Epilepsy & Behavior 75 (2017) 134–142

Contents lists available at ScienceDirect

Epilepsy & Behavior

journal homepage: www.elsevier.com/locate/yebeh

# The role of necroptosis in status epilepticus-induced brain injury in juvenile rats☆

Qianyun Cai [a,b,1], Jing Gan [a,b,1], Rong Luo [a,b], Yi Qu [a,b], Shiping Li [b], Chaomin Wan [a,b], Dezhi Mu [a,b,*]

[a] Department of Pediatrics, West China Second University Hospital, Sichuan University, Chengdu 610041, Sichuan, China
[b] Key Laboratory of Obstetric & Gynecologic and Pediatric Diseases and Birth Defects of Ministry of Education, Sichuan University, Chengdu 610041, Sichuan, China

ARTICLE INFO

*Article history:*
Received 7 December 2016
Revised 18 May 2017
Accepted 19 May 2017

*Keywords:*
Status epilepticus
Necroptosis
Brain injury
RIP3
MLKL
Cognitive impairment

ABSTRACT

*Purpose:* To study the role of necroptosis in status epilepticus (SE)-induced injury in the developing brain and the possible associations of necroptosis with epileptogenesis and cognitive dysfunction.
*Methods:* The lithium-pilocarpine epilepsy model was reproduced in male rats at postnatal day 25. Propidium iodide (PI) staining was used to detect cell death after SE. Transmission electron microscopy (TEM) was performed to observe morphological changes in injured neurons. Western blot and immunofluorescence (IF) staining were used to investigate the expression of receptor interacting protein kinase-3 (RIP3), mixed lineage kinase domain-like (MLKL), and p-MLKL after SE. EEG was monitored during the chronic epileptic period. The Morris water maze test was performed to evaluate spatial learning and memory in juvenile rats after SE.
*Results:* Massive PI-positive ($PI^+$) neurocytes were observed mainly in the amygdala and piriform cortex 24 h to 7 days after SE, with the most prominent changes observed after 72 h. Injured neurons observed via TEM exhibited necroptotic morphological features, including loss of ribosomes, autophagosome formations, deformed nuclei with condensed and marginated chromatin, and disruptive cell membranes. The expression of RIP3 and p-MLKL increased after 24 h, peaked at 72 h, and decreased 7 days after SE. In addition, IF staining revealed that MLKL was expressed in cell plasma membranes present in the amygdala and piriform cortex. This finding was concomitant with the fact that MLKL is involved in executing necroptosis by binding and disrupting the plasma membrane. During the chronic epileptic period, spontaneous recurrent seizures were observed behaviorally and interictal spikes and sharp waves were recorded by EEG in the SE group. The Morris water maze test revealed that in the place navigation test, the escape latency of the SE group was longer than that of the control group ($p < 0.05$). In the spatial probe test, the number of times the rats in the SE group passed through the original platform site was lesser than that of the rats in the control group ($p < 0.05$).
*Conclusion:* SE-induced brain injury leads to neuronal necroptosis in juvenile rats. MLKL may play a significant role in the execution of SE-induced necroptosis. Further studies are required to determine whether inhibiting necroptosis can prevent chronic epileptogenesis and improve cognitive ability for juvenile rats.


## 1. Introduction

Epilepsy is a common and chronic neurological disorder characterized by recurrent unprovoked seizures. Experimental models and clinical studies have confirmed that a prolonged seizure or status epilepticus (SE) can cause neuronal death in the brain [1]. Such brain injuries may contribute to epileptogenesis and impairments in cognitive function [2]. Delineating the molecular pathways underlying seizure-induced neuronal death may yield novel strategies for protecting the brain against prolonged or repetitive seizures.

There are two classical forms of cell death: apoptosis and necrosis. Research has identified apoptosis, a form of programmed cell death, as the mechanism for neuronal cell death after SE. TUNEL-positive staining was detected in tissue samples from the rat brain after prolonged seizures [3]. Degenerating, TUNEL-positive cells in the hippocampus of mice after seizures exhibit nuclear features of apoptosis [4]. Furthermore, programmed cell death mechanisms, such as activation of p53 and cell death promoting Bcl-2 family members and endonuclease-induced DNA laddering, were identified in SE-induced neuronal death [5–6].

