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Therapeutic Effects of Kv7 Channel Activator Retigabine against Seizures and Neurodegeneration in Kainic Acid-Induced Seizure Model

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Abstract: Aims: The current study evaluates whether Kv7 channel activator retigabine (RTG) could attenuate seizures and hippocampal neurodegeneration in the kainic acid (KA) induced seizure model. **Methods**: The anticonvulsant activity of RTG is evaluated by electroencephalography (EEG) recordings in KA-kindled rats. Tunel assay and immunofluorescence of NeuN are used to evaluate the neuroprotective effect of RTG in the hippocampus of KA-kindled rats. **Results**: RTG effectively reduces epileptiform discharges induced by KA injection. Tunel assay and immunofluorescence of NeuN show that RTG exerts neuroprotection in the hippocampus of KA-kindled rats. **Conclusion**: Taken together, this study demonstrates that RTG effectively alleviates seizures and hippocampal neurodegeneration in the KA-induced seizure model.

Keywords: Kv7 Channel, RTG, Kainic Acid, Neuroprotection, Seizure

1 Introduction

Epilepsy is a common chronic central nervous system disease caused by the abnormal synchronized discharge of neurons that afflicts more than 65 million people all over the world (Moshé et al., 2015). Patients with epilepsy often appear anxiety, depression, cognitive dysfunction, and other complications. Temporal lobe epilepsy (TLE) is a common refractory focal epilepsy, accounting for 40% of epilepsy patients (Tai et al., 2018). Spontaneous recurrent seizures and hippocampal sclerosis are the main features of TLE (Fernandes et al., 2015). Hippocampal sclerosis is characterized by massive neurodegeneration, which is closely related to spontaneous recurrent seizures (Sutula et al., 1989). Patients with TLE are resistant to existing antiepileptic drugs (White, 2003). Therefore, it is mandatory to discover the novel therapeutic pathways to meet an extensive clinical need.

Kainic acid (KA) is an ionotropic glutamate receptor agonist, which can cause intense membrane depolarization and lead to an abnormal discharge of neurons (Nadler, 1981; Wang et al., 2005). KA activates N-methyl-D-aspartic acid receptors and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors in the postsynaptic membrane of neurons and promotes the influx of sodium, potassium, and calcium ions. KA also induces strong depolarization of neurons for a long time, resulting in neuron necrosis and apoptosis (Vincent and Mulle, 2009). Intracerebroventricular injection of KA in rats can induce spontaneous recurrent seizures and hippocampal sclerosis, which are highly consistent with the symptoms of patients with TLE (Bloss and Hunter, 2010). Therefore, this model of KA-induced epilepsy has become the most widely accepted animal seizure model for the study of human TLE.

The relative imbalance between excitatory and inhibitory neurotransmission will result in abnormally frequent electrical discharges in neurons that subsequently initiate epilepsy (Bell et al., 2011). The Kv7 channels are voltage-gated potassium channels encoded by the KCNQ gene, which are formed by a homomeric or heteromeric complex of five different Kv7 subunits (Kv7.1–7.5, encoded by the KCNQ1–5 genes) (Greene and Hoshi, 2017). In addition to Kv7.1 found in the cardiac myocytes and pancreatic beta cells, other subunits are widely present in the central and peripheral nervous systems (Brown and Passmore, 2009; Wulff et al., 2009). Heterotetramers composed of Kv7.2 and Kv7.3 subunits are the molecular basis of neuronal M channels, which play a key role in controlling neuron excitability (Brown and Passmore, 2009; Mora and Tapia, 2005). Non-inactivated voltage-dependent potassium currents are generated by the Kv7/M channels activating continuously, making the membrane potential return to the resting state and thus reducing the excitability of neurons. Therefore, Kv7 channels have been regarded as an important therapeutic target for epilepsy, based on which, novel therapies against epilepsy have been developed.

