1521-0103/384/2/315–325$35.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

dx.doi.org/10.1124/jpet.122.001328
J Pharmacol Exp Ther 384:315–325, February 2023

The Journal of PHARMACOLOGY And Experimental Therapeutics

Attenuation of Epileptogenesis and Cognitive Deficits by a Selective and Potent Kv7 Channel Opener in Rodent Models of Seizures

Xiao-fei Zhuang,[1] Yu-xue Liu,[1] Zhi-hong Yang, Qin Gao, Lei Wang, Chuanxia Ju, and KeWei Wang

Department of Pharmacology, School of Pharmacy, Qingdao University Medical College (X.-F.Z., Y.-X.L., Z.-H.Y., Q.G., L.W., C.J., K.W.) and Institute of Innovative Drugs, Qingdao University, Qingdao, China (K.W.)

Received May 27, 2021; accepted October 20, 2022

ABSTRACT

Targeting neuronal Kv7 channels by pharmacological activation has been proven to be an attractive therapeutic strategy for epilepsy. Here, we show that activation of Kv7 channels by an opener SCR2682 dose-dependently reduces seizure activity and severity in rodent models of epilepsy induced by a GABAa receptor antagonist pentylenetetrazole (PTZ), maximal electroshock, and a glutamate receptor agonist kainic acid (KA). Electroencephalographic recordings of rat cerebral cortex confirm that SCR2682 also decreases epileptiform discharges in KA-induced seizures. Nissl and neuronal nuclei staining further demonstrates that SCR2682 also protects neurons from injury induced by KA. In Morris water maze navigation and Y-maze tests, SCR2682 improves PTZ- and KA-induced cognitive impairment. Taken together, our findings demonstrate that pharmacological activation of Kv7 by novel opener SCR2682 may hold promise for therapy of epilepsy with cognitive impairment.

SIGNIFICANCE STATEMENT

A neuronal Kv7 channel opener SCR2682 attenuates epileptogenesis and seizure-induced cognitive impairment in rodent models of seizures, thus possessing a developmental potential for effective therapy of epilepsy with cognitive impairment.

Introduction

Epilepsy is a chronic neurologic disorder characterized by abnormal synchronous neuronal discharge and recurrent epileptic seizures affecting more than 70 millions of people worldwide (Löscher et al., 2020). Among the patients with epilepsy, approximately 30% to 40% of them develop learning and memory impairments (Sayin et al., 2004), especially in the patients with temporal lobe epilepsy (TLE; Tai et al., 2018). Cognitive comorbidities are as detrimental as seizures severely affecting patients' quality of life (Bell et al., 2011; Brooks-Kayal et al., 2013). Therefore, it is necessary to develop novel therapeutic agents to meet a wide range of medical needs by mitigating recurrent seizures and improving cognitive deficits.

Pharmacotherapy has been a primary treatment strategy for epilepsy. Unfortunately, one-third of patients with epilepsy can develop resistance to existing antiepileptic drugs (AEDs; Löscher et al., 2020). The most common treatment of epilepsy primarily relies on AEDs that work by reducing abnormal electrical activity in the brain (Wang and Chen, 2019). Membrane ion channels are responsible for neuronal excitability, and the conventional AEDs targeting either sodium or calcium channels are still the mainstay for treatment of epilepsy. The only AED with a novel mechanism of action is a Food and Drug Administration–approved retigabine (RTG; or ezogabine) in 2011 that activates the voltage-gated Kv7/KCNQ/M-channels for the treatment of partial epilepsy (Weisenberg and Wong, 2011). Nevertheless, the AED RTG was recently withdrawn from the market due to its metabolites causing side effects such as vision change and discoloration of the skin (Rubi et al., 2017).

