doi: 10.4149/gpb_2024022

PVT1 regulates hippocampal neuron apoptosis and inflammation in epilepsy by miR-206-3p-dependent regulation of CAMK4

Chuankun Li[1], Ruichun Li[1], Xiaobin Bai[1] and Haitao Jiang[1]

[1] *Department of Neurosurgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China*

Abstract. This study was designed to dissect the function of plasmacytoma variant translocation 1 (PVT1) in hippocampal neuron injury in epilepsy and its possible molecular basis. Status epilepticus (SE) mouse model was built and primary hippocampal neurons were isolated. qRT-PCR and Western blot were applied to quantify the levels of related genes and proteins. Cell proliferation and apoptosis were examined by CCK-8, EdU, and flow cytometry assays. Inflammatory factors were detected using ELISA analysis. Dual-luciferase reporter and RIP assays were carried out to validate the relationship between miR-206-3p and PVT1 or CAMK4. PVT1 and CAMK4 were increased, and miR-206-3p was downregulated in the hippocampus and hippocampal neurons of SE mice. Knockdown of PVT1 or CAMK4 abated SE-induced proliferation inhibition, apoptosis, and inflammation in hippocampal neurons. Mechanistically, PVT1 could sponge miR-206-3p to upregulate the expression of CAMK4 in hippocampal neurons. Moreover, downregulation of miR-206-3p reversed the inhibitory effects of PVT1 knockdown on SE-induced apoptosis and inflammation in hippocampal neurons. Similarly, overexpression of CAMK4 abolished miR-206-3p-evoked arrest of apoptosis and inflammation in hippocampal neurons under SE condition. Collectively, PVT1 contributed to SE-induced apoptosis and inflammation in hippocampal neurons by modulating the miR-206-3p/CAMK4 axis, offering a novel insight into the prevention of epilepsy.

Key words: PVT1 — Hippocampal neurons — Epilepsy — Inflammation — Apoptosis

Abbreviations: CAMK4, calcium/calmodulin dependent protein kinase IV; CASC2, cancer susceptibility candidate 2; CCK-8, Cell Counting Kit-8; ceRNAs, competing endogenous RNA; EdU, 5-ethynyl-2'-deoxyuridine; lncRNAs, long noncoding RNAs; MUT, mutated; PVT1, plasmacytoma variant translocation 1; RIP, RNA immunoprecipitation; SE, status epilepticus; WT, wild-type.

Introduction

Epilepsy is a serious brain condition characterized by repeated unprovoked seizures that affect over 70 million people throughout the world and impair the life quality of these patients (Thijs et al. 2019). About 10 million people currently suffer from epilepsy, with about 400,000 new cases annually (Ding et al. 2021). Despite advances in the management of epilepsy, over 30% of patients continue to experience seizures and develop drug-resistant epilepsy (Mesraoua et al. 2019). It has been proposed that epilepsy is pathologically characterized by the disruption of hippocampal neurons and the impairment of the hippocampus (Liu et al. 2014). Thus, further clarification of the molecular mechanism of hippocampal neuronal injury may be helpful for the development of novel molecular therapies for epilepsy.

Long noncoding RNAs (lncRNAs) are one subtype of the noncoding RNAs typically longer than 200 nucleotides in length, which have been reported to be implicated in regulating large-scale molecular processes (Geisler and Coller 2013; Schmitz et al. 2016). Besides that, lncRNAs act as key

Electronic supplementary material. The online version of this article (doi: 10.4149/gpb_2024022) contains Supplementary material.

Correspondence to: Haitao Jiang, Department of Neurosurgery, The First Affiliated Hospital of Xi'an Jiaotong University, No. 277 Yanta West Road, Xi'an 710061, China
E-mail: jianght1016@163.com

players in a variety of physiological and pathophysiological processes involving growth, differentiation, metastasis, apoptosis, and immune responses (Yan et al. 2015; Ma et al. 2019; Statello et al. 2021). Moreover, recent findings implicate that lncRNAs are highly expressed in brain tissue and brain-specific lncRNAs is highly conserved across species (Derrien et al. 2012; Lin et al. 2014). Importantly, dysregulated lncRNA expression participates in the onset and development of neurological diseases, including glioma (Zheng et al. 2016), Alzheimer's disease (Massone et al. 2011), and epilepsy (Wan and Yang 2020). For example, Wang et al. showed that ZFAS1 deficiency could attenuate the oxidative, apoptotic, and inflammatory injury in hippocampal neurons in epilepsy (Wang et al. 2022). LncRNA CASC2 (cancer susceptibility candidate 2) exerted a protective effect and suppress epileptic seizure by reducing adenosine release and astrocyte activation (Zhu et al. 2020). LncRNA PVT1 (plasmacytoma variant translocation 1), located on chromosomal 8q24.21, is a significant carcinogenic lncRNA that is highly expressed in diverse malignancies and predicts poor outcomes (Pan et al. 2018; Martínez-Barriocanal et al. 2020). Additionally, Zhao et al. showed that PVT1 was elevated in hippocampus tissues of epileptic rats, and silencing of PVT1 improved functional recovery, attenuated neuronal loss, and suppressed astrocyte activation in epilepsy rats (Zhao et al. 2019). However, the underlying regulatory network of PVT1 in epilepsy is still vague.

It has been revealed that lncRNAs can act as the competing endogenous RNA (ceRNAs) for miRNAs, thus reducing the molecules available for binding to their mRNA targets to prevent the degradation of mRNAs (Salmena et al. 2011). Herein, this study aimed to investigate the role and underlying miRNA/mRNA network of PVT1 in hippocampal neuron apoptosis and inflammatory response during epilepsy.

Materials and Methods

Animal epilepsy model

Adult male mice (8–12 weeks, $n = 30$) were used to establish the model of epilepsy in this study. The pilocarpine epilepsy mice model was performed as previously described (Vezzani 2009; Zhao et al. 2020). In the epilepsy group (SE mice), adult mice were intraperitoneally injected with lithium chloride (125 mg/kg) for 20 h, followed by intraperitoneal administration of pilocarpine (20 mg/kg, Sigma-Aldrich). Atropine (2 mg/kg) was given 30 min before pilocarpine administration. The mice at behavioral seizures stage IV–V based on the Racine scale (Racine 1972) were regarded as successful modeling. The mice without obvious epilepsy were administered 10 mg/kg pilocarpine at the interval of 30 min for 6 times until reaching Racine IV–V grade. The diazepam (10 mg/kg, Sigma-Aldrich) was administrated after status epilepticus (SE) one hour for termination. The same volume of 0.9% saline (125 mg/kg, Sigma-Aldrich) instead of pilocarpine was injected into mice to establish the Sham group. Mice who did not receive any treatment served as the control group. After modeling for 30 days, all mice were euthanized using pentobarbital sodium anesthesia, then the hippocampus was collected, and frozen in liquid nitrogen until use.

Hippocampal neuron isolation and culture

Based on a previous report (He et al. 2018), the primary hippocampal neurons were isolated, and then cultured in DMEM with 10% FBS and 1% penicillin/streptomycin (all from Life Technology, Wuhan, China) at 37°C in 5% CO_2.

qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then reversely transcribed into cDNAs using ReverTra Ace qPCR RT Kit (TaKaRa, Tokyo, Japan). Then SYBR green (Takara) was added into the PCR reaction system to conduct qRT-PCR analysis. The relative fold changes were determined by CT value. Table 1 displayed the primers.

Vector construction and cell transfection

The specific siRNAs targeting PVT1 or CAMK4 (si-PVT1; 5'-CAGCAGATGTCACACAGACGATAAA -3' or si-CAMK4; 5'-CCTATGCTCTCAAAGTGTTAAAGAA-3'), pcDNA

Table 1. Primer sequences

Gene	Forward primer	Reverse primer
PVT1	5'-GAGGAGTGCGGATGATGCTT-3'	5'- GGGCACAGGCAACTATCCAA-3'
CAMK4	5'- TTCCCTGCCTGGTCTCTGC-3'	5'- CGTCGATCCAGTAATCCGGG-3'
U6	5'-CTCGCTTCGGCAGCACATATACT-3'	5'-ACGCTTCACGAATTTGCGTGTC-3'
GAPDH	5'-GGTGAAGGTCGGTGTGAACG-3'	5'-CTCGCTCCTGGAAGATGGTG-3'
Mmu-miR-206-3p	5'-GGGTGGAATGTAAGGAAGT-3'	5'-CAGTGCGTGTCGTGGAGT-3'
miR-206-3p RT	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCCACAC-3'	

3.1-CAMK4 overexpression plasmid (CAMK4), miR-206-3p mimic/inhibitor (miR-206-3p/anti-miR-206-3p) and their corresponding control (si-NC, pcDNA, miR-NC, or anti-miR-NC) were procured from Genechem (Shanghai, China). Then transient transfection was performed using Lipofectamine 2000 reagent provided by Invitrogen.

