RESEARCH ARTICLE

MicroRNA-219 alleviates glutamate-induced neurotoxicity in cultured hippocampal neurons by targeting calmodulin-dependent protein kinase II gamma

Ting Wang[1, 2], Qun Cai[3], Wen-Jie Yang[4], Hai-Hua Fan[4], Jian-Feng Yi[4], Feng Xu[1, *]

1 Department of Emergency, First Affiliated Hospital of Soochow University, Suzhou, Jiangsu Province, China
2 Department of Emergency, Affiliated Hospital of Nantong University, Nantong, Jiangsu Province, China
3 Department of Pediatrics, Affiliated Hospital of Nantong University, Nantong, Jiangsu Province, China
4 Medical College of Nantong University, Nantong, Jiangsu Province, China

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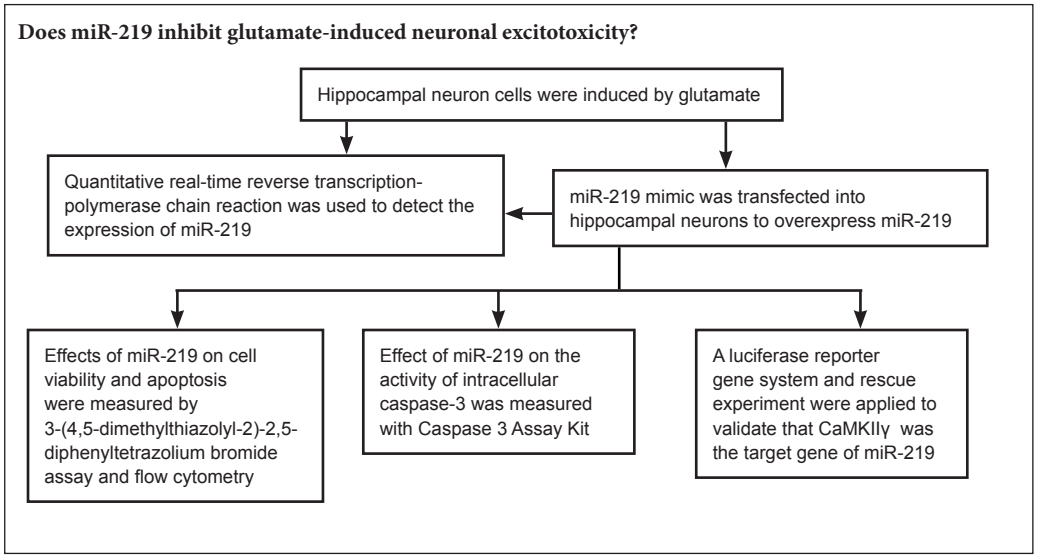
Graphical Abstract



Correspondence to:
Feng Xu, Ph.D., M.D., suda_xf@163.com.

orcid:
0000-0002-1496-9141 (Feng Xu)

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Abstract

Septic encephalopathy is a frequent complication of sepsis, but there are few studies examining the role of microRNAs (miRs) in its pathogenesis. In this study, a miR-219 mimic was transfected into rat hippocampal neurons to model miR-219 overexpression. A protective effect of miR-219 was observed for glutamate-induced neurotoxicity of rat hippocampal neurons, and an underlying mechanism involving calmodulin-dependent protein kinase II γ (CaMKIIγ) was demonstrated. miR-219 and CaMKIIγ mRNA expression induced by glutamate in hippocampal neurons was determined by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). After neurons were transfected with miR-219 mimic, effects on cell viability and apoptosis were measured by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry. In addition, a luciferase reporter gene system was used to confirm CaMKIIγ as a target gene of miR-219. Western blot assay and rescue experiments were also utilized to detect CaMKIIγ expression and further verify that miR-219 in hippocampal neurons exerted its effect through regulation of CaMKIIγ. MTT assay and qRT-PCR results revealed obvious decreases in cell viability and miR-219 expression after glutamate stimulation, while CaMKIIγ mRNA expression was increased. MTT, flow cytometry, and caspase-3 activity assays showed that miR-219 overexpression could elevate glutamate-induced cell viability, and reduce cell apoptosis and caspase-3 activity. Moreover, luciferase CaMKIIγ-reporter activity was remarkably decreased by co-transfection with miR-219 mimic, and the results of a rescue experiment showed that CaMKIIγ overexpression could reverse the biological effects of miR-219. Collectively, these findings verify that miR-219 expression was decreased in glutamate-induced neurons, CaMKIIγ was a target gene of miR-219, and miR-219 alleviated glutamate-induced neuronal excitotoxicity by negatively controlling CaMKIIγ expression.

Key Words: *nerve regeneration; brain injury; septic encephalopathy; miR-219; hippocampal neurons; glutamate; excitotoxicity; apoptosis; caspase-3; calmodulin-dependent protein kinase II γ; luciferase reporter gene system; neuroprotection; neural regeneration*

Introduction

Sepsis is a systemic inflammatory response syndrome caused by severe bacterial infection (Doyle and Forni, 2016; Matthay et al., 2017). In essence, it is an instinctive response of the body's organs and tissues against infective factors. However, it can become a potentially life-threatening complication of infections (Plante, 2016), as it triggers a cascade of physiological changes that can cause damage and failure in multiple organ systems (Long et al., 2017; Nishihara et al., 2017; O'Brien et al., 2017).