Contrarily, researchers also found that neuronal death in SE shows a necrotic morphology [7]. During SE, excessive NMDA receptor activation results in excessive calcium influx and calcium release from

☆ None of the authors has any conflict of interest to disclose.
* Corresponding author at: Department of Pediatrics, West China Second University Hospital, Sichuan University, Chengdu 610041, Sichuan, China.
*E-mail address:* mudz@scu.edu.cn (D. Mu).
[1] These authors contributed equally to this paper.

http://dx.doi.org/10.1016/j.yebeh.2017.05.025
1525-5050/

intracellular stores. This activates cytoplasmic proteases, generates free radicals, and causes significant reduction in ATP levels, resulting in the proteolysis of cytoskeletal proteins, rupture of cytoplasmic membranes, DNA damage, and eventually cell necrosis [1,6].

However, these two classical cell death pathways could not fully explain all the phenomena observed in SE-induced brain injury. Fujikawa et al. [8] found that in rat models with SE, induced by lithium-pilocarpine or kainic acid, neuronal death in the limbic structures exhibited necrotic morphology even though DNA laddering was observed in these regions 24 and 72 h after SE and some regions showed TUNEL-positive staining 72 h after SE. Therefore, they suggested that programmed cell death mechanisms may contribute to SE-induced neuronal necrosis. Nevertheless, the underlying mechanisms still need to be clarified.

Recent evidence reveals that necrotic cell death can be tightly regulated. Necroptosis mediated by receptor interacting protein kinase-3 (RIP3) protein and its substrate, mixed lineage kinase domain-like (MLKL) protein, is the best-characterized form of regulated necrosis [9–11]. A morphological pattern between classical apoptosis and classical necrosis was shown in cell necroptosis [12–13]. Necroptosis may occur in response to multiple stimuli, with tumor necrosis factor-α (TNF-α) being the most well-studied initiation signal. TNF-α binds to TNF-α receptor 1 (TNFR1) and triggers the binding of receptor interacting protein kinase-1 (RIP1) with RIP3 through the RIP homotypic interaction motif (RHIM), when caspase 8 is inhibited. RIP1 and RIP3 then form an amyloid-like structure termed necrosome that allows RIP3 dimerization. Activated RIP3 recruits and phosphorylates MLKL protein to promote its oligomerization and translocation to the plasma membrane, resulting in membrane rupture and necrosis [10–11,14–15].

Necroptosis has been claimed to contribute to many pathologies, including ischemia–reperfusion injuries, neurodegenerative diseases, and inflammatory processes [15–17]. However, the relationship between necroptosis and SE-induced injury to the immature brain has not been studied fully. In this study, we investigate the role of necroptosis in mediating SE-induced neuronal injury in juvenile rats and the possible interplay among necroptosis, epileptogenesis, and cognitive impairment.

## 2. Methods

### 2.1. Animals

Postnatal day 25 (P25) Sprague–Dawley rats (Sichuan Dashuo Animal Science and Technology Co., Ltd., China), weighing 60–75 g, were used for the study. All rats were housed in quiet, uncrowded facilities under standard laboratory conditions and 12 h:12 h light–dark cycle (lights on at 7:00 a.m.) in a room with controlled temperature of 22 ± 2 °C and humidity of 55–58%. Food and water were available ad libitum. Only male rats were used for the experiments to eliminate confounding effects of variable estrogen levels on neuronal excitability. All procedures used in this study conformed to the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and were approved by the Committee for Animal Care and Use at Sichuan University.