Kv7/KCNQ/M-channels are a subfamily of voltage-gated K^+ channels with five members, Kv7.1–7.5 (Gutman et al., 2005; Brown and Passmore, 2009; Greene and Hoshi, 2017). With the exception of Kv7.1, all other four subunits (Kv7.2–7.5) are expressed in the central nervous system (Brown and Passmore, 2009; Barrese et al., 2018). The Kv7.2 and Kv7.3 subunits can co-assemble into the heterotetrameric channels underlying the molecular basis of native M current in neurons (Wang et al., 1998). Pharmacological activation of Kv7/KCNQ/M-channels give rise to

This work was supported by research grants to C.J. and K.W. from Shandong Provincial Natural Science Foundation [ZR2020MH155], Shandong Province Higher Educational Science and Technology Program [J17KA236], Science and Technology Program of Guangdong [2018B030334001], and the Ministry of Science and Technology of China [2018ZX09711001-004-006].

No author has an actual or perceived conflict of interest with the contents of this article.

[1]These authors contributed equally.

dx.doi.org/10.1124/jpet.122.001328.

ABBREVIATIONS: AEDs, antiepileptic drugs; EEG, electroencephalography; ICV, intracerebroventricular; KA, kainic acid; MES, maximal electroshock; MWM, Morris water maze; NeuN, neuronal nuclei; PTZ, pentylenetetrazole; PSD, power spectral density; RTG, retigabine; SPT, spatial probe test; SRSs, spontaneous recurrent seizures; TLE, temporal lobe epilepsy.

the non-inactivating voltage-dependent K^+ currents that suppress neuronal hyperexcitability, thus serving as an attractive therapeutic strategy for epilepsy (Mora and Tapia, 2005).

We have recently identified a novel small compound, 2, 6-dimethyl-4-(piperidin-yl) phenyl)-amide derivative SCR2682 that potently and selectively activates neuronal Kv7 channels (Zhang et al., 2019). The channel opener SCR2682 also enhances native M-current from hippocampal and cortical neurons in rat (Zhang et al., 2019). In this study, we tested the effects of SCR2682 on seizures and cognitive impairment in rodent models of seizures and found that activation of neuronal Kv7 currents by the channel opener SCR2682 attenuates epileptogenesis and seizure-induced cognitive impairment.

Materials and Methods

Reagents

Kv7/KCNQ/M-channel opener SCR2682 (Simcere Pharmaceuticals Co. Ltd., Shanghai, China) was dissolved in Tween-80 (10 mg/ml) and stored at −20°C. The chemical structure of SCR2682 (4-(2-bromo-6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)-2,6-dimethylphenyl)-3,3-dimethylbutanamide) was previously reported (Zhang et al., 2019). AED RTG (Zhimeng Biopharma Co. Ltd., Shanghai, China) was dissolved in DMSO at 10 mg/ml and stored at −20°C. Compounds such as XE991, pentylenetetrazole (PTZ) and kainic acid (KA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). XE991 was dissolved in DMSO at 5 mg/ml and stored at −20°C. PTZ was freshly prepared with saline prior to injections. KA was dissolved in aerobic artificial cerebral spinal fluid (10 mg/ml) and stored at −20°C before use.

Animals

Kunming male mice (approximately 18–22 g, about 8 weeks old) were obtained from Experimental Animal Center of Shandong Lukang Pharmaceutical Co, Ltd. Wistar male rats (approximately 170–190 g, about 8 weeks old) and Sprague-Dawley male rats (approximately 170–190 g, about 8 weeks old) were from Beijing Vital River Laboratory Animal Technology Co., Ltd. Before experiments, animals were placed to adapt to the environment for one week in a quiet temperature-controlled (22 ± 3°C) and humidity-controlled (65 ± 5%) rooms on a 12 hour light/dark cycle (7:00–19:00 hour light) at the Experimental Animal Center of Qingdao University Medical College. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Qingdao University Medical College and performed in accordance with institutional and national guidelines for the use and care of animals for experiments.

PTZ-Induced Seizures

Wistar rats were administrated with 35 mg/kg PTZ (intraperitoneally) for six times at an interval of 48 hours until the development of seizures (Kaur et al., 2015). All rats were monitored and recorded for their seizures according to previous reports (Corda et al., 1990; Hashemian et al., 2017), and the seizure stages were classified according to Racine's scales (Racine, 1972). Rats exhibiting stage 2 or more severe seizures for five consecutive tests were considered as kindled rats, which were selected for following experiments.

