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

Ting Wang1,2, Qun Cai3, Wen-Jie Yang1, Hai-Hua Fan1, Jian-Feng Yi4, Feng Xu1,*

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

Funding: This study was supported by the National Natural Science Foundation of China, No. 81101159; the Natural Science Foundation of Jiangsu Province of China, No. BK20151268.

Graphical Abstract

Does miR-219 inhibit glutamate-induced neuronal excitotoxicity?

Hippocampal neuron cells were induced by glutamate

Quantitative real-time reverse transcription-polymerase chain reaction was used to detect the expression of miR-219

miR-219 mimic was transfected into hippocampal neurons to overexpress miR-219

Effects of miR-219 on cell viability and apoptosis were measured by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay and flow cytometry

Effect of miR-219 on the activity of intracellular caspase-3 was measured with Caspase 3 Assay Kit

A luciferase reporter gene system and rescue experiment were applied to validate that CaMKIIγ was the target gene of miR-219

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 CaMKIIy 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 CO₂ 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 resuspended. Cells in exponential growth phase were harvested for following manipulations. All cells were cultured in an incubator with 5% CO₂ 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 Locke’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 CO₂ 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 \( \mu \)M glutamate and transfected with miR-219 mimic (miR-219 mimic + 125 \( \mu \)M glutamate) or mimic control (mimic control + 125 \( \mu \)M glutamate). The remaining group was used as a normal control without any treatment.

### 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 \( 5 \times 10^3/\text{mL} \) were seeded into 96-well plates and subjected to glutamate treatment at various concentrations for 15 minutes. Afterwards, 10 \( \mu \)L of MTT solution was added to each well and the plate was incubated at 37°C for 4 hours. Next, 150 \( \mu \)L 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™ CT 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 \( \mu \)g 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^(-ΔΔCT) 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 \( \mu \)M 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 \( \mu \)L of cold lysis buffer and incubated on ice for 10 minutes. Protein concentration was measured and adjusted to 100 \( \mu \)g of protein per 50 \( \mu \)L of cell lysis buffer for each sample. Next, 50 \( \mu \)L of 2x reaction buffer containing 10 mM DTT and 200 \( \mu \)M 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 \( 3 \times 10^5 \) cells/mL (100 \( \mu \)L 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-
served 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 pGLO 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), recombinant 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 (Perrell 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
Wang T, Cai Q, Yang WJ, Fan HH, Yi JF, Xu F (2018) MicroRNA-219 alleviates glutamate-induced neurotoxicity in cultured hippocampal neurons by targeting calmodulin-dependent protein kinase II gamma. Neural Regen Res 13(7):1216-1224. doi:10.4103/1673-5374.235059

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.
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-219 mimic after treatment with 125 μM glutamate compared with similarly treated mimic control group neurons ($P < 0.01$; Figure 5).

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
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 expression 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 expression 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.
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.

Acknowledgments: We thank Associate Professor Mao-rong Jiang from Nantong University of China for assistance in paper preparation.

Author contributions: FX designed this study. TW, QC, WJY, HHF and JFY performed experiments. TW and QC analyzed data. TW wrote the paper. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Financial support: This study was supported by the National Natural Science Foundation of China, No. 81101159; the Natural Science Foundation of Jiangsu Province of China, No. BK20151268. The funding bodies played no role in the study design, in the collection, analysis and interpretation of data, in the writing of the paper, and in the decision to submit the paper for publication.

Institutional review board statement: The study protocol was approved by the Animal Ethics Committee of Nantong University of China (approval No. 20150304-007). The experimental procedure followed the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985).

Copyright license agreement: The Copyright License Agreement has been signed by all authors before publication.

Data sharing statement: Datasets analyzed during the current study are available from the corresponding author on reasonable request.

Plagiarism check: Checked twice by iThenticate.

Peer review: Externally peer reviewed.