Bacteria that cause infection can seriously disturb the body's functions, such as changing body temperature, heart rate, and blood pressure (Abir et al., 2017). In addition, sepsis may lead to complications of kidney, lung, brain, and heart tissues, and can result in death (Gomez and Kellum, 2016; Trevelin et al., 2017). To improve chances for survival, early treatment of sepsis usually provides antibiotics and large amounts of intravenous fluids (Simpson et al., 2016; Wittayachamnankul et al., 2016; Girardot et al., 2017).

Septic encephalopathy, also known as sepsis-associated encephalopathy, is the most common complication of sepsis (Gao et al., 2017; Tauber et al., 2017). Patients suffering from septic encephalopathy show brain dysfunction, such as cognitive impairments and disturbance of consciousness, caused by systemic inflammation or sepsis (Lu et al., 2016; Savio et al., 2016; Zhu et al., 2016). Excitatory amino acids are also excitatory neurotransmitters, and the excitotoxicity of excitatory amino acids plays a role in septic encephalopathy (Chaudhry and Duggal, 2014; Tauber et al., 2017). Even though great progress has been made for treatment of septic encephalopathy, the lack of effective therapeutic strategies remains an important social problem (Lyu et al., 2015; Wang et al., 2015; Kaur et al., 2016). Therefore, it is necessary to develop more useful treatments for septic encephalopathy.

MicroRNA (miR) is a class of small non-coding RNA that suppress target gene expression by complementary binding to the messenger 3′-untranslated region (UTR) (Li and Tang, 2016; Gradilone, 2017). Previous studies have focused on roles of miRNAs as key regulators in neuronal development and nervous system diseases (Chang et al., 2017; Fang et al., 2017; Molasy et al., 2017). miR-219, an evolutionarily conserved type of miRNA, is generally expressed throughout rodent and human brain tissues (Murai et al., 2016), and reportedly participates in various physiological and pathological processes (Pan et al., 2014). It has been implicated in regulation of circadian rhythm, development and progression of Alzheimer's disease and schizophrenia (Shi et al., 2013; Denk et al., 2015; Zhang et al., 2015), and is necessary for oligodendrocyte differentiation and myelination (Dugas et al., 2010; Pusic and Kraig, 2014; Diao et al., 2015). Furthermore, miR-219 was identified to be anti-oncogenic and down-regulated in various tumor types, such as respiratory, digestive, and nervous system tumors (Xiong et al., 2015; Garufi et al., 2016; Zhi et al., 2016). However, biological roles of miR-219 in glutamate-induced neurotoxicity are unknown.

This study examined effects of miR-219 on protecting primary hippocampal neurons against glutamate-induced neurotoxicity. First, miR-219 and CaMKIIγ mRNA expression were detected in glutamate-treated hippocampal neurons. Second, miR-219 was overexpressed to examine its influence on the viability and apoptosis of neurons treated with glutamate. Third, potential mechanisms of miR-219 for alleviating glutamate-induced neurotoxicity were investigated. Above all, this research provides a practical basis to examine the potentially beneficial effects of miR-219 as a reagent for the treatment of septic encephalopathy.

Materials and Methods

Cell culture

Animal experiments strictly abided to the Institutional Animal Care Guidelines of Nantong University of China (20150304-007). All Sprague-Dawley rats were purchased from the Laboratory Animal Center at Nantong University [SYXK (Su) 2012-0031].

Pregnant rats at 18–19 days post-fertilization were decapitated, and whole brains were removed from embryos and carefully placed into a dish using sterile scissors. Hippocampi were harvested in Hank's Balanced Salt Solution (Gibco; ThermoFisher, Waltham, MA, USA) under a dissecting microscope. Hippocampal tissues were mechanically mixed and dissociated with 0.25% trypsin at 37°C for 15 minutes into cell suspensions. After centrifugation, cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (Gibco), and cultured in poly-L-lysine-coated six-well plates for 4 hours in a CO_2 incubator. Following cell attachment to the bottom of plates, media were replaced with Neurobasal Plating Media supplemented with 2% B-27, 0.5 mM glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin (all components from Gibco). Cells were cultured for 7–8 days, which was necessary for the growth of hippocampal neurons to maturity, with half-renewal of media every 3 days.

Human embryonic kidney 293 (HEK293) cells were obtained from the Shanghai Cell Bank of Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences, and grown in DMEM containing 10% fetal calf serum. When HEK293 cultures achieved 70% to 80% density, cells were digested with 0.25% trypsin and subcultured. Cells in exponential growth phase were harvested for following manipulations. All cells were cultured in an incubator with 5% CO_2 and saturated humidity at 37°C, and growth was observed using an inverted microscope.

Cell treatment

The excitotoxicity of primary hippocampal neurons was accomplished by previously reported protocols (Chen et al., 2008; Zhou et al., 2008). Briefly, hippocampal neurons were exposed to glutamate by replacing Neurobasal Plating Media with Lock's solution containing 10 μM glycine and a certain concentration of glutamate (Sigma-Aldrich, St. Louis, MO, USA), and incubating for 15 minutes in a CO_2 incubator.

The experiment was divided into four groups (n = 6 per group). Neurons in three groups were treated with 62.5, 125, or 250 μM glutamate, respectively. The remaining group was

used as a normal control without any treatment. After excitotoxicity was induced, cells were washed with Lock's solution to remove any remaining glutamate and then cultured in Neurobasal Plating Media for indicated time periods.