Cell Counting Kit-8 (CCK-8) assay

Hippocampal neurons were reacted with 10 μl CCK-8 reagent (Beyotime, Shanghai, China) in a 96-well plate. 2 h later, the absorbance was measured at 450 nm using a microplate reader (Tecan, NANOQUANT, Switzerland).

5-Ethynyl-2'-deoxyuridine (EdU) assay

Hippocampal neurons were reacted with 50 μmol/l EdU (RiboBio) for 2 h, followed by dyeing with Apollo solution for 30 min. Cell nucleus staining was conducted using DAPI. Finally, the EdU-positive cells were analyzed.

Flow cytometry

The apoptosis of hippocampal neurons was determined in line with the protocol of Annexin V- FITC/PI Staining Kit (BD Biosciences, San Diego, CA, USA), and apoptotic cells were finally examined by the flow cytometry.

Western blotting

The extracted proteins were boiled and run on 10% SDS-PAGE, transferred onto the polyvinylidene fluoride membranes (Millipore, Darmstadt, Germany) and then sealed with skim milk at 37°C for 1 h. Thereafter, the membrane was incubated with primary antibodies against Bax (ab32503, 1:1000), Bcl-2 (ab182858, 1:2000), Caspase-3 (ab13847, 1:500), Cleaved-caspase-3 (ab2302, 1:1000), and CAMK4 (ab3557, 1:1000) at 4°C overnight, and subsequently probed with HRP-conjugated secondary antibodies at 37°C for 2 h. At last, blot bands were observed by ECL reagent and the gray value was analyzed with ImageJ software. All antibodies were obtained from Abcam (Cambridge, MA, USA).

ELISA analysis

The culture media of hippocampal neurons were collected and then the levels of TNF-α and IL-6 were detected based on the instructions of the commercial ELISA kits (Abcam).

Dual-luciferase reporter assay

The wild-type (WT) fragments of PVT1 or CAMK4 3'UTR possessing miR-206-3p binding sites and mutated (MUT) fragments were amplified and cloned into pmirGLO luciferase reporters (Promega, Madison, WI, USA). Later on, miR-206-3p mimic and negative control were then separately co-transfected into hippocampal neurons at 50 mM with 100 ng of recombinant luciferase reporter vectors (WT-PVT1, WT-CAMK4 3'UTR, MUT-PVT1, or MUT-CAMK4 3'UTR). 48 h later, the detection of luciferase activity analysis was performed by adopting the Dual Luciferase Reporter Gene Assay Kit (Promega).

RNA immunoprecipitation (RIP) assay

Hippocampal neurons were homogenized in complete RNA lysis buffer, and then incubated with magnetic beads (Millipore) coupled with negative control IgG antibody or Ago2 antibody at 37°C for 4 h. After treated with Proteinase K for 30 min, the RNA immunoprecipitation was isolated for qRT-PCR analysis.

Statistical analysis

All data were presented as the mean ± SD. Each experiment was repeated at least three times. Statistical analysis and graph drawing were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Comparisons were carried out by using Student's *t*-test, Mann-Whitney, or analysis of variance. $p < 0.05$ suggests a statistically significant difference.

Results

Knockdown of PVT1 relieves SE-induced apoptosis and inflammation in hippocampal neurons

As shown in Figure 1A, PVT1 expression was higher in the hippocampus of SE mice than those in the Sham group and the control group, and there was no statistically significant difference in PVT1 expression between the two groups. Thereafter, hippocampal neurons were isolated from the hippocampus of SE mice or the control group. As expected, PVT1 expression was also increased in hippocampal neurons from SE mice compared with the control (Fig. 1B). Then PVT1 siRNA was designed and transfected into SE hippocampal neurons to conduct loss-of-function experiments. The introduction of si-PVT1 significantly reduced PVT1 expression level in hippocampal neurons of SE mice (Fig. 1C). Functionally, the viability and DNA synthesis activity of hippocampal neurons in SE mice were suppressed, while this condition was attenuated by PVT1 knockdown (Fig. 1D,E). Meanwhile, flow cytometry showed a higher apoptosis rate of hippocampal neurons in the SE group, which was reduced after PVT1 knockdown (Fig. 1F), and the inhibition

on SE hippocampal neuron apoptosis after PVT1 down-regulation was also confirmed by the decrease of Bax and Cleaved-caspase-3 as well as the increase of Bcl-2 (Fig. 1G). Besides that, silencing PVT1 protected against SE-induced inflammation in hippocampal neurons, evidenced by the reduction of TNF-α and IL-6 levels (Fig. 1H,I).

PVT1 acts as a sponge for miR-206-3p

It has been proposed that lncRNA located in the cytoplasm can function as a sponge for miRNA to exert its effects (Salmena et al. 2011). Then we determined the subcellular localization of PVT1 by nuclear-cytoplasmic fractiona-