Open access statement: This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-Non-Commercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

References
Abir G, Akgadali S, Butwick A, Carvalho B (2017) Clinical and microbiological features of maternal sepsis: a retrospective study. Int J Obstet Anesth 29:26-33.
Aow J, Dore K, Malinov R (2015) Conformational signaling required for synaptic plasticity by the NMDA receptor complex. Proc Natl Acad Sci U S A 112:14711-14716.
Chang HL, Wang HC, Chunag YT, Chou CW, Lin IL, Lai CS, Chang LL, Cheng KI (2017) miRNA expression change in dorsal root ganglia after peripheral nerve injury. J Mol Neurosci 61:169-177.
Chaudhry N, Duggal AK (2014) Sepsis associated encephalopathy. Adv Med 2014;762320.
Chen X, Liu J, Gu XS, Ding F (2008) Saldiosoreid attenuates glutamate-induced apoptotic cell death in primary cultured hippocampal neurons of rats. Brain Res 1238:189-198.
Cheng A, Hou Y, Mattson MP (2010) Mitochondria and neuroplasticity. ASN Neuro 2:e00045.
Denk J, Boelmins K, Siegismund C, Lassner D, Arlt S, Jahn H (2015) MicroRNA profiling of CSF reveals potential biomarkers to detect Alzheimer’s disease. PLoS One 10:e0126623.
Diao HJ, Low WC, Lu QB, Chew SY (2015) Topographical effects on fiber-mediated microRNA delivery to control oligodendrogial precursor cells development. Biomaterials 70:105-114.
Doyle JF, Forni LG (2016) Update on sepsis-associated acute kidney injury: emerging targeted therapies. Biologics 10:149-156.
Dugas JC, Cuellar TL, Scholze A, Ason B, Ibrahim A, Emery B, Zamanian JL, Foo LC, McManus MT, Barres BA (2010) Dicer1 and miR-219 Are required for normal oligodendrocyte differentiation and myelination. Nature 65:597-611.
Fang J, Lian Y, Xie K, Cai S, Wen P (2014) Epigenetic modulation of neuronal apoptosis and cognitive functions in sepsis-associated encephalopathy. Neurosci 35:283-288.
Fang Q, Xu T, Wu C, Zhou S, Sun H (2017) Biotargets in neural regeneration. Biotarget. doi: 10.21037/biotarget.2017.05.01.
Galvan EJ, Gutierrez R (2017) Target-dependent compartmentalization of the corelease of glutamate and GABA from the mossy fibers. J Neurosci 37:701-714.
Gao R, Ji MH, Gao DP, Yang RH, Zhang SG, Yang JJ, Shen JC (2017) Neuroinflammation-Induced downregulation of hippocampal neuregulin 1-erbB4 signaling in the parvalbumin interneurons might contribute to cognitive impairment in a mouse model of sepsis-associated encephalopathy. Inflammation 40:387-400.
Garuti C, Giacomini É, Torsello A, Sperduti I, Melucci E, Mottolese M, Zeuli M, Ettorre GM, Ricciardi T, Cognetti F, Magnani M, Ruzzo A (2016) Gender effects of single nucleotide polymorphisms and miRNAs targeting clock-genes in metastatic colorectal cancer patients (miCRC). Sci Rep 6:4006.
Girardot T, Rimmele T, Venet F, Monneret G (2017) Apoptosis-induced lymphopenia in sepsis and other severe injuries. Apoptosis 22:295-305.
Gomez H, Kellum JA (2016) Sepsis-induced acute kidney injury. Curr Opin Crit Care 22:546-553.
Gradilone SA (2017) Extracellular vesicles as therapeutic carriers of microRNAs for cholangiocarcinoma. Hepatology 65:404-406.
Kaur J, Singh P, Singh S, Malhi P, Saini AG (2016) Neurodevelopmental and behavioral outcomes in children with sepsis-associated encephalopathy admitted to pediatric intensive care unit: a prospective case control study. J Child Neurol 31:683-690.
Kim JJ, Kang YJ, Shin SA, Bak DH, Lee JW, Lee KB, Yoo YC, Kim DK, Lee BH, Kim DW, Lee J, Jo EK, Yuk JM (2016) Phlorofucofuroeckol improves glutamate-induced neurotoxicity through modulation of oxidative stress-mediated mitochondrial dysfunction in PC12 cells. PLoS One 11:e0163433.
Li Y, Tang PF (2016) Can exosomal micro-RNAs be as biomarkers of diseases? Zhongguo Zuzhi Gongcheng Y anjiu 20:7738-7745.
Long B, Koyfman A, Modissett KL, Woods CJ (2017) Practical considerations in sepsis resuscitation. J Emerg Med 52:472-483.