To investigate the effects of miR-219 on hippocampal neurons induced by glutamate, the experiment was divided into three groups. Neurons in two groups (n = 6 per group) were treated with 125 μM glutamate and transfected with miR-219 mimic (miR-219 mimic + 125 μM glutamate) or mimic control (mimic control + 125 μM glutamate). The remaining group was used as a normal control without any treatment.

Table 1 Primer sequence for quantitative real-time reverse transcription-polymerase chain reaction

Gene	Sequence (5′–3′)	Product size (bp)
CaMKIIγ	Forward: CCT CGT GTT TGA CCT TGT T	122
	Reverse: GGA TGT GGT TGA CGC TCT	
GAPDH	Forward: ATC CCA GAG CTG AAC GGG A	104
	Reverse: ATC ACG CCA CAG CTT TCC	

CaMKIIγ: Calmodulin-dependent protein kinase II γ; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay

An MTT assay was performed to examine cell viability according to the manufacturer's protocol (Sigma-Aldrich). Primary hippocampal neurons (5×10^6/mL) were seeded into 96-well plates and subjected to glutamate treatment at various concentrations for 15 minutes. Afterwards, 10 μL of MTT solution was added to each well and the plate was incubated at 37°C for 4 hours. Next, 150 μL of 20% dimethyl sulfoxide was added to each well to dissolve the formazan for 20 hours. An Epoch™ Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) was used to read optical density values at 490 nm. The experiment was repeated in triplicate.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

To analyze miR-219 expression, cells were transfected with either miR-219 mimic or mimic control for 48 hours using a riboFECT™ CP transfection kit according to the manufacturer's protocol (RiboBio, Guangzhou, China) before extracting total RNA from neurons using a mirVana miRNA Isolation kit (Thermo Fisher, Waltham, MA, USA). To analyze CaMKIIγ mRNA expression, total RNA was extracted from neurons using Trizol reagent (Invitrogen). A NanoDropND-1000 spectrophotometer (NanoDrop Tech, Wilmington, DE, USA) was used to measure RNA concentrations. One μg of RNA was reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (TaKaRa, Dalian, China).

Expression of miR-219 was examined using qRT-PCR with a Bulge-Loop miRNA qRT-PCR kit (RiboBio) and miR-219–specific primers. qRT-PCR parameters were as follows: 95°C for 10 seconds, 40 cycles of 95°C for 5 seconds and 60°C for 20 seconds. Primers used for qRT-PCR (synthesized by Sangon Biotech, Shanghai, China) of CaMKIIγ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA are listed in **Table 1**. The experiment was repeated in triplicate. Expression of miR-219 was calculated as relative expression to internal reference U6, while expression of CaMKIIγ mRNA was calculated as relative expression to internal reference GAPDH. The $2^{-\Delta\Delta Ct}$ method was utilized to analyze data (Yu et al., 2012).

Flow cytometry assay

The role of miR-219 in glutamate-induced cell apoptosis was measured by flow cytometry with an Annexin V-FITC Apoptosis Detection Kit (Roche, New York, NY, USA). In brief, after 48-hour treatment, neurons were harvested *via* trypsinization, washed twice with cold phosphate-buffered saline (PBS), and stained with Annexin V-FITC and propidium iodide (PI) staining solution. The staining reaction was conducted in darkness for 15 minutes. $FITC^+/PI^-$ cells were considered to be early apoptotic cells. Cell apoptosis was measured using a flow cytometer and analyzed with flow cytometry software (BD Bioscience, Franklin Lakes, NJ, USA). All samples were filtered with a filter screen.

Caspase-3 activity assay

Intracellular caspase-3 activity was measured with a colorimetric Caspase-3 Assay Kit (ab39401; Abcam, Cambridge, UK). Treated cells were exposed to 125 μM glutamate for 12 hours, while control cultures were concurrently carried out without induction. Rat hippocampal neurons were further maintained in Neurobasal medium (Invitrogen) for 6 hours at 37°C. Subsequently, cells were suspended in 50 μL of cold lysis buffer and incubated on ice for 10 minutes. Protein concentration was measured and adjusted to 100 μg of protein per 50 μL of cell lysis buffer for each sample. Next, 50 μL of 2× reaction buffer containing 10 mM DTT and 200 μM DEVD-p-NA substrate was added to the lysate. The reaction was incubated at 37°C for 60–120 minutes. An ElX-800 absorbance spectrophotometer (Bio-Tek Instruments) was then applied to measure optical density values at 400–405 nm.

Luciferase reporter assay

HEK293 cells were cultured at a density of 3×10^5 cells/mL (100 μL per well) in a 96-well plate for 24 hours. After cells reached 70% confluence, the culture medium was removed and cells were transfected using Lipofectamine 2000 (Invitrogen). Wild-type 3′-UTR of CaMKIIγ, mutant 3′UTR of CaMKIIγ, or their respective negative controls were co-transfected into HEK293 cells with miR-219 mimic or a mimic negative control. After 48 hours of incubation, luciferase was assayed with a Bio-Tek Synergy Microplate Reader (Bio-Tek Instruments) (Nicolas, 2011).