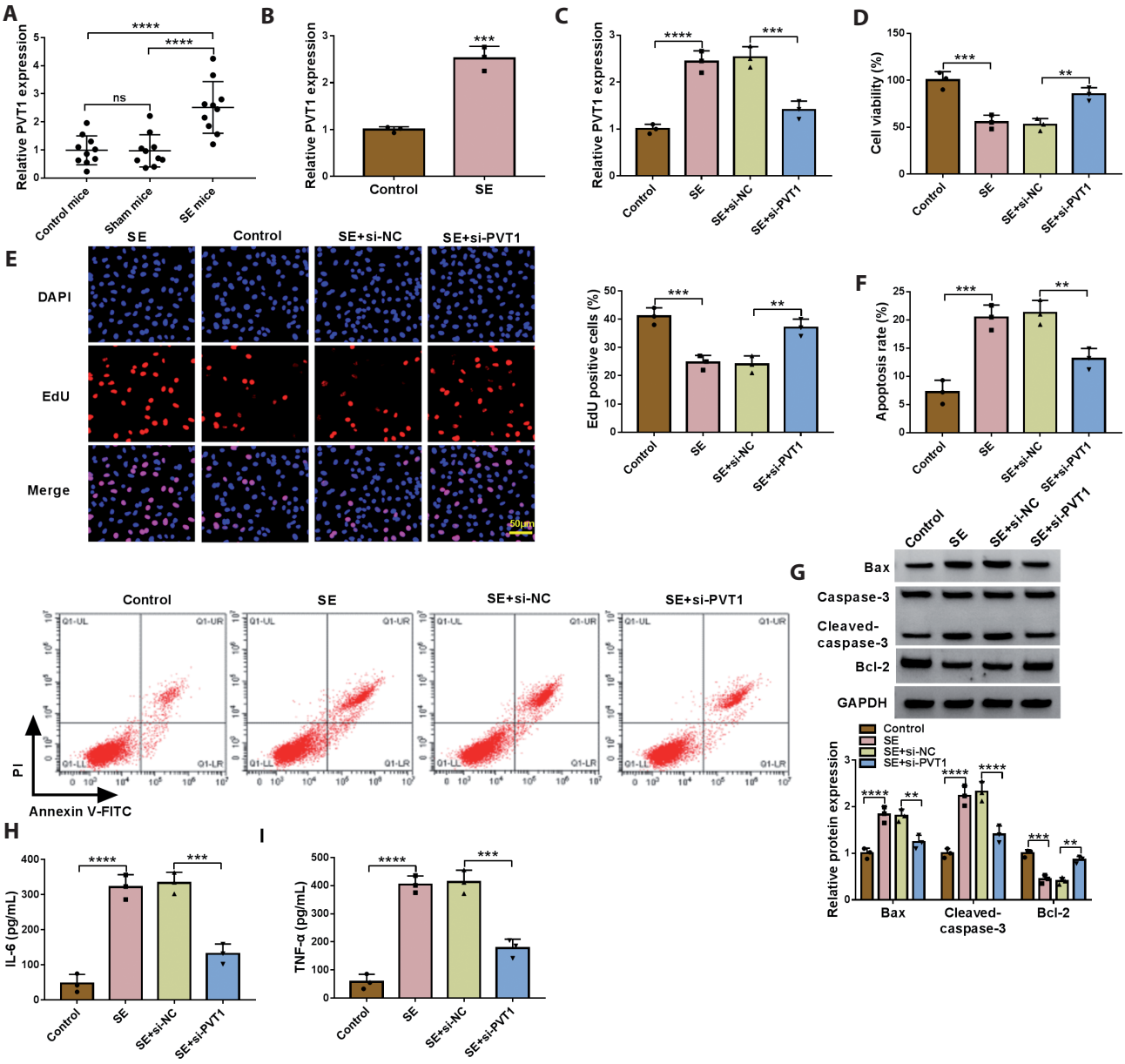

Figure 1. Knockdown of PVT1 relieves SE-induced apoptosis and inflammation in hippocampal neurons. **A, B.** The expression level of PVT1 detected in the hippocampus (n = 10, F = 16.32) and in hippocampal neurons (n = 3, t = 9.797) by qRT-PCR. SE hippocampal neurons were transfected with si-PVT1 or si-NC (n = 3; C–I). **C.** qRT-PCR analysis of PVT1 expression after transfection (n = 3, F = 45.84). **D, E.** Cell proliferation analyzed using CCK-8 (F = 27.6) and EdU (F = 26.8) assays (n = 3). **F.** Flow cytometry for cell apoptosis (n = 3, F = 30.09). **G.** Western blotting analysis for the protein level of Bax, Bcl-2, Caspase-3 and Cleaved-caspase-3 in cells (n = 3, F = 36.03). **H, I.** ELISA analysis for the levels of IL-6 (F = 66.14) and TNF-α (F = 82.4) in cell culture media (n = 3). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

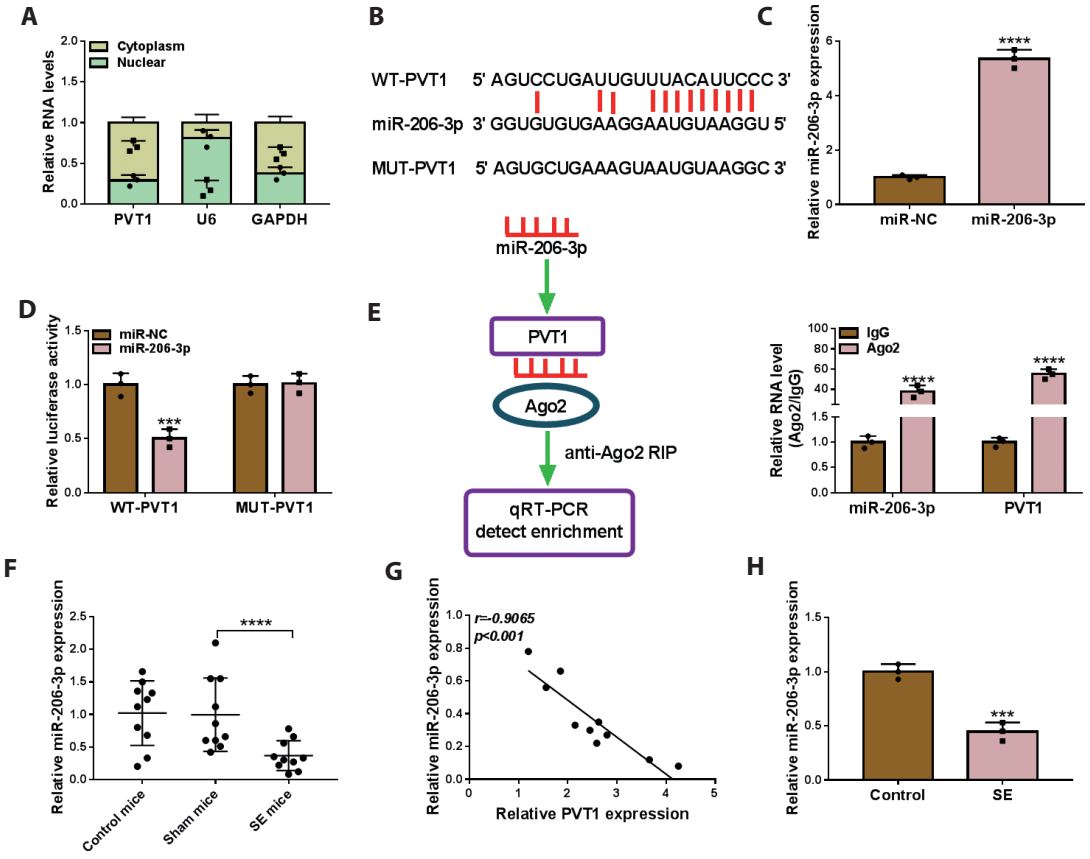

Figure 2. PVT1 acts as a sponge for miR-206-3p. **A.** Nuclear-cytoplasmic fractionation assay showed that PVT1 was mainly localized in the cytoplasm of hippocampal neurons ($n = 3$, F = 69.05). **B.** The putative conserved target site of miR-206-3p on PVT1 was predicted by starbase database. **C.** The transfection efficiency of miR-206-3p mimic and the mimic control was validated using qRT-PCR in hippocampal neurons obtained from the SE group ($n = 3$, t = 21.91). **D.** Dual-luciferase reporter assay for the luciferase activity of wild-type and mutated PVT1 reporter after miR-206-3p overexpression in hippocampal neurons ($n = 3$, F = 23.74). **E.** qRT-PCR analysis of miR-206-3p and PVT1 after RIP assay ($n = 3$, F = 14.21). **F.** qRT-PCR analysis of miR-206-3p expression in hippocampus from SE mice, Sham group or control group ($n = 10$, F = 6.687). **G.** The correlation analysis between miR-206-3p and PVT1 in hippocampus from SE mice (r = 0.9065). **H.** qRT-PCR analysis of miR-206-3p expression in hippocampal neurons from SE mice or control group ($n = 3$, t = 8.701). *** $p < 0.001$, **** $p < 0.0001$.

tion assay, and the results exhibited that PVT1 was mainly distributed in the cytoplasm of SE hippocampal neurons (Fig. 2A). Therefore, the potential miRNAs interaction with PVT1 was investigated. According to the prediction of the starbase database, there is a binding region between PVT1 and miR-206-3p (Fig. 2B). qRT-PCR showed that the level of miR-206-3p was significantly elevated by miR-206-3p mimic compared with the negative control in hippocampal neurons obtained from the SE group (Fig. 2C) or Sham group (Fig. S1 in Supplementary material). Then the dual-luciferase reporter assay was conducted, and the results showed that miR-206-3p mimic overtly reduced the luciferase activity of wild-type PVT1 reporter vector in SE hippocampal neurons but not the mutant one (Fig. 2D). Furthermore, RIP assay suggested that Ago2 combined with PVT1 and miR-206-3p was significantly increased compared with the IgG negative control (Fig. 2E). These results verified that PVT1 could bind to miR-206-3p. The expression of miR-206-3p was found to be decreased in the hippocampus of SE mice than those in the Sham group (Fig. 2F), which was negatively correlated with PVT1 (Fig. 2G). Similarly, a decreased expression of miR-206-3p was also observed in hippocampal neurons isolated from the hippocampus of SE mice compared with the control group (Fig. 2H).