For evaluation of the time- and dose-dependent antiepileptic activities of SCR2682, the kindled rats were divided into different groups of PTZ (saline, intraperitoneally), RTG (7 mg/kg, i.p.) and SCR2682 (0.4, 0.8, 1.2 and 1.6 mg/kg, i.p.). Rats in each group were injected with the corresponding drugs. After the different time (15, 30, 45, 60, and 75 minute), each group of rats was injected with PTZ (35 mg/kg, i.p.) and SCR2682 or RTG. The seizure stages of kindled rats were observed and recorded within 1 hour after PTZ injection.

Maximal Electroshock

Mouse seizure model of maximal electroshock (MES) was induced using an electroconvulsive apparatus (YLS-9A Electrical Stimulation Instrument, Beijing Zhongshi Dichuang Technology Development Co. Ltd., China). The stimulation parameters were set as follows: continuous wave output, positive square waves (20 ms) + interval (10 ms) + negative square waves (20 ms). The wave number was 75, the constant current was 4 mA and the voltage was 90 V. Compound SCR2682 at different doses (0.25, 0.5, 1, and 2 mg/kg), RTG (10 mg/kg), or saline were administered through intraperitoneal injections 30 minutes before seizure induction. XE991 (3 mg/kg, i.p.) was injected 5 minutes before administering of SCR2682 (2 mg/kg, i.p.). The mouse hind limb extension was observed to consider a successful induction of electroconvulsion (Davoren et al., 2015; Sakkaki et al., 2016).

KA-Induced Acute Seizures

Rats (Sprague–Dawley) with body weight about 190–210g) were anesthetized by injection of 10% chloral hydrate (400 mg/kg, i.p.). There were three holes drilled in the surface of the skull by a dental drill with the coordinates as the following: anteroposterior = 0.8 mm posterior to bregma, mediolateral = 1.5 mm lateral to bregma, and dorsoventral = 3.8 mm. For intracerebroventricular (ICV) microinjections, a stainless-steel guide cannula (RWD Life Science, Shenzhen, China) was implanted into the left lateral ventricle of the brain. Seven days after the surgery, rats were divided into the groups of KA (saline, intraperitoneally) as control, RTG (7 mg/kg, i.p.), and different doses of SCR2682 (0.4, 0.8, and 1.6 mg/kg, i.p.). Forty-five minutes after compound administrations, rats in all groups were injected with 1 μg KA by an infusion cannula inserted into the left lateral ventricle through the guide cannula. Subsequently, the rats were recorded for 1 hour by video (Hikvision Digital Technology, Hangzhou, China).

KA-Induced Chronic Spontaneous Recurrent Seizures

A total of 1 μg KA was slowly injected into the left lateral ventricle using a microsyringe. The control rats were injected with aerobic artificial cerebral spinal fluid. Most rats underwent at least 3 hours of acute status epilepticus in Racine stages of 3, 4, and 5, and subsequently developed spontaneous recurrent seizures (SRSs) 2 to 3 weeks after the KA injection, which is similar to symptoms of patients with TLE (Lévesque and Avoli, 2013). Rats developing SRSs were divided into the groups of KA (saline, intraperitoneally), RTG (7 mg/kg, i.p.), and SCR2682 in different doses (0.4, 0.8, and 1.6 mg/kg, i.p.). For the baseline recording of spontaneous seizures, rats were recorded for 7 consecutive days using a video monitoring system. The kindled rats were intraperitoneally administered with SCR2682, RTG, or saline once a day and they were continuously monitored for 7 days. Seizure frequency and duration were measured by observing video recordings.