Pharmacotherapy is regarded as the primary treatment for patients with epilepsy. Retigabine (RTG) as a classical Kv7 channel activator has been used in the treatment of epilepsy. RTG is authorized by the US Food

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and Drug Administration as adjuvant therapy for partial seizures, and since then, RTG-induced augmentation of the Kv7-current has seen great success as an antiepileptic therapy (Fattore and Perucca, 2011; Weisenberg et al., 2011). RTG activates most subunits of the Kv7 channel family, such as Kv7.2-7.5, thus reducing the excitability of neurons (Gunthorpe et al., 2012). In the present study, the antiepileptic property and neuroprotection of RTG were evaluated in the KA-induced seizure model. This study demonstrated RTG possessed a potently anti-epileptic effect in the KA-induced seizure model, which is also capable of ameliorating neurodegeneration associated with epilepsy.

2 Material and methods

2.1 Drugs

Retigabine (RTG) was purchased from Targsense scientific Co., Ltd (Shanghai, China), freshly dissolved in dimethylsulfoxide to prepare the stock solution (10 mg/ml). Kainic acid (KA) was purchased from Aladdin (Shanghai, China), and dissolved in the artificial cerebral spinal fluid (ACSF) (1 mg/ml). Normal ACSF has the following composition: 125 mM NaCl, 2.5 mM KCl, 21.4 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 11.1 mM glucose.

2.2 Animals

Adult male Sprague-Dawley rats (180~210 g) used in this study were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Before any experiments, the rats were kept in cages containing 5 rats per cage and maintained under standard housing conditions (23 ± 2 °C, 12/12 h light/dark cycle) for one week to adapt to the environment. All animals were allowed free access to food and water. All animal experimental procedures were performed in accordance with guidelines of the Institutional Animal Care and Use Committee of Qingdao University Medical College.

2.3 KA-induced seizure model in rats

The rats were injected intraperitoneally with 10% chloral hydrate at a dose of 400 mg/kg to achieve narcotism. The rats were put into deep coma and mounted on a stereotaxic instrument. The head skin was incised longitudinally from the midpoint of the junction of the two eyes to the midpoint of the junction of the two external auditory canals. A small hole was drilled above the left lateral ventricle on the surface of the skull. 1 μg KA was slowly infused into the left lateral ventricle of rats (coordinates: 1 mm posterior to the bregma, 1.5 mm lateral to the midline, and 3.7 mm under the dura) by using a micro syringe inserted in the lateral ventricle. The needle remained *in situ* for 5 min to allow KA to be fully absorbed after injection before being slowly extracted. Intraoperatively, 3-5% hydrogen peroxide and topical penicillin were used to prevent contamination and infection. Rats in the control group were treated with an equal volume of ACSF instead of KA using the same method. All rats injected KA were monitored to assess their seizure stages according to Racine's scales as follows (Racine, 1972): Stage 0, no response; Stage 1, ear and facial twitching; Stage 2, head nodding and wet dog shakes; Stage 3, unilateral forelimb clonus and myoclonic jerks; Stage 4, bilateral forelimb clonus and rearing and Stage 5, generalized tonic-clonic seizures, rearing and falling. Most rats exhibited continuous epilepticus of Racine stages of 3 or higher for at least 3 consecutive hours after the KA injection. Rats with Racine stages of below 3 were excluded from the experiment.

2.4 EEG recording and treatment

After rats were injected with KA or ACSF in the lateral ventricle, electrodes were immediately implanted in the cerebral cortex before seizures occurred in kindled rats. Two 1 mm diameter holes were drilled on the skull surface, one hole above the cortex for recording electrode fixation, and the other hole above the cerebellum for reference electrode fixation. The electrodes were fixed into the corresponding hole with dental cement so that the bottom of the electrode in contact with the dura mater (Jimenez-Pacheco et al., 2013). The rats were placed in cages with free access to water and food, and their electrodes were connected to the electroencephalograph (Blackrock Microsystems, USA) by a special wire. All rats were randomly divided into three groups (n = 3): control group (saline), KA group (saline), KA + RTG group (RTG, 7 mg/kg). The rats were injected intraperitoneally with corresponding drugs once a day and then recorded 2 hours a day by electroencephalograph for 10 days continuously. EEG of epileptic discharge was analyzed by using NeuroExplorer 5 software.