Knockdown of PVT1 relieves SE-induced apoptosis and inflammation by regulating miR-206-3p in hippocampal neurons

Subsequently, whether miR-206-3p mediated the action of PVT1 on hippocampal neuron apoptosis and inflammation was investigated. SE hippocampal neurons were

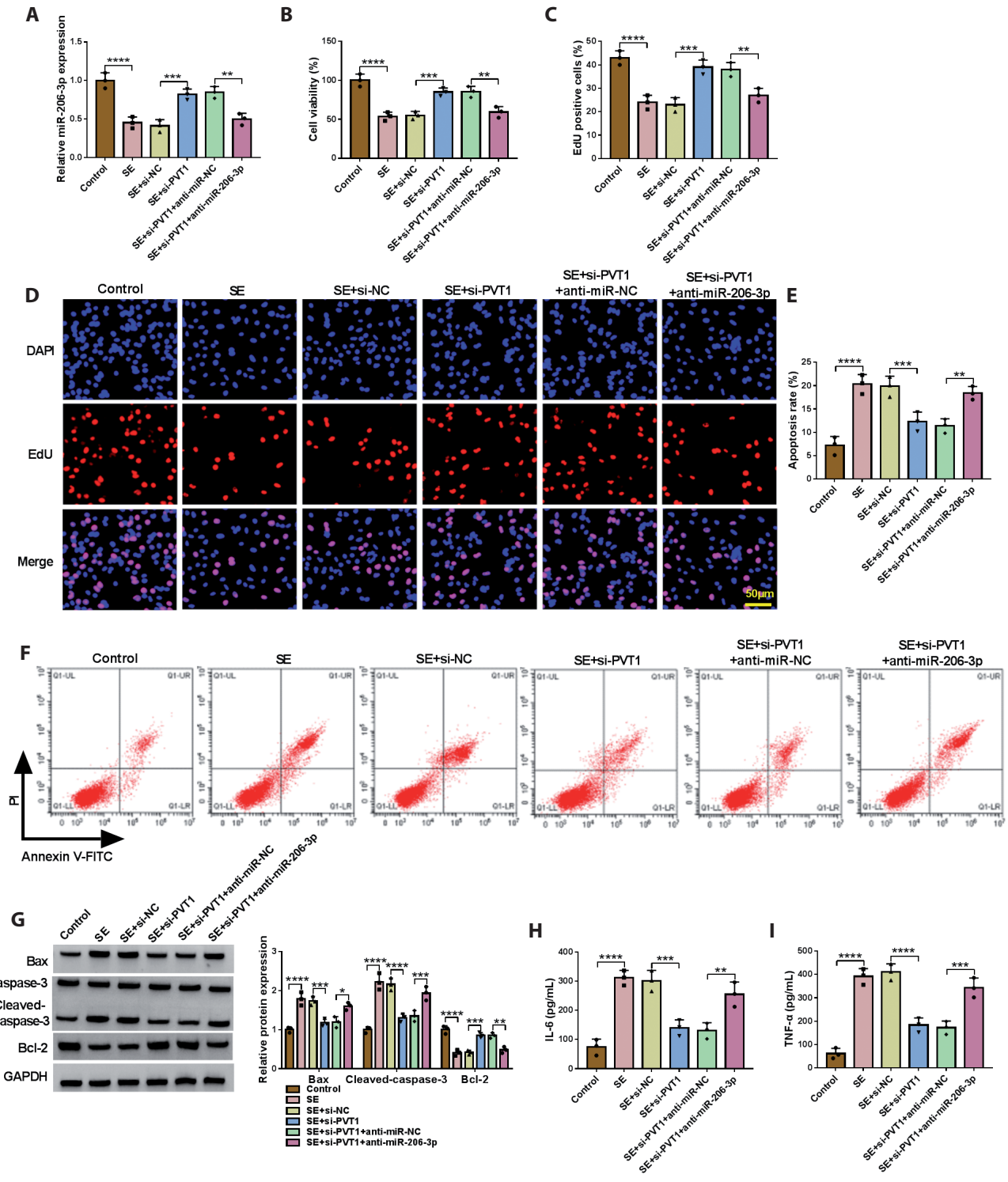

Figure 3. Knockdown of PVT1 relieves SE-induced apoptosis and inflammation by targeting miR-206-3p in hippocampal neurons. SE hippocampal neurons were co-transfected with si-NC, si-PVT1, si-PVT1+anti-miR-NC, or si-PVT1+anti-miR-206-3p. **A.** qRT-PCR analysis of miR-206-3p expression after transfection ($n = 3$, F = 28.6). **B.–D.** Cell proliferation was analyzed using CCK-8 (F = 16.06) and EdU (F = 25.02) assays ($n = 3$). **E, F.** Flow cytometry assay for cell apoptosis ($n = 3$, F = 24.73). **G.** Western blotting analysis for the protein level of Bax, Bcl-2, Caspase-3, and Cleaved-caspase-3 in cells ($n = 3$, F = 34.77). **H, I.** ELISA analysis for the levels of IL-6 (F = 36.03) and TNF-α (F = 30.54) in cell culture media ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

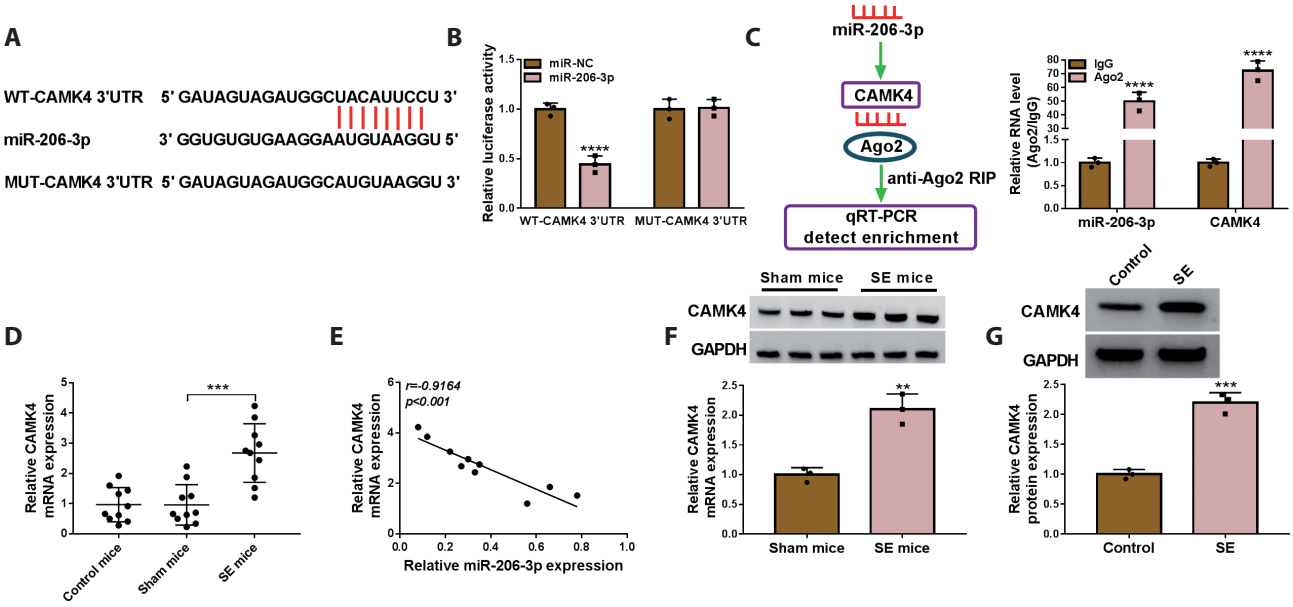

Figure 4. CAMK4 is a target of miR-206-3p. **A.** The putative binding site between CAMK4 and miR-206-3p. **B.** Dual-luciferase reporter assay for the luciferase activity of wild-type and mutated CAMK4 reporter after miR-206-3p overexpression in hippocampal neurons (n = 3, F = 34.36). **C.** qRT-PCR analysis of miR-206-3p and CAMK4 after RIP assay (n = 3, F = 16.2). **D.** qRT-PCR analysis of CAMK4 expression in hippocampus from SE mice, Sham group or control group (n = 10, F = 17.18). **E.** The correlation analysis between miR-206-3p and CAMK4 in hippocampus from SE mice (r = 0.9164). **F, G.** Western blotting analysis of CAMK4 expression in hippocampus from SE mice or Sham group (n = 10, t = 6.802), as well as in hippocampal neurons from SE mice or control group (n = 3, t = 11.22). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

co-transfected with PVT1 siRNA and/or miR-206-3p inhibitor, and qRT-PCR showed that the introduction of miR-206-3p inhibitor attenuated si-PVT1-caused elevation of miR-206-3p in SE hippocampal neurons (Fig. 3A). Thereafter, it was proved that miR-206-3p inhibition attenuated PVT1 silencing-evoked cell proliferation enhancement (Fig. 3B–D), apoptosis arrest (Fig. 3E–G), and inflammation inhibition (Fig. 3H,I) in SE hippocampal neurons.