Vector construction and rescue experiment

Based on the CaMKIIγ sequence (GenBank: NM_133605), the 3′-UTR sequence of CaMKIIγ was amplified and in-

Table 2 Primer sequence for polymerase chain reaction

Restriction enzyme cutting site	Sequence (5′–3′)	Product size (bp)
Eco RI	Forward: CG GAATTC ATG GCC ACC ACC GCC ACC TG	1587
Bam HI	Reverse: 5′-CG GGATCC CTG CAG CGG TGC AGC AGG GG	

The cutting sites are underlined.

serted into the luciferase reporter vector pGL3 (Promega, Madison, WI, USA) at an appropriate restriction enzyme cleavage site. Sequences of wild-type and mutant 3′-UTR were confirmed by DNA sequencing. Using specific primers, different products of PCR amplification of CaMKIIγ 3′-UTR were inserted into pGL3 vectors.

Primers used for PCR-based construction of recombinant expression vector pcDNA3.1-CaMKIIγ, are listed in **Table 2**. Primers were synthesized by Shanghai Invitrogen Corporation. Total RNA was extracted from rat cells with Trizol reagent and reverse transcribed into cDNA using reverse transcriptase (Invitrogen). cDNA was PCR amplified with the above primers, and products were cloned into a pGEM-T vector. After sequencing, the fragment was subcloned into pcDNA3.1 (Invitrogen).

For the rescue experiment, hippocampal neurons were divided into four groups. Neurons in three groups were treated with 125 μM glutamate and transfected with vector control (vector control + 125 μM glutamate), recombined vector pcDNA3.1-CaMKIIγ (pcDNA3.1-CaMKIIγ + 125 μM glutamate), or pcDNA3.1-CaMKIIγ and miR-219 mimic (pcDNA3.1-CaMKIIγ + miR-219 mimic + 125 μM glutamate). The remaining group was used as a normal control without any treatment.

Western blot assay

After transfection with miR-219 mimic or mimic negative control for 48 hours, cells were washed with pre-cooled PBS and lysed in cell lysis buffer containing protease inhibitors. Next, total proteins were quantified with a Bradford Protein Assay Kit (Promega). Cell lysis buffer, protease inhibitors, and Bradford Protein Assay Kit were all obtained from Beyotime Biotechnology (Jiangsu, China). Protein blotting was performed using standard protocols. Briefly, blotted polyvinylidene fluoride membranes were blocked with blocking buffer for 1 hour at room temperature and then washed three times in Tris-buffered saline containing Tween 20. Membranes were then reacted with a rabbit anti-CaMKIIγ polyclonal antibody (1:2000; Abcam) and mouse anti-β-actin monoclonal antibody (1:5000; Sigma-Aldrich) overnight at 4°C. Afterwards, membranes were reacted with appropriate horseradish peroxidase-conjugated secondary goat anti-rabbit or goat anti-mouse antibodies (1:2000; Abcam) for 2 hours at room temperature. Immunoreactive proteins were visualized by an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA). Relative CaMKIIγ contents are shown as the gray scale of CaMKIIγ relative to β-actin, with the gray scale being measured using Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All data are expressed as the mean ± standard deviation (SD) of three independent experiments (each in duplicate). Student's *t*-test and one-way analysis of variance followed by a Scheffe post-hoc test were used for statistical analysis with SPSS 19.0 software (IBM, Armonk, NY, USA) and GraphPad Prism 6 (La Jolla, CA, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Expression of miR-219 and CaMKIIγ mRNA in glutamate-induced primary cultured hippocampal neurons

Previous studies reported dose-dependent excitotoxicity induced by glutamate (0.1–1000 μM) in cultured cortical neurons (Perrella and Bhavnani, 2005) and hippocampal neurons (Chen et al., 2008); so we chose glutamate (62.5–250.0 μM) to induce neuronal cell injury in the following experiments. The results of MTT assay reflected decreased cell viability induced by glutamate (**Figure 1A**). Compared with normal controls, the cell viability of the 125-μM glutamate-treated group decreased significantly ($P < 0.01$). Therefore, treatment with 125 μM glutamate for 15 minutes was used to induce excitotoxicity in subsequent experiments.

To characterize expression of miR-219 and CaMKIIγ mRNA in glutamate-induced hippocampal neurons, qRT-PCR for miR-219 (relative to U6 snRNA) and CaMKIIγ (relative to GAPDH) was performed. As illustrated in **Figure 1B**, expression of miR-219 gradually declined after treatment with 62.5–250.0 μM glutamate compared with normal control ($P < 0.05$). However, expression of CaMKIIγ mRNA was increased and continued to be up-regulated during 62.5–250 μM glutamate treatment. These results suggested that miR-219 and CaMKIIγ played essential roles in causing glutamate-induced damage.

Effect of miR-219 on glutamate-induced cell viability

To determine whether miR-219 could alleviate glutamate-induced neurotoxicity, an MTT assay was performed in primary cultured hippocampal neurons. Neurons were transfected with miR-219 mimic or mimic control by transfection reagent, and qRT-PCR was used to detect miR-219 after 48 hours. As shown in **Figure 2**, miR-219 expression was significantly up-regulated (four-fold compared with other hippocampal neurons) after 48-hour transfection with miR-219 mimic. Moreover, MTT results showed that miR-219 overexpression effectively rescued the decreased viability of cells induced by 125 μM glutamate in normal or mimic controls ($P < 0.05$; **Figure 3**).