### 2.2. Induction of SE

Animals were randomly divided into an experimental group (SE group, n = 78) and a control group (normal saline group, n = 40). Eighteen to 20 h before the pilocarpine treatment, an intraperitoneal (IP) injection of lithium chloride was administered at a dose of 127 mg/kg (Sigma, USA). Thirty minutes before the administration of pilocarpine, the rats were pretreated with an IP injection of 1 mg/kg methylscopolamine-bromide (Shangdong Weifang Pharmaceutical Co., Ltd., China) to counteract the peripheral cholinomimetic effects of pilocarpine. The rats were then administered a single 30 mg/kg IP injection of pilocarpine (Sigma, USA). After the first injection of pilocarpine, the rats received repeated IP injections of pilocarpine (10 mg/kg) every 30 min until they developed SE that could be classified as stage 4 or 5 according to the Racine grading (1972) [18]. The maximum dose of pilocarpine was set to 60 mg/kg. Sixty minutes after SE, rats were given 10 mg/kg diazepam (Tianjin Pharmaceutical Co., Ltd., China) to curtail the seizures and reduce morbidity and mortality. For rats in the control group, all the injections were performed using the same volume of normal saline at the same time points.

### 2.3. Detection of cell death in brain tissue using propidium iodide staining

Propidium iodide (PI) staining was used to detect cell necrosis at 24 h, 48 h, 72 h, and 7 days after SE. For in vivo PI staining, PI (3 mg/kg, Sigma, USA) was administered via tail vein, 3–5 h prior to sacrifice. After transcardial perfusion, the brains were harvested and fixed in 4% paraformaldehyde at 4 °C for 48 h. Brain samples were embedded in 4% agarose and cut into 40-μm thick coronal plane sections with an oscillating tissue slicer. Following tissue sectioning, DAPI (1:5000, Beyotime, China) nuclear stain was applied for 5 min. Next, all sections were mounted onto glass slides and cover-slipped with antifade mounting medium (Beyotime, China). Brain sections were imaged using a laser scanning confocal microscope (Olympus, Japan). Brain regions were defined according to the stereotaxic atlas of rat brain. PI-positive and DAPI-positive cells were counted by Image-Pro Plus (Media Cybernetics, USA). Cell counts were performed twice on three adjacent sections for each region by one observer blinded to the different groups of rat. The cell counts were averaged in each region.

### 2.4. Transmission electron microscopy

Brain tissue samples were fixed in a buffer containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS) at 4 °C for 24–36 h. Afterwards, they were post-fixed in 3% glutaraldehyde and 1% phosphate-buffered osmium tetroxide, dehydrated in series acetone, and embedded in Epon812. Ultrathin sections (90 nm) were stained with uranyl acetate and lead citrate, and observed under an H-600IV transmission electron microscope (Hitachi, Japan).

### 2.5. Western blotting

The rat brain cortex was homogenized immediately by ultrasonication after isolating it in RIPA solution supplemented with proteases and phosphatase inhibitors. Brain tissue homogenates were maintained on ice for 30 min and then centrifuged at 14,000 rpm for 30 min at 4 °C; the supernatant was collected. Protein concentration was determined by BCA protein assay (Sigma, USA). Sixty micrograms of protein per lane was subjected to 8% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membrane was then washed, put in blocking solution, and probed with rabbit RIP3 polyclonal antibody (1:1000, Abcam, USA) or phospho-MLKL (p-MLKL) monoclonal antibody (1:1000, Abcam, USA). β-actin (1:5000, Sigma, USA) was used as a loading control. Bands were visualized with enhanced chemiluminescence (ECL, Millipore, USA) using a gel imaging analysis system (Bio-RAD, USA). The intensity of each band was measured using Gel-Pro Image Analyzer Software (Gel-Pro 4.0, Media Cybernetics, USA).

### 2.6. Immunofluorescence staining

After the tissue was sectioned on an oscillating slicer and rinsed with PBS, a blocking solution composed of PBS, 2% fetal calf serum, and 0.2% Triton X-100 was applied to the sections for 1 h. The sections were incubated in the primary antibody, rabbit anti-MLKL polyclonal antibody (1:500, Abcam, USA), overnight at 4 °C. After washing, they were