2.5 Immunofluorescence

After 10 days of treatment, the rats were sacrificed to detect hippocampal neurons. The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate at a dose of 400 mg/kg. In about 15 min, the rats entered into a deep coma and lost responses when their toes were pinched. Each rat was rapidly perfused with 150 ml saline followed by 150 ml 4% paraformaldehyde (PFA) solution to ventriculus sinister by infusion apparatus. Brains were quickly removed and postfixed in 4% PFA for over 12 hours at 4°C for fixation. Then, the brains were gradually dehydrated with alcohol and embedded in paraffin. According to the stereotactic map of the brain, 5 μm-thick transverse sections of the rats' hippocampus were cut on a rotary microtome, which was prepared for immunofluorescence and Tunel staining.

Paraffin-embedded tissue sections were first deparaffinized in xylene and rehydrated in graded ethanol before antigen retrieval of sections was performed by microwave heating method. The sections were sequentially treated with 0.4% Triton-X100

(Solarbio, China) and blocked with 2% bovine serum albumin buffer. Tissue sections were further incubated with primary antibodies anti-NeuN (Abcam, UK) in 2% bovine serum albumin buffer overnight at 4°C. The slices were washed with PBS and next immunostained by the secondary antibody Alexa fluor 647 IgG (Abcam, UK) in the dark for 1 h. A mounting medium containing DAPI nuclear counterstain was applied to the sections before sections were coverslipped. The fluorescence images were captured and analyzed by a scanning laser microscope (Leica, Germany) to assess the degree of nerve cell loss within the hippocampal CA1 region.

2.6 Terminal dUTP Nick-end Labeling (TUNEL) Assay

The brain sections were first dewaxed by xylene and dehydrated by gradient ethanol, and then treated with 20 μg/mL DNase-free protease K for over 15 min (Beyotime, China). After three washes in PBS, brain sections were first incubated in DNase I buffer at room temperature for 10 min and then in Tunel detection solution at 37°C for 60 min. The brain sections were incubated with DAPI for 10 min before coverslips were mounted using an anti-fluorescence quenching agent. Finally, the brain sections were inspected by a confocal microscope (Leica, Germany). Fluorescence intensity was quantified by using the Image J software.

3 Statistical analysis

The GraphPad Prism was used for statistical analysis. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical comparisons of multiple groups. All values were expressed as the mean ± s.e.m. (standard error of the mean). The statistically significant differences were considered as the *p*-value of less than 0.05.

4. Results

4.1 Protection against KA-induced seizures by RTG

The rats were treated with saline or RTG (7 mg/kg) once a day by intraperitoneal injection and monitored continuously by EEG recordings for 10 days. We examined the seizure activities in EEG and found that epileptiform discharges of rats were discovered every day after the development of seizures induced by KA. The EEG was stable without obvious spike-wave in the control. The EEG of the KA group was abnormal, with predominant fast sharp rhythmic activity (Figures 2). Compared with the KA group, we found that the administration of RTG dramatically suppressed continuous fast sharp rhythmic activity. In addition, RTG also significantly decreased the frequency of spikes and reduced the duration of epileptiform discharges (Figure 2). Our data showed that RTG suppressed electrographic seizure activity in the KA-induced seizure model.

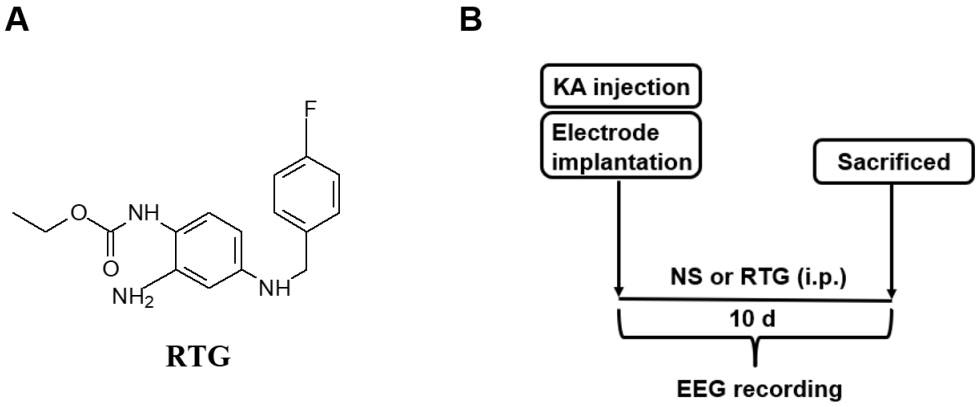

Figure 1. The chemical structure of retigabine (RTG) and a schematic representation of this study
(A), The diagram of the chemical structure of RTG.
(B), A schematic representation for KA-induced seizure model in rats and RTG or saline treatment.