CAMK4 is a target of miR-206-3p

Thereafter, we further investigated the underlying targets of miR-206-3p. The Starbase database predicted that miR-206-3p had many targeted mRNAs. Through the literature search, four mRNAs (TLR4, CAMK4, ROCK2, and OXSR1) that were highly expressed in epilepsy and promoted the progression of epilepsy were selected. As shown in Figure S2 in Supplementary material, we found that miR-206-3p overexpression reduced the expression of TLR4 and CAMK4 in hippocampal neurons, especially CAMK4, but not affected ROCK2 and OXSR1. Thus, we selected CAMK4 and further confirmed whether CAMK4 is a target of miR-206-3p. The putative binding site of CAMK4 on miR-206-3p was shown in Figure 4A. Dual-luciferase reporter assay showed that miR-206-3p overexpression overtly reduced the luciferase activity of wild-type CAMK4 reporter vector in SE hippocampal neurons, but did not affect the luciferase activity of the mutant one (Fig. 4B). RIP assay suggested that CAMK4 and miR-206-3p were significantly pulled down by Ago2 antibody relative to the IgG negative control (Fig. 4C), further verifying the binding between CAMK4 and miR-206-3p. We then found that CAMK4 mRNA was increased in the hippocampus of SE mice than those in the Sham group (Fig. 4D) and was negatively correlated with miR-206-3p (Fig. 4E). Besides that, the protein level of CAMK4 was also elevated in the hippocampus of SE mice and SE hippocampal neurons (Fig. 4F,G).

Knockdown of CAMK4 relieves SE-induced apoptosis and inflammation in hippocampal neurons

To study the action of CAMK4 in hippocampal neuron apoptosis and inflammation, we designed the siRNA targeting CAMK4 to knock down CAMK4 in hippocampal neurons, and the knockdown efficiency of si-CAMK4 was validated by Western blotting (Fig. 5A). Functionally, CAMK4 knockdown reversed SE-induced proliferation arrest (Fig. 5B–D) and apoptosis promotion (Fig. 5E,F) in hippocampal neurons. Besides that, the levels of Bax and Cleaved-caspase-3 were reduced, while Bcl-2 expression was increased by CAMK4 knockdown in SE hippocampal neurons (Fig. 5G,H). Meanwhile, ELISA analysis suggested

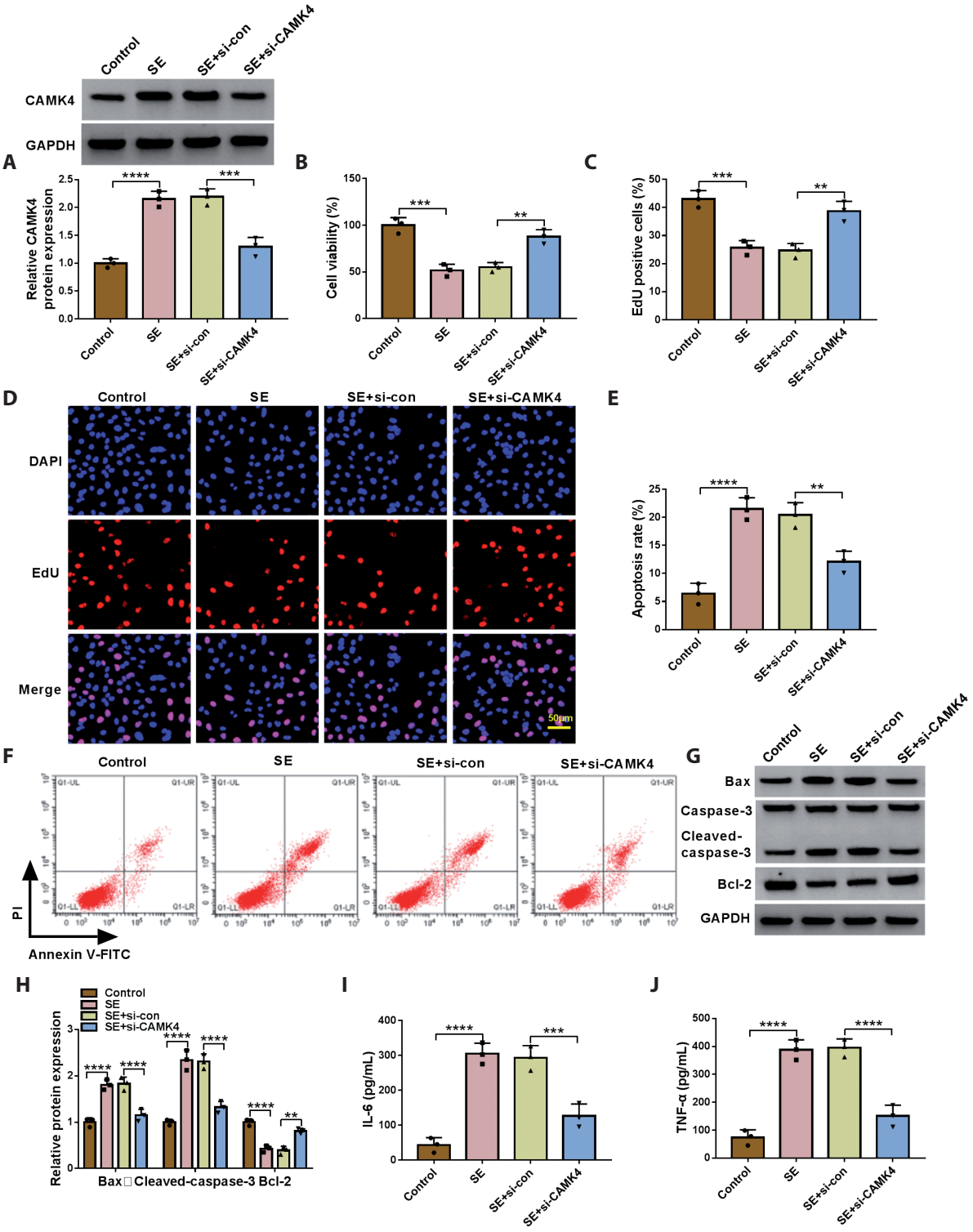

Figure 5. Knockdown of CAMK4 relieves SE-induced apoptosis and inflammation in hippocampal neurons. SE hippocampal neurons were transfected with si-CAMK4 or si-NC. **A.** qRT-PCR analysis of CAMK4 expression after transfection (n = 3, F = 58.27). **B–D.** Cell proliferation was analyzed using CCK-8 (F = 36.12) and EdU (F = 30.04) assays (n = 3). **E, F.** Flow cytometry for cell apoptosis (n = 3, F = 38.75). **G, H.** Western blotting analysis for the protein level of Bax, Bcl-2, Caspase-3 and Cleaved-caspase-3 in cells (n = 3, F = 52.01). **I, J.** ELISA analysis for the levels of IL-6 (F = 30.09) and TNF-α (F = 72.14) in cell culture media (n = 3). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