Cognitive Tests

Morris Water Maze Test for PTZ-Induced Seizures. The apparatus of Morris water maze (MWM) is composed of a circular pool with 50 cm in height and 160 cm in diameter and a video-tracking system powered by SMART 3.0 software (Panlab Harvard Apparatus, Spain). The water temperature in circular pool is set about 23 ± 1°C, and the water depth is 30 cm with a hidden escape platform below the water surface about 2 cm in quadrant 3. The MWM task consists of a place navigation test (4 consecutive days from day 1 to day 4) and a spatial probe test (SPT; day 5). Rats were randomly divided into the groups of Control (saline, intraperitoneally), PTZ (saline, intraperitoneally), RTG (7 mg/kg, i.p.), and different doses of SCR2682 (0.4, 0.8 and 1.6 mg/kg, i.p.). For MWM experiments, rats were administered with their corresponding compounds once

a day for consecutive 5 days, and the test was carried out 45 minutes after the injection. The time for rats from entering the water to discovering the escape platform was recorded within 60 seconds. The time for rats to find the platform was defined as the escape latency. If rats were unable to identify the escape platform within 60 seconds, rats were allowed to stay on the platform for 20 seconds, and the escape latency was 60 seconds. On the day 5, a SPT was performed without a platform. Each rat was allowed to enter the water from the quadrant 1. Rats crossing the number of times of platform location within 60 seconds were recorded and subsequently analyzed.

Y-Maze for KA-Induced Chronic Spontaneous Recurrent Seizures. The short-term spatial working memory in KA-induced chronic SRSs was evaluated using a Y-maze test in rats. The Y-maze apparatus consists of three arms of equal size at 43 cm (length) × 12 cm (width) × 27 cm (height), as starting arm, familiar arm and novel arm, respectively. Rats were allowed to move freely in the start arm and familiar arm for 10 minutes to achieve habituation in the first trial. After rest in the cage for 6 hours, a second trial was performed. During the trial, rats were allowed to conduct free exploration for 5 minutes in all three arms (home, familiar, and novel; Featherby et al., 2008). The duration of movement in all three arms was recorded and analyzed by video-tracking systems.

Electroencephalography Recordings

Electroencephalography (EEG) recordings were carried out for KA-induced acute or chronic SRSs of rats after behavioral tests. Three cortical EEG electrodes were skull-mounted, including two recording electrodes and one grounding electrode, and connected to a female header connector by wires. Installation of EEG electrodes, connector, and guide cannula was carried out using dental cement (Jimenez-Pacheco et al., 2013; Hu et al., 2019). Rats were placed in observational cages made of transparent glass with electrodes on their heads connected to an EEG apparatus through a wire (Blackrock Microsystems, Utah, USA). For EEG baseline, rats were recorded for 30 minutes before KA at 1 μg was infused through an cannula inserted into the left lateral ventricle, and rats were subsequently recorded for EEG of another 1 hour. For quantitative analysis of EEG recordings, EEG data were processed by NeuroExplorer 5 software (Blackrock Microsystems, Utah, USA). The power spectral density (PSD) was analyzed using the "EEG spectrogram analysis" function.

For EEG recordings of KA-induced chronic SRSs, a baseline of spontaneous seizures was recorded for 10 hours a day for 5 consecutive days after a 3-day recovery from the surgery of electrode implantation. Rats were administrated corresponding compounds/drugs once a day and monitored for ten hours a day for 5 consecutive days. The data of EEG were analyzed for duration of daily epileptiform discharges using NeuroExplorer 5 software.

Histology

Nissl Staining. After development of SRSs induced by KA, rats were intraperitoneally injected with SCR2682 (1.6 mg/kg), RTG (7 mg/kg), or saline once a day for 7 consecutive days. Rats were quickly performed cardiac perfusion with ice-cold saline and followed by paraformaldehyde. The brains were made into paraffin-embedded tissue blocks and cut into coronal sections (Feng et al., 2021). The sections were stained with Nissl Solution (Beyotime, C0117, China) and photographed with a light microscope (Nikon, Japan). After scanning of brain tissue sections, area measuring tools were used to draw circles around the injured areas. The average value was calculated from three to four different hippocampal CA3 subfields (Zheng et al., 2021). Cell counts of the hippocampal fields were assessed.