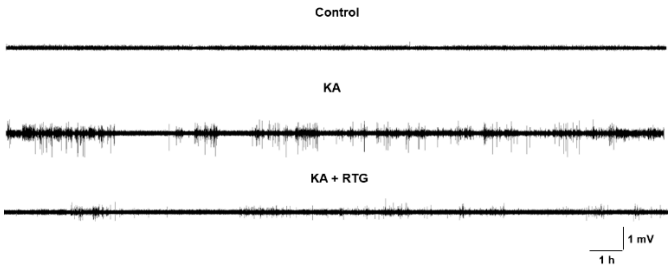

Figure 2. The representative EEG recordings for 1 h in the control, KA, and KA + RTG (7 mg/kg) group.

4.2 Neuroprotection against KA-induced seizures by RTG

Fluorescence immunostaining was exerted for the mature neuronal marker NeuN in hippocampal tissues. NeuN staining was found in both the nucleus and soma of hippocampal neurons. The sections of the KA group exhibited extensively neuronal loss in the hippocampal CA1 region, which demonstrated that there was severe neuronal damage induced by KA (Figure 3A). Quantity analysis of fluorescence indicated that RTG enhanced significantly the number of NeuN positive neurons in the hippocampal CA1 region compared with the KA group (Figure 3B). To examine whether retigabine attenuates

the cell apoptosis induced by KA, we assessed the cell apoptosis in brain sections using the Tunel assay. The KA group showed the greatest amount of apoptosis in the hippocampal CA3 region (Figure 4A). Cell apoptosis was higher in the KA group than in the control group, but RTG treatment rescued cell apoptosis in the hippocampal CA3 region (Figure 4B). These data suggested that RTG treatment could reduce cell apoptosis induced by KA in rats. In conclusion, our data demonstrated that RTG exerted neuroprotection in the KA-induced seizure model.

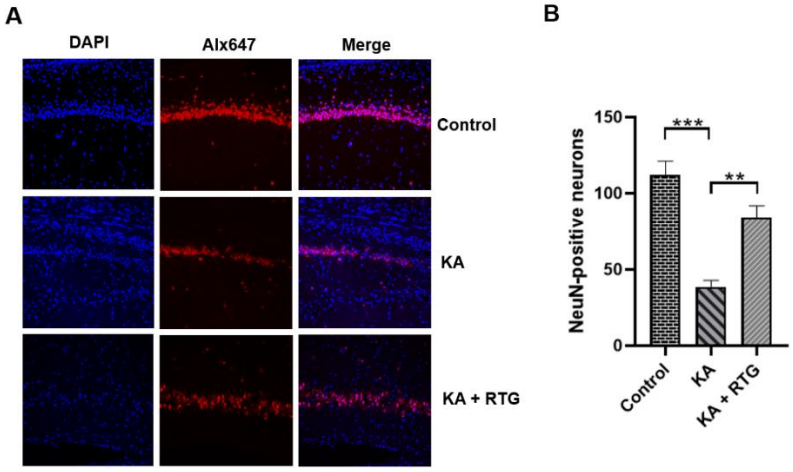

Figure 3. Neuroprotection of RTG on hippocampal neurons from KA-kindled rats by fluorescence immunostaining.
(A), Representative NeuN-immunostaining from control, KA and KA + RTG (7 mg/kg) group in the hippocampal CA1 area. Scale bar: 100 μm.
(B), Quantitative analysis of the number of NeuN positive neurons in control, KA, and KA + RTG (7 mg/kg) group. $^{**}p < 0.01$, $^{***}p < 0.001$ vs KA group, n=3.