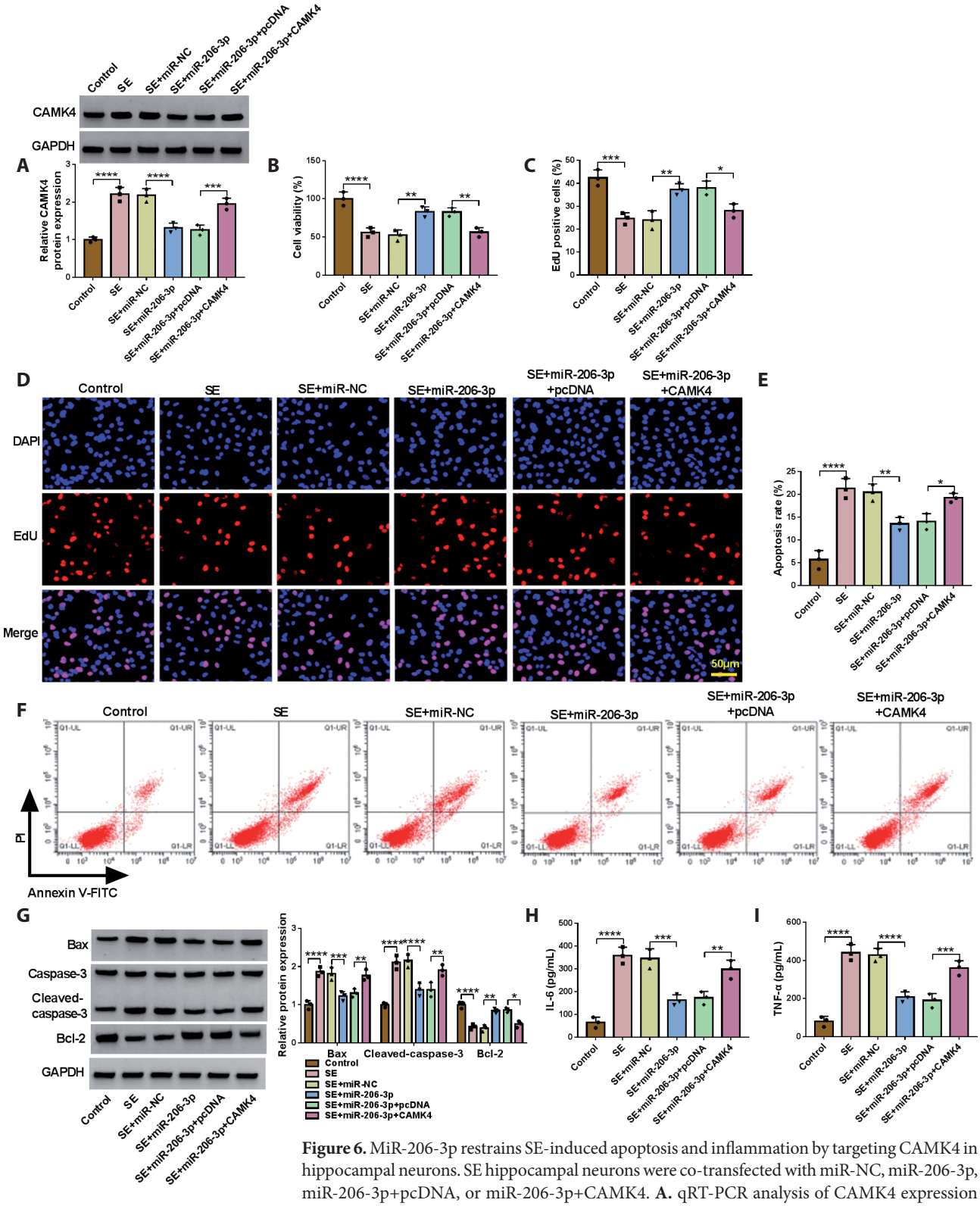

Figure 6. MiR-206-3p restrains SE-induced apoptosis and inflammation by targeting CAMK4 in hippocampal neurons. SE hippocampal neurons were co-transfected with miR-NC, miR-206-3p, miR-206-3p+pcDNA, or miR-206-3p+CAMK4. **A.** qRT-PCR analysis of CAMK4 expression after transfection ($n = 3$, F = 39.16). **B–D.** Cell proliferation was analyzed using CCK-8 (F = 24.87) and EdU (F = 18.55) assays ($n = 3$). **E, F.** Flow cytometry for cell apoptosis ($n = 3$, F = 34.35). **G.** Western blotting analysis for the protein level of Bax, Bcl-2, Caspase-3 and Cleaved-caspase-3 in cells ($n = 3$, F = 28.98). **H, I.** ELISA analysis for the levels of IL-6 (F = 37.14) and TNF-α (F = 30.54) in cell culture media ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

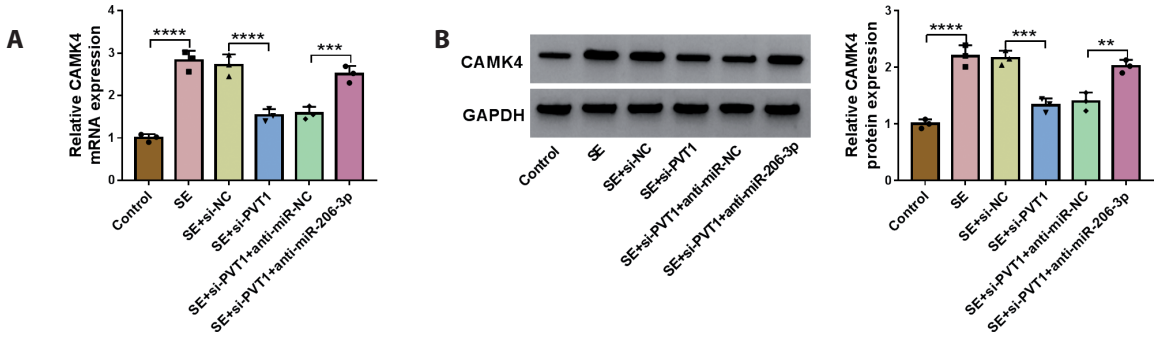

Figure 7. The identification of PVT1/miR-206-3p/CAMK4 axis in hippocampal neurons. **A, B.** qRT-PCR and Western blotting analysis of CAMK4 expression in SE hippocampal neurons transfected with si-NC, si-PVT1, si-PVT1+anti-miR-NC, or si-PVT1+anti-miR-206-3p (n = 3, A: F = 47.86, B: F = 40.88). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

that CAMK4 silencing reduced the release of TNF-α and IL-6 in SE hippocampal neurons (Fig. 5I,J).

MiR-206-3p restrains SE-induced hippocampal neuron apoptosis and inflammation by CAMK4

Next, the role of miR-206-3p/CAMK4 axis in SE-induced hippocampal neuron injury was explored. SE hippocampal neurons were co-transfected with miR-206-3p mimic and/or CAMK4 overexpression plasmid, and Western blotting showed that miR-206-3p mimic led to a decrease of CAMK4 expression, which was rescued by CAMK4 plasmid transfection in cells (Fig. 6A). Thereafter, we demonstrated that miR-206-3p suppressed SE-induced hippocampal neuron proliferation inhibition (Fig. 6B–D) and apoptosis enhancement (Fig. 6E–G), which were mitigated by CAMK4 up-regulation (Fig. 6B–G). Additionally, the release of TNF-α and IL-6 was decreased after miR-206-3p re-expression in SE hippocampal neurons, while CAMK4 up-regulation counteracted this action (Fig. 6H,I).

The identification of PVT1/miR-206-3p/CAMK4 axis in hippocampal neurons

Bivariate correlation analysis was processed to determine the interactions among PVT1, miR-206-3p, and CAMK4. As exhibited in Figure 7A and B, PVT1 knockdown caused a decrease of CAMK4 expression, which was rescued by miR-206-3p down-regulation in SE hippocampal neurons, indicating the PVT1/miR-206-3p/CAMK4 regulatory pathway in hippocampal neurons.

