, Baram TZ (2005): Interleukin-1beta contributes to the generation of experimental febrile seizures. Ann. Neurol. **57,** 152-155 https://doi.org/10.1002/ana.20358

Duong BH, Onizawa M, Oses-Prieto JA, Advincula R, Burlingame A, Malynn BA, Ma A (2015): A20 restricts ubiquitination of pro-interleukin-1β protein complexes and suppresses NLRP3 inflammasome activity. Immunity **42,** 55-67 https://doi.org/10.1016/j.immuni.2014.12.031

Eggen BJL, Raj D, Hanisch UK, Boddeke HWGM (2013): Microglial phenotype and adaptation. J. Neuroimmune Pharmacol. **8,** 807-823 https://doi.org/10.1007/s11481-013-9490-4

Elger CE, Helmstaedter C, Kurthen M (2004): Chronic epilepsy and cognition. Lancet Neurol. **3,** 663-672 https://doi.org/10.1016/S1474-4422(04)00906-8

Farrell JS, Wolff MD, Teskey GC (2017): Neurodegeneration and pathology in epilepsy: clinical and basic perspectives. Adv. Neurobiol. **15,** 317-334 https://doi.org/10.1007/978-3-319-57193-5_12

Goldberg EM, Coulter DA (2013): Mechanisms of epileptogenesis: a convergence on neural circuit dysfunction. Nat. Rev. Neurosci. **14,** 337-349 https://doi.org/10.1038/nrn3482

Haneklaus M, O'Neill LAJ, Coll RC (2013): Modulatory mechanisms controlling the NLRP3 inflammasome in inflammation: recent developments. Curr. Opin. Immunol. **25,** 40-45 https://doi.org/10.1016/j.coi.2012.12.004

Hjelmeland AB, Wu Q, Wickman S, Eyler C, Heddleston J, Shi Q, Lathia JD, Macswords J, Lee J, McLendon RE, et al. (2010): Targeting A20 decreases glioma stem cell survival and tumor growth. PLoS Biol. **8,** e1000319 https://doi.org/10.1371/journal.pbio.1000319

Ho YH, Lin YT, Wu CW, Chao YM, Chang AY, Chan JY (2015): Peripheral inflammation increases seizure susceptibility via the induction of neuroinflammation and oxidative stress in the hippocampus. J. Biomed. Sci. **22,** 46 https://doi.org/10.1186/s12929-015-0157-8

Jensen CJ, Massie A, De Keyser J (2013): Immune players in the CNS: the astrocyte. J. Neuroimmune Pharmacol. **8,** 824-839 https://doi.org/10.1007/s11481-013-9480-6

Keller SS, Glenn GR, Weber B, Kreilkamp BAK, Jensen JH, Helpern JA, Wagner J, Barker GJ, Richardson MP, Bonilha L (2017): Preoperative automated fibre quantification predicts postoperative seizure outcome in temporal lobe epilepsy. Brain **140,** 68-82 https://doi.org/10.1093/brain/aww280

Lee EG, Boone DL, Chai S, Libby SL, Chien M, Lodolce JP, Ma A (2000): Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. Science **289,** 2350-2354 https://doi.org/10.1126/science.289.5488.2350

Liu SS, Lv XX, Liu C, Qi J, Li YX, Wei XP, Li K, Hua F, Cui B, Zhang XW, et al. (2019): Targeting degradation of the transcription factor C/EBPβ reduces lung fibrosis by restoring activity of the ubiquitin-editing enzyme A20 in macrophages. Immunity **51,** 522-534 https://doi.org/10.1016/j.immuni.2019.06.014

Man SM, Kanneganti T-D (2015): Regulation of inflammasome activation. Immunol. Rev. **265,** 6-21 https://doi.org/10.1111/imr.12296

Maroso M, Balosso S, Ravizza T, Iori V, Wright CI, French J, Vezzani A (2011): Interleukin-1β biosynthesis inhibition reduces acute seizures and drug resistant chronic epileptic activity in mice. Neurotherapeutics **8,** 304-315 https://doi.org/10.1007/s13311-011-0039-z

Meng XF, Tan L, Tan MS, Jiang T, Tan CC, Li MM, Wang HF, Yu JT (2014): Inhibition of the NLRP3 inflammasome provides neuroprotection in rats following amygdala kindling-induced status epilepticus. J. Neuroinflammation **11,** 212 https://doi.org/10.1186/s12974-014-0212-5

Miao HS, Yu LY, Hui GZ, Guo LH (2005): Antiapoptotic effect both in vivo and in vitro of A20 gene when transfected into rat hippocampal neurons. Acta Pharmacol. Sin. **26,** 33-38 https://doi.org/10.1111/j.1745-7254.2005.00002.x

Mohseni-Moghaddam P, Sadr SS, Roghani M, Arabzadeh S, Khamse S, Zamani E, Hosseini M, Moradi F (2019): Huperzine A ameliorates cognitive dysfunction and neuroinflammation in kainic acid-induced epileptic rats by antioxidant activity and NLRP3/caspase-1 pathway inhibition. Clin. Exp. Pharmacol. Physiol. **46,** 360-372 https://doi.org/10.1111/1440-1681.13064

Musto AE, Rosencrans RF, Walker CP, Bhattacharjee S, Raulji CM, Belayev L, Fang Z, Gordon WC, Bazan NG (2016): Dysfunctional epileptic neuronal circuits and dysmorphic dendritic spines are mitigated by platelet-activating factor receptor antagonism. Sci. Rep. **6,** 30298 https://doi.org/10.1038/srep30298

Neligan A, Hauser WA, Sander JW (2012): The epidemiology of the epilepsies. Handb Clin. Neurol. **107,** 113-133 https://doi.org/10.1016/B978-0-444-52898-8.00006-9