Neuronal Nuclei Immunostaining. After brain tissue sections were dewaxed and dehydrated, antigen retrieval was implemented using 10 × Tris EDTA (pH = 9.0; 1:10, Solarbio, C1038, China) in a pressure cooker. The cell membrane was broken using 0.3% Triton-X100 (Solarbio, T8200, China) before incubated with 1% bovine serum albumin blocking buffer at room temperature. The primary antibody rabbit anti-NeuN (1:2000, Abcam, ab177487, UK) was incubated with tissue slices for over 12 hours at 4°C. After washed with PBS, the slices were incubated with the secondary antibodies Alexa fluor 647 Goat anti-rabbit IgG (H + L; 1:200, Abcam, ab150079, UK) for 1 hour in the dark at room temperature. After treated with DAPI (Beyotime, C1006, China) for 10 minutes, the cover slips were mounted using antifluorescence quenching agent. Confocal fluorescence images of CA3 hippocampal structures from different groups were obtained using a scanning laser microscope (Nikon, Japan) with a ×20 objective and measured the optical density using the Image J software.

Statistical Analysis

Statistics was analyzed using GraphPad Prism 8.0 software for paired Student's t test, Fisher's exact test, one-way ANOVA followed by Dunnett's test, or Kruskal–Wallis H test. The P value < 0.05 was considered to be statistically significant. All data are expressed as the means ± S.E.M.

Results

Protective Effects of Kv7 Opener SCR2682 on Seizures Induced by PTZ or MES

The rat model of seizures induced by PTZ was used to assess the time- and dose-dependent effects of Kv7 opener SCR2682 on seizures stages (Fig. 1A). As shown in Fig. 1B, intraperitoneal injections of SCR2682 (0.4 mg/kg) reduced the seizure severity and achieved the maximum anticonvulsive effect 45 minutes post administration, as comparted with the PTZ group alone. Similarly, RTG at 7 mg/kg also exhibited the anticonvulsive effect. We further tested the dose-dependent effect of SCR2682 on the stages of seizures. Co-administrations of PTZ (35 mg/kg, i.p.) with different doses of SCR2682 (0.4, 0.8, 1.2, and 1.6 mg/kg, i.p.) reduced the seizure severity of PTZ kindling rats to seizure stage 1 from the stage 4 in a dose-dependent manner, as compared with the PTZ group (Fig. 1C). As a positive control, administration of RTG (7 mg/kg, i.p.) also reduced the seizure severity to the stage 3 from the stage 4 in PTZ kindling rats (Fig. 1C).

To further confirm the observation, we tested the effect of SCR2682 on seizures induced by MES in mice (Fig. 1D). SCR2682 (0.25, 0.5, 1, 2, mg/kg, i.p.) dose-dependently protected against seizures from 44% to 100%, as compared with the vehicle control without protection or RTG (10 mg/kg, i.p.) with 66.7% protection (Fig. 1E). SCR2682-mediated protective effect was partially reversed about 33% by co-intraperitoneal injections of SCR2682 (2 mg/kg) with Kv7 specific blocker XE991 (3 mg/kg) (Fig. 1E).

SCR2682 Protects Against KA-Induced Acute or Chronic Spontaneous Recurrent Seizures

To further confirm the effects of SCR2682 on attenuating seizures, we recorded the KA-induced seizures in rats pretreated with different doses of SCR2682 or RTG 45 minutes before ICV injection of 1 μg KA in the left lateral ventricle of the brain (Fig. 2A). Intraperitoneal injections of different doses of SCR2682 at 0.4, 0.8, or 1.6 mg/kg showed a dose-dependent reduction of the Racine scores as compared with the saline control of KA group (Fig. 2A). Similarly, RTG at 7 mg/kg (i.p.) also exhibited the antiepileptic effects (Fig. 2A). In EEG recordings, there were frequent spikes with increased amplitudes in the KA group as compared with the baseline