Effect of miR-219 on glutamate-induced cell apoptosis

To investigate whether miR-219 could inhibit glutamate-induced cell apoptosis, flow cytometry was performed. The

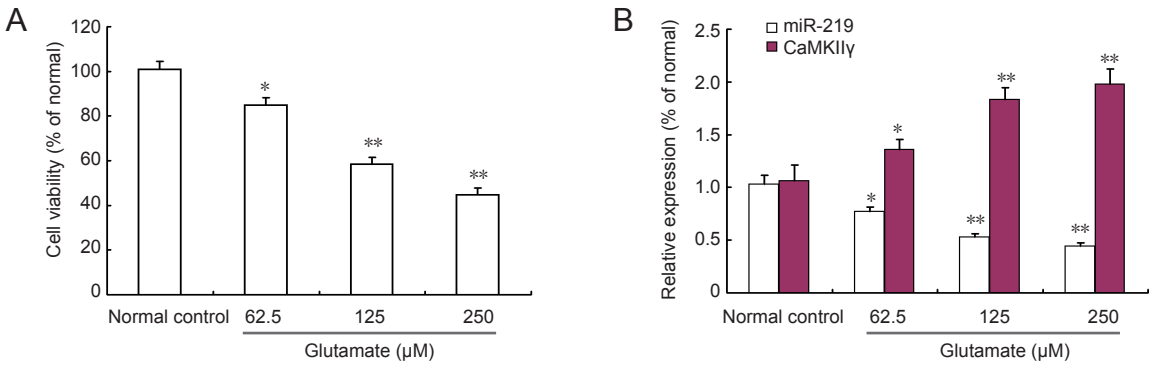

Figure 1 Cell viability and expression of miR-219 and CaMKIIγ mRNA in primary hippocampal neurons induced by glutamate in a dose-dependent manner.
(A) Cell viability was tested using MTT assay. (B) Expression of miR-219 and CaMKIIγ mRNA were detected by quantitative real-time reverse transcription-polymerase chain reaction. **P* < 0.05, ***P* < 0.01, *vs.* normal control group (mean ± SD, *n* = 6, one-way analysis of variance followed by Scheffe *post-hoc* test). Glutamate 62.5, 125 and 250 μM group: Hippocampal neurons were treated with 62.5, 125 or 250 μM glutamate, respectively. Normal control group: without any treatment. Experiment was conducted in triplicate. CaMKIIγ: Calmodulin-dependent protein kinase II γ.

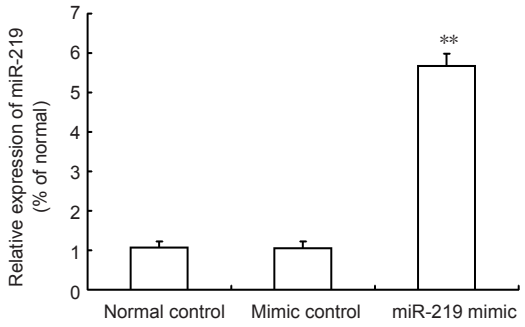

Figure 2 Expression of miR-219 detected by quantitative real-time reverse transcription-polymerase chain reaction after transfection.
***P* < 0.01, *vs.* normal control and mimic control (mean ± SD, *n* = 6, one-way analysis of variance followed by Scheffe *post-hoc* test). miR-219 mimic group, miR-219 mimic + 125 μM glutamate; mimic control, mimic control + 125 μM glutamate; normal control group, without any treatment. Experiment was conducted in triplicate.

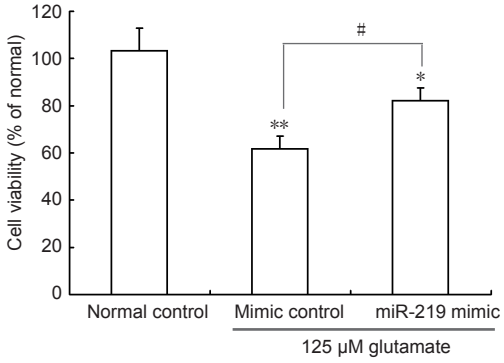

Figure 3 Effect of miR-219 on cell viability in hippocampal neurons treated with glutamate.
Cell viability was measured using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay after transfection. **P* < 0.05, ***P* < 0.01, *vs.* normal control group (mean ± SD, *n* = 6, one-way analysis of variance followed by Scheffe *post-hoc* test). #*P* < 0.05. miR-219 mimic group, miR-219 mimic + 125 μM glutamate; mimic control, mimic control + 125 μM glutamate; normal control group, without any treatment. Experiment was conducted in triplicate.