Lu CX, Qiu T, Tong HS, Liu ZF, Su L, Cheng B (2016) Peripheral T-lymphocyte and natural killer cell population imbalance is associated with septic encephalopathy in patients with severe sepsis. Exp Ther Med 11:1077-1084.

Lyu J, Zheng G, Chen Z, Wang B, Tao S, Xiang D, Xie M, Huang J, Liu C, Zeng Q (2015) Sepsis-induced brain mitochondrial dysfunction is associated with altered mitochondrial Src and PTP1B levels. Brain Res 1620:130-138.

Marcello KL, Means AR, York B (2016) The Ca(2+)-Calmodulin/CalM-KK axis: nature's metabolic carbam. Trends Endocrinol Metab 27:706-718.

Mathay MA, Pati S, Lee JW (2017) Concise Review: mesenchymal stem (stromal) cells: biology and preclinical evidence for therapeutic potential for organ dysfunction following trauma or sepsis. Stem Cells 35:316-324.

Mauger O, Lemoine F, Scheiffele P (2016) Targeted intron retention and excision for rapid gene regulation in response to neuronal activity. Neuron 92:1266-1278.

McCullough TD, Tarabishy S, Liu L, Benashski S, Xu Y, Ribar T, Means A, Li J (2013) Inhibition of calcium/calmodulin-dependent protein kinase beta and calcium/calmodulin-dependent protein kinase IV is detrimental in cerebral ischemia. Stroke 44:2559-2566.

Molassy M, Walczak A, Szalik J, Szalik JP, Majsterek I (2017) MicroRNAs in glaucoma and neurodegenerative diseases. J Hum Genet 62:105-112.

Moskovitz A, Omar Y, Chase M, Lokhandwala S, Patel P, Andersen LW, Cocchi MN, Donnino MW (2016) Reasons for death in patients with sepsis and septic shock. J Crit Care 38:284-288.

Murai K, Sun G, Ye P, Tian E, Yang S, Cui Q, Sun G, Trinh D, Sun O, Hong T, Wen Z, Kalkum M, Riggs AD, Song H, Ming GL, Shi Y (2016) The TLX-miR-219 cascade regulates neural stem cell proliferation in neurodevelopment and schizophrenia (PSI) model. Nat Commun 7:10965.

Nicolas FE (2011) Experimental validation of microRNA targets using a luciferase reporter system. Methods Mol Biol 732:139-152.

Nishihara Y, Dangor Z, French N, Madhi S, Heyderman R (2017) Molecular and synaptic plasticity. Mil Med Res 3:26.

Pan Z, Zhou L, Li QY, Hao LY, Yin C, Yang JX, Guo Y, Zhang S, Hua L, Xue ZY, Zhang H, Cao JL (2014) Epigenetic modification of spinal miR-219 expression regulates chronic inflammation pain by targeting CamKIIgamma. J Neurosci 34:9476-9483.

Perrella J, Bhavnani BR (2005) Protection of cortical cells by equine estrogens against glutamate-induced excitotoxicity is mediated through a calcium independent mechanism. BMC Neurosci 6:34.

Plante LA (2016) Management of sepsis and septic shock for the obstetrician-gynecologist. Obstet Gynecol Clin North Am 43:659-678.

Pusic AD, Kraig RP (2014) Youth and environmental enrichment generate serum exosomes containing miR-219 that promote CNS myelination. Glia 62:284-299.

Rosen LG, Sun N, Rushlow W, Laviolette SR (2015) Molecular and neuronal plasticity mechanisms in the amygdala-prefrontal cortical circuit: implications for opiate addiction memory formation. J Perinatol 37:10965.

Savio LE, Andrade MG, de Andrade Mello P, Santana PT, Moreira-Souza AC, Kolling J, Feldbrugge L, Wu Y, Wyse AT, Robson SC, Coutinho-Silva R (2016) P2X7 receptor signaling contributes to sepsis-associated brain dysfunction. Mol Neurobiol 9:399.

Shi F, Chen X, Fu A, Hansen J, Stevens R, Tjonneland A, Vogel UB, Zheng T, Zha Y (2013) aberrant DNA methylation of miR-219 promoter in long-term night shiftworkers. Environ Mol Mutagen 54:406-413.