Discussion

To date, a large amount of lncRNAs have been discovered in the hippocampus and are involved in the dysfunction of hippocampal neurons in epilepsy patients. For example, Nespas lncRNA was found to suppress the loss of hippocampal neurons in epilepsy (Feng et al. 2021). Hu et al. showed that ZFAS1 promoted the autophagy and apoptosis of hippocampal neurons in epilepsy by inducing PI3K/AKT pathway activation through sequestering miR-421 (Hu et al. 2020). Geng's team suggested that UCA1 could suppress hippocampal neuron apoptosis by elevating Nrf2 *via* miR-495 in epilepsy (Geng et al. 2018). Therefore, lncRNAs may be potential therapeutic biomarkers for epilepsy prevention. In our study, silencing of PVT1 reversed SE-evoked proliferation arrest, apoptosis promotion, and proinflammatory cytokine in hippocampal neurons. Bax is a pro-apoptotic protein and the core regulator of intrinsic pathway of apoptosis that can activate the cascade of reactions by releasing cytochrome c from the mitochondria, leading to the activation of caspases to cause cell death (Peña-Blanco and García-Sáez 2018). When Bax expression is high, cells are sensitive to death signals and promote apoptosis, while Bcl-2 is an anti-apoptotic protein, it can form heterodimer with Bax and inhibit apoptosis, the intracellular Bcl-2/Bax ratio plays an important role in determining the sensitivity of apoptosis (Kulsoom et al. 2018). Caspase-3 is the main effector caspase that plays a key role in apoptosis, Cleaved caspase-3 is the active form of caspase-3 during apoptosis, which can proteolytically cleave and activate other caspases, or other relevant targets in cells to promote apoptosis (Liu et al. 2018). IL-6 and TNF-α are major proinflammatory cytokines and have been detected in a variety of brain diseases, and they are found to reinforce inflammatory and immune reactions, which can lead to the death of oligodendrocytes and neurons in the nervous system, or neuronal death in the hippocampus (Ishijima and Nakajima 2021). In this study, we also found that PVT1 knockdown led to the decreases of Bax and Cleaved caspase-3 protein levels, as well as IL-6 and TNF-α levels, but the increase of Bcl-2 protein levels in hippocampal neurons, further suggesting the inhibition of

PVT1 on neuronal apoptosis and inflammation. These data suggested that PVT1 siRNA may have roles in the prevention of epilepsy.

Currently, the role lncRNA/miRNA/mRNA network in the genesis and progression of epilepsy has gradually emerged (Geng et al. 2018; Li et al. 2019). Our study showed the cytoplasmic location of PVT1 in hippocampal neurons, and then identified the PVT1/miR-206-3p/CAMK4 regulatory axis in cells. The involvement of miRNAs in epilepsy biology has been widely discussed (Henshall et al. 2016). A previous study showed that miR-206 re-expression could protect against neuronal loss and inflammation in epileptic rat models *via* binding to CCL2 (Wu et al. 2019). Therefore, we speculated that miR-206-3p might also protect against epilepsy-induced brain injury. As expected, miR-206-3p overexpression suppressed apoptosis and inflammatory response in SE hippocampal neurons, accompanied by decreased Bax, Cleaved caspase-3, IL-6 and TNF-α levels, as well as increased Bcl-2 levels. Interestingly, the knockdown of miR-206-3p reversed the protective action of PVT1 siRNA on hippocampal neurons. CAMK4, a vital mediator of calcium-mediated dynamics and activity, has been shown to be implicated in neuronal homeostasis and brain development (Zech et al. 2018). Besides that, altered CAMK4 expression was reported to attenuate miR-125a-5p-caused inhibition of inflammation and seizure in epilepsy rats (Liu et al. 2019). Thus, this study investigated the function of CAMK4 in epilepsy and found that CAMK4 knockdown could protect hippocampal neurons from SE-induced apoptotic and inflammatory injuries by elevating Bcl-2 levels and reducing Bax, Cleaved caspase-3, IL-6 and TNF-α levels. Importantly, CAMK4 overexpression could abolish the protective effects of miR-206-3p on hippocampal neurons.

In conclusion, our work first clarified that PVT1 silencing could alleviate SE-evoked apoptosis and inflammatory response in hippocampal neurons by miR-206-3p/CAMK4 axis. However, there are still some limitations. The brainwaves should be recorded, and the morphological change in the hippocampus should be observed by using HE staining and Nissl's staining to investigate the effects of PVT1 on epileptic mice. In addition, the function of PVT1 siRNA in healthy hippocampal neurons or mice should be measured to probe the application of PVT1 siRNA in clinical use in the future. Even so, this research also may fill the gap in knowledge about the molecular contribution of PVT1 in epilepsy development.

Data availability statement. The datasets analysed during the current study are available from the corresponding author.

Conflicts of interest. The authors declare that they have no conflicts of interest.

References

Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, Guernec G, Martin D, Merkel A, Knowles DG, et al. (2012): The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res. **22,** 1775-1789 https://doi.org/10.1101/gr.132159.111

Ding D, Zhou D, Sander JW, Wang W, Li S, Hong Z (2021): Epilepsy in China: major progress in the past two decades. Lancet Neurol. **20,** 316-326 https://doi.org/10.1016/S1474-4422(21)00023-5

Feng H, Gui Q, Wu G, Zhu W, Dong X, Shen M, Fu X, Shi G, Luo H, Yang X, et al. (2021): Long noncoding RNA Nespas inhibits apoptosis of epileptiform hippocampal neurons by inhibiting the PI3K/Akt/mTOR pathway. Exp. Cell Res. **398,** 112384 https://doi.org/10.1016/j.yexcr.2020.112384

Geisler S, Coller J (2013): RNA in unexpected places: long noncoding RNA functions in diverse cellular contexts. Nat. Rev. Mol. Cell. Biol. **14,** 699-712 https://doi.org/10.1038/nrm3679

Geng JF, Liu X, Zhao HB, Fan WF, Geng JJ, Liu XZ (2018): LncRNA UCA1 inhibits epilepsy and seizure-induced brain injury by regulating miR-495/Nrf2-ARE signal pathway. Int. J. Biochem. Cell Biol. **99,** 133-139 https://doi.org/10.1016/j.biocel.2018.03.021

He W, Yuan QH, Zhou Q (2018): Histamine H3 receptor antagonist Clobenpropit protects propofol-induced apoptosis of hippocampal neurons through PI3K/AKT pathway. Eur. Rev. Med. Pharmacol. Sci. **22,** 8013-8020

Henshall DC, Hamer HM, Pasterkamp RJ, Goldstein DB, Kjems J, Prehn JHM, Schorge S, Lamottke K, Rosenow F (2016): MicroRNAs in epilepsy: pathophysiology and clinical utility. Lancet Neurol. **15,** 1368-1376 https://doi.org/10.1016/S1474-4422(16)30246-0

Hu F, Shao L, Zhang J, Zhang H, Wen A, Zhang P (2020): Knockdown of ZFAS1 inhibits hippocampal neurons apoptosis and autophagy by activating the PI3K/AKT pathway via up-regulating miR-421 in epilepsy. Neurochem. Res. **45,** 2433-2441 https://doi.org/10.1007/s11064-020-03103-1

Ishijima T, Nakajima K (2021): Inflammatory cytokines TNFα, IL-1β, and IL-6 are induced in endotoxin- stimulated microglia through different signaling cascades. Sci. Prog. **104,** 368504211054985 https://doi.org/10.1177/00368504211054985

Kulsoom B, Shamsi TS, Afsar NA, Memon Z, Ahmed N, Hasnain SN (2018): Bax, Bcl-2, and Bax/Bcl-2 as prognostic markers in acute myeloid leukemia: are we ready for Bcl-2-directed therapy? Cancer Manag. Res. **10,** 403-416 https://doi.org/10.2147/CMAR.S154608

Li X, Giri V, Cui Y, Yin M, Xian Z, Li J (2019): LncRNA FTX inhibits hippocampal neuron apoptosis by regulating miR-21-5p/SOX7 axis in a rat model of temporal lobe epilepsy. Biochem. Biophys. Res. Commun. **512,** 79-86 https://doi.org/10.1016/j.bbrc.2019.03.019

Lin N, Chang KY, Li Z, Gates K, Rana ZA, Dang J, Zhang D, Han T, Yang CS, Cunningham TJ, et al. (2014): An evolutionarily

conserved long noncoding RNA TUNA controls pluripotency and neural lineage commitment. Mol. Cell. **53,** 1005-1019 https://doi.org/10.1016/j.molcel.2014.01.021

Liu Q, Wang L, Yan G, Zhang W, Huan Z, Li J (2019): MiR-125a-5p alleviates dysfunction and inflammation of pentylenetetrazol-induced epilepsy through targeting calmodulin-dependent protein kinase IV (CAMK4). Curr. Neurovasc. Res. **16,** 365-372 https://doi.org/10.2174/1567202616666190906125444

Liu X, Jiang S, Tian X, Jiang Y (2018): Expression of cleaved caspase-3 predicts good chemotherapy response but poor survival for patients with advanced primary triple-negative breast cancer. Int. J. Clin. Exp. Pathol. **11,** 4363-4373