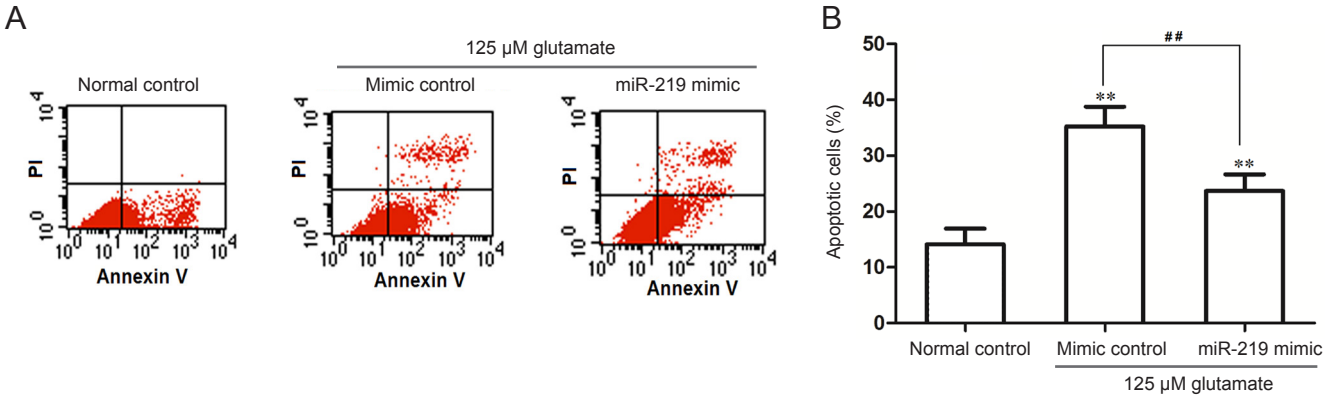

Figure 4 Effect of miR-219 on apoptosis in hippocampal neurons treated with glutamate.
Cell apoptosis was detected using flow cytometry after transfection. ***P* < 0.01, *vs.* normal control group (mean ± SD, *n* = 6, one-way analysis of variance followed by Scheffe *post-hoc* test). ##*P* < 0.01. miR-219 mimic group, miR-219 mimic + 125 μM glutamate; mimic control, mimic control + 125 μM glutamate; normal control group, without any treatment. Experiment was conducted in triplicate.

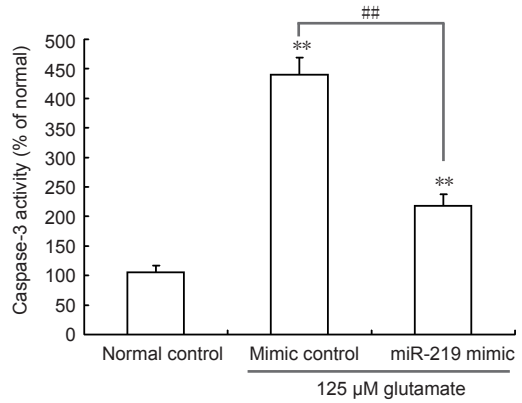

Figure 5 Effect of miR-219 on intracellular caspase-3 activity in glutamate-induced hippocampal neurons.
Intracellular caspase-3 activity was analyzed with a Caspase-3 Activity Assay Kit after transfection. $^{**}P < 0.01$, *vs.* normal control group (mean ± SD, $n = 6$, one-way analysis of variance followed by Scheffe *post-hoc* test). $^{\#\#}P < 0.01$. miR-219 mimic group, miR-219 mimic + 125 μM glutamate; mimic control, mimic control + 125 μM glutamate; normal control group, without any treatment. Experiment was conducted in triplicate.

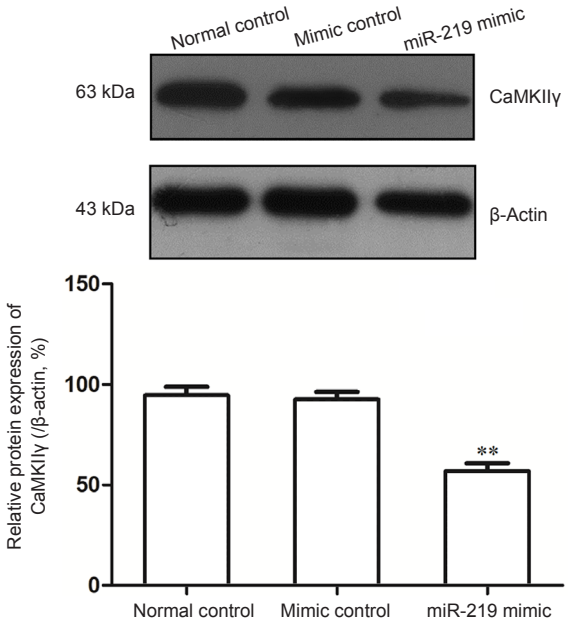

Figure 7 Effect of miR-219 on CaMKIIγ expression in hippocampal neurons.
Relative protein expression of CaMKIIγ examined using western blot assay after transfection shown in gray scale relative to β-actin. $^{**}P < 0.01$, *vs.* normal control and mimic control (mean ± SD, $n = 6$, one-way analysis of variance followed by Scheffe post-hoc test). miR-219 mimic group, miR-219 mimic + 125 μM glutamate; mimic control, mimic control + 125 μM glutamate; normal control group, without any treatment. Experiment was conducted in triplicate. CaMKIIγ: calmodulin-dependent protein kinase II γ.

results of flow cytometry indicated a significantly higher percentage of apoptotic neurons in the 125 μM glutamate-treated group ($P < 0.01$) compared with the normal control group. However, a significantly lower ratio of apoptotic cells was observed in neurons transfected with miR-