Simpson SQ, Gaines M, Husein Y, Badgett RG (2016) Early goal-directed therapy for severe sepsis and septic shock: a living systematic review. J Crit Care 36:43-48.

Song H, Kim W, Kim SH, Kim KT (2016) VRR3-mediated nuclear localization of HSP70 prevents glutamate excitotoxicity-induced apoptosis and Abeta accumulation via enhancement of ERK phosphatase VRH activity. Sci Rep 6:38452.

Tan WY, Zhang SJ, Hoffmann T, Bading H (2012) Increasing levels of wild-type CREB up-regulates several activity-regulated inhibitor of death (AID) genes and promotes neuronal survival. BMC Neurosci 13:48.

Tauber SC, Eiffert H, Bruck W, Nau R (2017) Septic encephalopathy and septic encephalitis. Expert Rev Anti Infect Ther 15:121-132.

Trevellin SC, Carlos D, Beretta M, da Silva JS, Cunha FQ (2017) Diabetes mellitus and sepsis: a challenging association. Shock 47:276-287.

Wang H, Peng RY (2016) Basic roles of key molecules connected with NMDAR signaling pathway on regulating learning and memory and synaptic plasticity. Mil Med Res 3:26.

Wang H, Hong LJ, Huang JY, Jiang Q, Tao RR, Tan C, Lu NN, Wang CK, Ahmed MM, Lu YM, Liu ZR, Shi WX, Lai EY, Wilcox CS, Han F (2015) P2RX7 sensitizes Mac-1/ICAM-1-dependent leukocyte-endothelial adhesion and promotes neurovascular injury during septic encephalopathy. Cell Res 25:674-690.

Wang S, Garcia M, Hankins C (2017) Impact of neonatal early-onset sepsis calculator on antibiotic use within two tertiary healthcare centers. J Perinatol 37:394-397.

Wittayachammankul B, Chentanakij B, Suramsiri K, Chattipakorn N (2016) The role of central venous oxygen saturation, blood lactate, and central venous-to-arterial carbon dioxide partial pressure difference as a goal and prognosis of sepsis treatment. J Crit Care 36:223-229.

Xiog GB, Zhang GN, Xiao Y, Niu BZ, Qiu HZ, Wu B, Lin GL, You L, Shu H (2015) MicroRNA-219-5p functions as a tumor suppressor partially by targeting platelet-derived growth factor receptor alpha in colorectal cancer. Neoplasma 62:855-863.

Youssef KA, Lang CH (1994) Modulation of endotoxin-induced changes in hemodynamics and glucose metabolism by an N-methyl-D-aspartate receptor antagonist. Shock 1:335-342.

Yu B, Zhou S, Wang Y, Qian T, Ding G, Ding F, Gu X (2012) miR-221 and miR-222 promote Schwann cell proliferation and migration by targeting LASS2 after sciatric nerve injury. J Cell Sci 125:2675-2683.

Zhang S, Li H, Zhang L, Li J, Wang R, Wang M (2017) Effects of troxerutin on cognitive deficits and glutamate cysteine ligase subunits in the hippocampus of streptozotocin-induced type I diabetes mellitus rats. Brain Res 1657:355-360.

Zhang Y, Fan M, Wang Q, He G, Fu Y, Li H, Yu S (2015) Polymorphisms in MicroRNA Genes and genes involving in NMDAR signaling and schizophrenia: a case-control study in chinese han population. Sci Rep 5:12984.

Zhi F, Shao N, Li B, Xue L, Deng D, Xu Y, Lan Q, Peng Y, Yang Y (2016) A serum 6-miRNA panel as a novel non-invasive biomarker for meningioma. Sci Rep 6:32067.

Zhou S, Yang Y, Wu X, Ding F (2008) Chito oligosaccharides protect cultured hippocampal neurons against glutamate-induced neurotoxicity. Neurosci Lett 444:270-274.

Zhu SZ, Huang LP, Han YL, Han QP, Zha GF, Wen MY, Deng YY, Zeng HK (2016) Huperzine A protects sepsis associated encephalopathy by promoting the deficient cholinergic nervous function. Neurosci Lett 631:70-78.

Ziaja M (2013) Septic encephalopathy. Curr Neurol Neurosci Rep 13:383.