Liu YQ, Yu F, Liu WH, He XH, Peng BW (2014): Dysfunction of hippocampal interneurons in epilepsy. Neurosci. Bull. **30,** 985-998 https://doi.org/10.1007/s12264-014-1478-4

Ma Y, Zhang H, Li G, Hu J, Liu X, Lin L (2019): LncRNA ANRIL promotes cell growth, migration and invasion of hepatocellular carcinoma cells via sponging miR-144. Anticancer Drugs **30,** 1013-1021 https://doi.org/10.1097/CAD.0000000000000807

Martínez-Barriocanal Á, Arango D, Dopeso H (2020): PVT1 long non-coding RNA in gastrointestinal cancer. Front. Oncol. **10,** 38 https://doi.org/10.3389/fonc.2020.00038

Massone S, Vassallo I, Fiorino G, Castelnuovo M, Barbieri F, Borghi R, Tabaton M, Robello M, Gatta E, Russo C, et al. (2011): 17A, a novel non-coding RNA, regulates GABA B alternative splicing and signaling in response to inflammatory stimuli and in Alzheimer disease. Neurobiol. Dis. **41,** 308-317 https://doi.org/10.1016/j.nbd.2010.09.019

Mesraoua B, Deleu D, Kullmann DM, Shetty AK, Boon P, Perucca E, Mikati MA, Asadi-Pooya AA (2019): Novel therapies for epilepsy in the pipeline. Epilepsy Behav. **97,** 282-290 https://doi.org/10.1016/j.yebeh.2019.04.042

Pan X, Zheng G, Gao C (2018): LncRNA PVT1: a novel therapeutic target for cancers. Clin. Lab. **64,** 655-662 https://doi.org/10.7754/Clin.Lab.2018.171216

Peña-Blanco A, García-Sáez AJ (2018): Bax, Bak and beyond - mitochondrial performance in apoptosis. FEBS J. **285,** 416-431 https://doi.org/10.1111/febs.14186

Racine RJ (1972): Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr. Clin. Neurophysiol. **32,** 281-294 https://doi.org/10.1016/0013-4694(72)90177-0

Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP (2011): A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell **146,** 353-358 https://doi.org/10.1016/j.cell.2011.07.014

Schmitz SU, Grote P, Herrmann BG (2016): Mechanisms of long noncoding RNA function in development and disease. Cell Mol. Life Sci. **73,** 2491-2509 https://doi.org/10.1007/s00018-016-2174-5

Statello L, Guo CJ, Chen LL, Huarte M (2021): Gene regulation by long non-coding RNAs and its biological functions. Nat. Rev. Mol. Cell. Biol. **22,** 96-118 https://doi.org/10.1038/s41580-020-00315-9

Thijs RD, Surges R, O'Brien TJ, Sander JW (2019): Epilepsy in adults. Lancet **393,** 689-701 https://doi.org/10.1016/S0140-6736(18)32596-0

Vezzani A (2009): Pilocarpine-induced seizures revisited: what does the model mimic? Epilepsy Curr. **9,** 146-148 https://doi.org/10.1111/j.1535-7511.2009.01323.x

Wan Y, Yang ZQ (2020): LncRNA NEAT1 affects inflammatory response by targeting miR-129-5p and regulating Notch signaling pathway in epilepsy. Cell Cycle **19,** 419-431 https://doi.org/10.1080/15384101.2020.1711578

Wang Z, Na Z, Cui Y, Wei C, Wang S (2022): LncRNA ZFAS1 regulates the hippocampal neurons injury in epilepsy through the miR-15a-5p/OXSR1/NF-κB pathway. Metab. Brain Dis. **37,** 2277-2290 https://doi.org/10.1007/s11011-022-01013-5

Wu Z, Liu Y, Huang J, Huang Y, Fan L (2019): MiR-206 inhibits epilepsy and seizure-induced brain injury by targeting CCL2. Cytotechnology **71,** 809-818 https://doi.org/10.1007/s10616-019-00324-3

Yan B, Yao J, Liu JY, Li XM, Wang XQ, Li YJ, Tao ZF, Song YC, Chen Q, Jiang Q (2015): lncRNA-MIAT regulates microvascular dysfunction by functioning as a competing endogenous RNA. Circ. Res. **116,** 1143-1156 https://doi.org/10.1161/CIRCRESAHA.116.305510

Zech M, Lam DD, Weber S, Berutti R, Poláková K, Havránková P, Fečíková A, Strom TM, Růžička E, Jech R, et al. (2018): A unique de novo gain-of-function variant in CAMK4 associated with intellectual disability and hyperkinetic movement disorder. Cold Spring Harb. Mol. Case Stud. **4,** a003293 https://doi.org/10.1101/mcs.a003293

Zhao MW, Qiu WJ, Yang P (2020): SP1 activated-lncRNA SNHG1 mediates the development of epilepsy via miR-154-5p/TLR5 axis. Epilepsy Res. **168,** 106476 https://doi.org/10.1016/j.eplepsyres.2020.106476

Zhao T, Ding Y, Li M, Zhou C, Lin W (2019): Silencing lncRNA PVT1 inhibits activation of astrocytes and increases BDNF expression in hippocampus tissues of rats with epilepsy by downregulating the Wnt signaling pathway. J. Cell. Physiol. **234,** 16054-16067 https://doi.org/10.1002/jcp.28264

Zheng J, Liu X, Wang P, Xue Y, Ma J, Qu C, Liu Y (2016): CRNDE promotes malignant progression of glioma by attenuating miR-384/PIWIL4/STAT3 axis. Mol. Ther. **24,** 1199-1215 https://doi.org/10.1038/mt.2016.71

Zhu H, Xu H, Ma H, Luo L, Yang L, Chen F, Qu X, Liu H, Zhang R (2020): LncRNA CASC2 inhibits astrocytic activation and adenosine metabolism by regulating PTEN in pentylenetetrazol-induced epilepsy model. J. Chem. Neuroanat. **105,** 101749 https://doi.org/10.1016/j.jchemneu.2020.101749

Received: February 2, 2024
Final version accepted: April 11, 2024

doi: 10.4149/gpb_2024022

Supplementary Material

PVT1 regulates hippocampal neuron apoptosis and inflammation in epilepsy by miR-206-3p-dependent regulation of CAMK4

Chuankun Li[1], Ruichun Li[1], Xiaobin Bai[1] and Haitao Jiang[1]

[1] *Department of Neurosurgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China*

Supplementary Figures

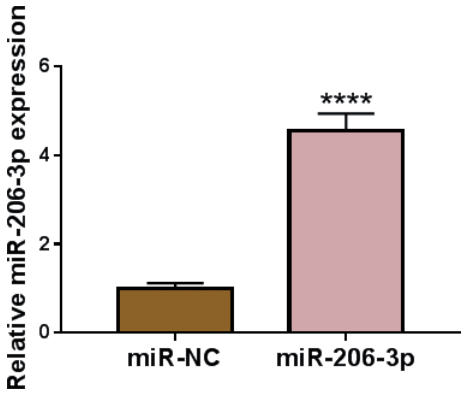

Figure S1. The transfection efficiency of miR-206-3p mimic and the mimic control. The transfection efficiency of miR-206-3p mimic and the mimic control was validated using qRT-PCR in hippocampal neurons obtained from Sham group (n = 3, t = 15.6). **** $p < 0.0001$.

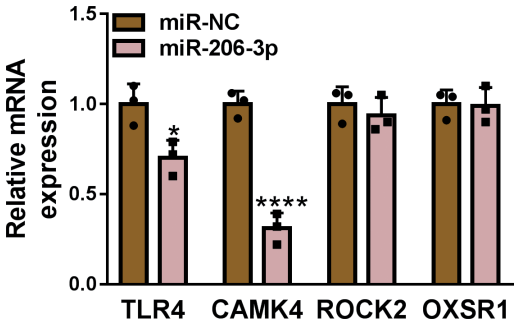

Figure S2. The effects of miR-206-3p mimic on the expression of predicted mRNAs. The expression levels of four predicted mRNAs (TLR4, CAMK4, ROCK2, and OXSR1) were detected using qRT-PCR in hippocampal neurons transfected with miR-206-3p mimic or miR-NC (n = 3, F = 16.5). * $p < 0.05$, **** $p < 0.0001$.