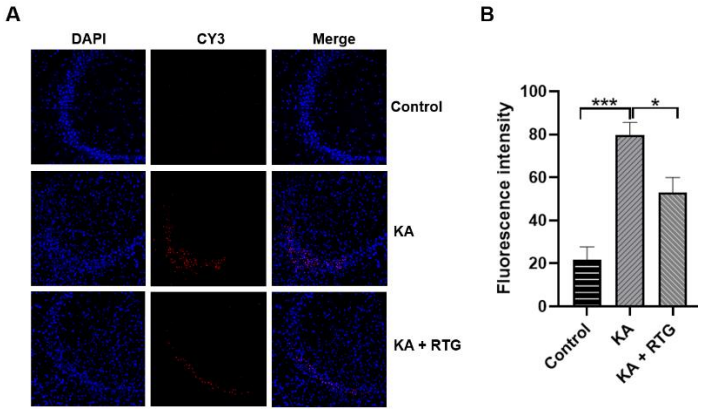

Figure 4. The attenuation of RTG on cell apoptosis from KA-kindled rats by Tunel staining.
(A), Cell apoptosis of hippocampal CA3 sections was detected by Tunel assay with or without RTG (7 mg/kg) treatment. Scale bar: 100 μm.
(B), Quantitative analysis of the fluorescence intensity from Control, KA and KA + RTG (7 mg/kg) group in the hippocampal CA3 region. $^{*}p < 0.05$, $^{***}p < 0.001$ vs KA group, n=3.

5 Discussion

Traditional seizure models induced by pentylenetetrazole and maximal electroshock are classical animal models for the evaluation of novel antiepileptic drugs, which provide an effective means for rapid identification of potential antiepileptic drugs (Barker-Haliski and Steve White, 2020). However, these acute seizure models cannot effectively evaluate antiepileptic drugs that are used for the treatment of drug-resistant epilepsy (Singh et al., 2021). The KA-induced animal model with spontaneous seizures solves this problem well. Previous studies have shown that RTG dramatically reduced spontaneous burst frequency in KA-treated slices of medial entorhinal cortex-hippocampal, which indicated the potential of RTG to prevent spontaneous seizures following status epilepticus (Porter et al., 2007; Smith et al., 2007). Although these results were less relevant to study in vivo, the apparent potency of RTG suggests that the Kv7 mechanism remains viable and effective in the KA-induced seizure model with spontaneous seizures. In this study, we investigated whether RTG plays a role in the KA-induced seizure model characterized by spontaneous recurrent seizures and neurodegeneration. Our results of EEG recording demonstrated RTG treatment significantly decreased the time of

epileptiform discharges and the number of spikes. The antiepileptic effect of RTG was confirmed in the KA-induced seizure model in vivo.

After being given intracerebral KA injection, rats develop neuropathological changes in the hippocampus such as neurodegeneration and neuronal cell death, which are similar to patients with chronic TLE and hippocampal sclerosis (Bear et al., 1996; Du et al., 1995; Sutula et al., 1989). The death of these neurons occurs rapidly after the KA injection and remains localized to the dorsal hippocampus (Venceslas and Corinne, 2017). Previous studies have shown that the RTG exerted a neuroprotective effect in rat organotypic hippocampal slices exposed to glutamic acid homolog (Boscia et al., 2006). In vitro studies that explored the neuroprotective properties of RTG are considered promising, but studies in the KA-induced seizure model will be useful in providing information of more valuable clinical relevance. Our study showed that extensive neuronal loss or apoptosis was observed in the KA group compared with the control group, supporting the idea that spontaneous recurrent seizures induced by KA can produce a strong, deleterious effect on the neurons of rats. However, RTG treatment alleviated significantly neuronal loss in the KA-induced seizure model. Our results indicated that RTG exerted neuroprotection in rats treated with KA by activating Kv7 channel, which appears to be advantageous over some currently available drugs.

Chemical activation of neuronal Kv7 channels has been considered an effectively treatment tactic for hyperexcitability-related diseases such as epilepsy. RTG is considered a potent Kv7 channel opener and is representative of a class of drugs that exert anti-epilepsy effects. In addition to hyperexcitability states are counteracted, RTG also decreases the progression of slowly-developing neurodegenerative diseases in the KA-induced seizure model. However, the specific mechanisms underlying the neuroprotective effects of RTG are not well understood in this study, and these questions require further exploration. Developing potent and specific openers of Kv7 channels, and putting them into clinical application, will be a large challenge ahead.

6 Conclusion

Retigabine, a small molecule that activates the Kv7 channel, reduces seizure activity and protects hippocampal neurons in the KA-induced seizure model with spontaneous recurrent seizures and neurodegeneration. The specific activation of Kv7 may provide a prospective therapeutic pathway to improve the treatment of patients with TLE.

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