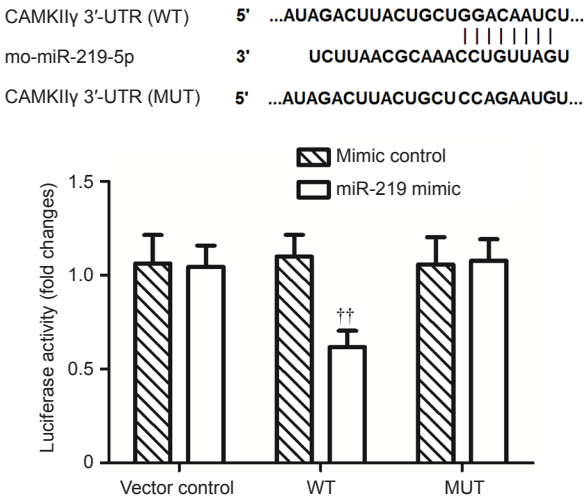

Figure 6 Dual-luciferase reporter gene assay confirmed that the predicted gene CaMKIIγ was a target of miR-219.
Upper panel shows schematic representation of a section of the CaMKIIγ 3′-UTR representing the predicted miR-219–reactive site. Lower panel shows the resulting histogram indicating relative luciferase activity in each group. $^{\dagger\dagger}P < 0.01$, *vs.* mimic control (mean ± SD, $n = 6$, Student's *t*-test). miR-219 mimic group, miR-219 mimic + 125 μM glutamate; mimic control, mimic control + 125 μM glutamate. Experiment was conducted in triplicate. WT: Wild type; MUT: mutant; CaMKIIγ: calmodulin-dependent protein kinase II γ.

219 mimic after treatment with 125 μM glutamate compared with similarly treated mimic control group neurons ($P < 0.01$; **Figure 4**).

Effect of miR-219 on caspase-3 activity in glutamate-induced cells

Kim et al. (2016) reported that activation of pro-apoptotic proteins including caspases was involved in glutamate-induced neurotoxicity. Caspase-3 activity was increased four-fold in neurons after a 15-minute exposure to 125 μM glutamate compared with the normal control group. However, glutamate-induced caspase-3 activity was decreased by half in neurons transfected with miR-219 mimic compared with mimic control (**Figure 5**).

CaMKIIγ is a direct target gene of miR-219

To explore potential targets for miR-219, the online miRNA target prediction algorithm TargetScan (http://www.targetscan.org/) was applied. Results of this analysis showed that CaMKIIγ, a central regulating protein in Ca^{2+} signaling cascade mediated by N-methyl-D-aspartic acid (Aow et al., 2015), was a possible target gene of miR-219. A dual-luciferase reporter gene assay was applied to analyze interactions between miR-219 and CaMKIIγ. The recombined CaMKIIγ 3′UTR (wild-type or mutant) reporter gene plasmid and the miR-219 mimic (or mimic control) were co-transfected into HEK293 cells. We found that luciferase activity was significantly decreased by co-transfection of the vector containing CaMKIIγ 3′UTR (wild-type) and miR-219 mimic compared with similarly co-transfected mimic control (**Figure 6**). The

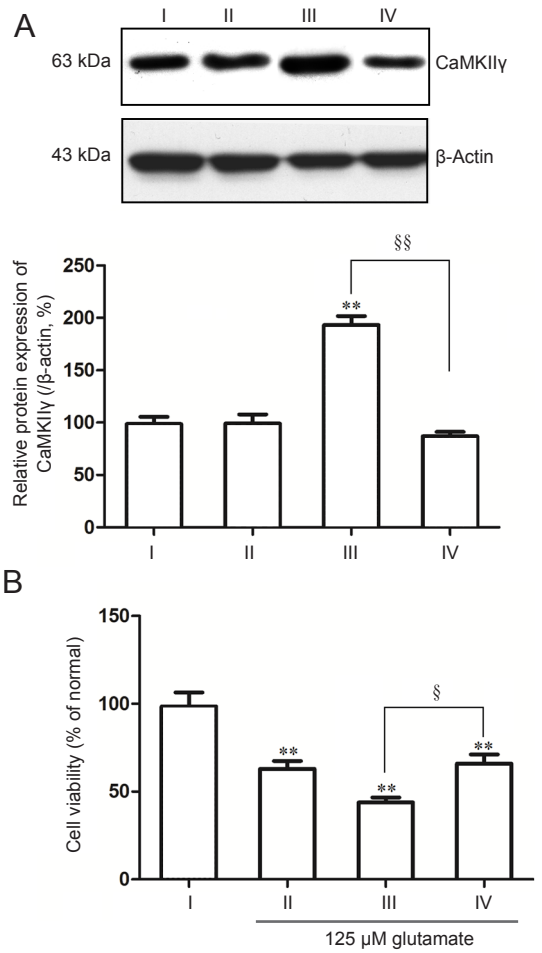

Figure 8 Rescue experiment verified the role of miR-219 in neurons to be specific for regulation of the target gene CaMKIIγ.
(A) CaMKIIγ expression was detected by western blot assay after transfection. Relative protein expression of CaMKIIγ is shown in gray scale relative to β-actin. (B) Cell viability was measured using MTT assay after transfection. $^{**}P < 0.01$, *vs.* normal control group (mean ± SD, $n = 6$, one-way analysis of variance followed by Scheffe *post-hoc* test). §$P < 0.05$, §§$P < 0.01$. miR-219 mimic group, miR-219 mimic + 125 μM glutamate; mimic control, mimic control + 125 μM glutamate; normal control group, without any treatment. Experiment was conducted in triplicate. I: Normal control; II: Vector control; III: pcDNA3.1-CaMKIIγ; IV: pcDNA3.1-CaMKIIγ+miR-219 mimic. MTT: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; CaMKIIγ: calmodulin-dependent protein kinase II γ.

results verified that the forecasted target location of miR-219 was in the CaMKIIγ 3′UTR.