Perga S, Montarolo F, Martire S, Bonaldo B, Bono G, Bertolo J, Magliozzi R, Bertolotto A (2021): Overexpression of the ubiquitin-editing enzyme A20 in the brain lesions of Multiple Sclerosis patients: moving from systemic to central nervous system inflammation. Brain Pathol. **31,** 283-296 https://doi.org/10.1111/bpa.12906

Pitkänen A, Nissinen J, Nairismägi J, Lukasiuk K, Gröhn OHJ, Miettinen R, Kauppinen R (2002): Progression of neuronal damage after status epilepticus and during spontaneous seizures in a rat model of temporal lobe epilepsy. Prog. Brain Res. **135,** 67-83 https://doi.org/10.1016/S0079-6123(02)35008-8

Pitkänen A, Lukasiuk K, Dudek FE, Staley KJ (2015): Epileptogenesis. Cold Spring Harbor Perpect. Med. **5,** a022822 https://doi.org/10.1101/cshperspect.a022822

Polikov VS, Tresco PA, Reichert WM (2005): Response of brain tissue to chronically implanted neural electrodes. J. Neurosci. Meth. **148,** 1-18 https://doi.org/10.1016/j.jneumeth.2005.08.015

Rathinam VAK, Zhao Y, Shao F (2019): Innate immunity to intracellular LPS. Nat. Immunol. **20,** 527-533 https://doi.org/10.1038/s41590-019-0368-3

Sanz P, Garcia-Gimeno MA (2020): Reactive glia inflammatory signaling pathways and epilepsy. Int. J. Mol. Sci. **21,** 4096 https://doi.org/10.3390/ijms21114096

Sheizaf B, Mazor M, Landau D, Burstein E, Bashiri A, Hershkovitz R (2007): Early sonographic prenatal diagnosis of seizures. Ultrasound Obstet. Gynecol. **30,** 1007-1009 https://doi.org/10.1002/uog.5153

Shembade N, Harhaj E (2010): A20 inhibition of NFκB and inflammation: targeting E2:E3 ubiquitin enzyme complexes. Cell Cycle **9,** 2481-2482 https://doi.org/10.4161/cc.9.13.12269

Spencer S, Huh L (2008): Outcomes of epilepsy surgery in adults and children. Lancet Neurol. **7,** 525-537 https://doi.org/10.1016/S1474-4422(08)70109-1

Stoyanova II, Lazarov NE (2005): Localization of nitric oxide synthase in rat trigeminal primary afferent neurons using NADPH-diaphorase histochemistry. J. Mol. Histol. **36,** 187-193 https://doi.org/10.1007/s10735-005-1694-3

Sun SC, Chang JH, Jin J (2013): Regulation of nuclear factor-κB in autoimmunity. Trends Immunol. **34,** 282-289 https://doi.org/10.1016/j.it.2013.01.004

Sunaga K, Sugaya E, Kajiwara K, Tsuda T, Sugaya A, Kimura M (2004): Molecular mechanism of preventive effect of peony root extract on neuron damage. J. Herb Pharmacother. **4,** 9-20 https://doi.org/10.1080/J157v04n01_02

Thurman DJ, Beghi E, Begley CE, Berg AT, Buchhalter JR, Ding D, Hesdorffer DC, Hauser WA, Kazis L, Kobau R, et al. (2011): Standards for epidemiologic studies and surveillance of epilepsy. Epilepsia **52,** 2-26 https://doi.org/10.1111/j.1528-1167.2011.03121.x

Usta IM, Adra AM, Nassar AH (2007): Ultrasonographic diagnosis of fetal seizures: a case report and review of the literature. BJOG **114,** 1031-1033 https://doi.org/10.1111/j.1471-0528.2007.01398.x

Vande Walle L, Van Opdenbosch N, Jacques P, Fossoul A, Verheugen E, Vogel P, Beyaert R, Elewaut D, Kanneganti T-D, van Loo G, et al. (2014): Negative regulation of the NLRP3 inflammasome by A20 protects against arthritis. Nature **512,** 69-73 https://doi.org/10.1038/nature13322

Veng LM, Granholm AC, Rose GM (2003): Age-related sex differences in spatial learning and basal forebrain cholinergic neurons in F344 rats. Physiol. Behav. **80,** 27-36 https://doi.org/10.1016/S0031-9384(03)00219-1

Voet S, Mc Guire C, Hagemeyer N, Martens A, Schroeder A, Wieghofer P, Daems C, Staszewski O, Vande Walle L, Jordao MJC, et al. (2018): A20 critically controls microglia activation and inhibits inflammasome-dependent neuroinflammation. Nat. Commun. **9,** 2036 https://doi.org/10.1038/s41467-018-04376-5

Waisman A, Liblau RS, Becher B (2015): Innate and adaptive immune responses in the CNS. Lancet Neurol. **14,** 945-955 https://doi.org/10.1016/S1474-4422(15)00141-6

Wilder BJ, Ramsay RE, Willmore LJ, Feussner GF, Perchalski RJ, Shumate JB (1977): Efficacy of intravenous phenytoin in the treatment of status epilepticus: kinetics of central nervous system penetration. Ann. Neurol. **1,** 511-518 https://doi.org/10.1002/ana.410010602

Zhao Q, Yin C, Yuan Y, Zhang H, Teng L (2019): Down-regulation of Mir-145 improves learning and memory abilities in epileptic rats by regulating hippocampal neuron apoptosis. World Neurosurg. **122,** e1432-e1438 https://doi.org/10.1016/j.wneu.2018.11.080

Received: March 2, 2022
Final version accepted: July 13, 2022