Western blot assay was performed to confirm the role of miR-219 in regulating CaMKIIγ expression. The results of western blot assay indicated suppression of CaMKIIγ in neurons overexpressing miR-219 compared with normal and mimic control groups ($P < 0.01$; **Figure 7**). These results demonstrated that miR-219 suppressed CaMKIIγ expression in a post-transcriptional manner.

Finally, to ensure that the role of miR-219 in neurons was specific to regulation of the target gene CaMKIIγ, a rescue experiment was performed. A recombinant CaMKIIγ vector was constructed to investigate the effect of CaMKIIγ overexpression on cell survival of glutamate-induced neurons transfected with miR-219 mimic. Western blot assay results demonstrated that CaMKIIγ expression doubled after transfected with pcDNA3.1-CaMKIIγ compared with untransfected and vector controls ($P < 0.01$; **Figure 8A**). CaMKIIγ expression was expected to be significantly reduced when pcDNA3.1-CaMKIIγ and miR-219 mimic were co-transfected. As shown in **Figure 8B**, decreased cell viability was observed in glutamate-induced neurons transfected with pcDNA3.1-CaMKIIγ compared with normal and vector controls ($P < 0.05$). Combined with results shown in **Figure 3**, this finding indicated that CaMKIIγ and miR-219 co-overexpression treatment inhibited the role of single miR-219 overexpression in rescuing cell viability of glutamate-induced neurons.

Discussion

Sepsis is a systemic inflammatory response caused by infection. Septic encephalopathy is a severe complication of sepsis, and the pathogenesis of this disease is still unclear (Moskowitz et al., 2016; Warren et al., 2017). Oxidative stress, changes in blood-brain barrier permeability, and increased cytokine production are involved in septic encephalopathy. Excitatory amino acids, which also act as excitatory neurotransmitters, play an excitotoxic role in septic encephalopathy (Chaudhry and Duggal, 2014; Tauber et al., 2017). Increased concentrations of glutamate in neural cells, as well as increased expression of glutamate-activated N-methyl-D-aspartic acid receptors, after treatment with endotoxin (lipopolysaccharide) lead to neuronal injury (Yousef and Lang, 1994). In fact, the brain dysfunction observed during septic encephalopathy is probably the consequence of co-action of numerous factors (Ziaja, 2013).

Glutamate, the most abundant excitatory amino acid in the brain, takes a central part in nerve generation in the embryo, as well as various excitatory synaptic transmission processes and synaptic plasticity in adulthood (Galvan and Gutierrez, 2017). Glutamate concentration increases rapidly in brain tissue after cerebral ischemia, leading to excessive activation of glutamate receptors (especially N-methyl-D-aspartic acid receptor) in the postsynaptic membrane and extracellular Ca^{2+} influx (Song et al., 2016). Excessive stimulation of glutamate receptors induced excitotoxicity, and participated in nerve injury and septic encephalopathy (Ziaja, 2013).

To provide a new therapy for septic encephalopathy, we measured the expression of miR-219 and CaMKIIγ mRNA by qRT-PCR in glutamate-induced hippocampal neurons. We observed an obvious change of miR-219 and CaMKIIγ mRNA in glutamate-treated neurons, indicating that miR-219 and CaMKIIγ may be involved in septic encephalopathy.

An overdose of glutamate induced nerve injury in neuronal cultures (Zhang et al., 2017). Overdose of glutamate caused decreased cell viability in a dose-dependent manner. During septic encephalopathy, signs of apoptosis could be observed in neurons, which exhibited shrunken nuclei and damaged cell membranes (Fang et al., 2014). Thus, reducing

apoptosis in neurons is key to treating septic encephalopathy. Effects of miR-219 overexpression on cell survival/viability and apoptosis were detected by MTT assay and flow cytometry. miR-219 overexpression could promote cell survival, attenuate glutamate-induced apoptosis, and inhibit caspase-3 activity. These data support a positive protective role of miR-219 as an antagonist of glutamate-induced excitotoxicity in primary hippocampal neurons.

Our TargetScan results predicted that CaMKIIγ could be a target gene of miR-219. CaMKII, an important protein kinase in the brain, is an abundantly expressed protein in neurons (Wang and Peng, 2016). Many studies have identified regulatory roles for CaMKII across multiple neural cell systems, including equilibrium and dynamics of calcium ions, cellular transport, cellular morphology, neurite growth, long-term synaptic plasticity, and learning and memory consolidation (Rosen et al., 2015; Marcelo et al., 2016; Mauger et al., 2016). Moreover, CaMKII plays an important role in the pathogenesis of neuronal diseases such as cerebral ischemia, Alzheimer's disease, and Parkinson's disease, which made CaMKII a new drug target for neuroprotection or myocardiac protection (Cheng et al., 2010; Tan et al., 2012; McCullough et al., 2013). Use of a fluorescence reporter gene system and rescue experiments confirmed that CaMKIIγ was regulated by miR-219 for neuroprotective attenuation of neurotoxicity. CaMKIIγ expression was repressed by miR-219, providing a compensatory mechanism to maintain N-methyl-D-aspartic acid receptor function during excessive glutamate stimulation. The effects of miR-219 on calmodulin or calcium-dependent upstream factors were not reported previously. However, we may do some researches about this in the future.

In conclusion, miR-219 may exert a neuroprotective effect on glutamate-induced hippocampal neurons by inhibiting caspase-3 activity and regulating CaMKIIγ. The positive protective effect of miR-219 might be applied in the future as an antagonist of excitotoxicity for septic encephalopathy.

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