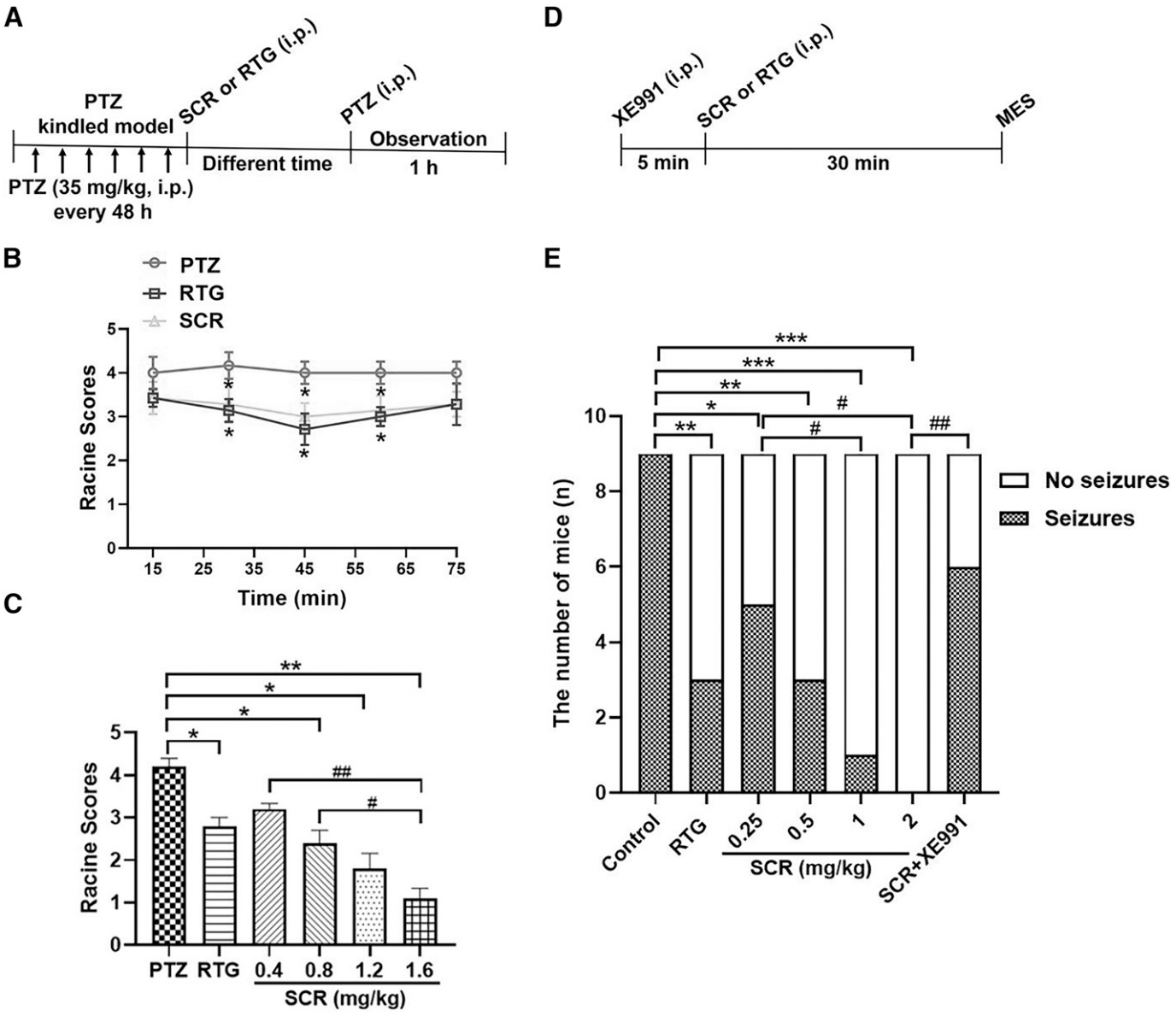

Fig. 1. Protection of PTZ-induced seizures and MES seizure by SCR2682 in rodents. (A) A schematic diagram for the rat model of PTZ-induced seizures and intraperitoneal injections of compounds. (B) Time course of PTZ-induced seizure severity in Racine's scale before and after administrations of saline (intraperitoneally), SCR2682 (0.4 mg/kg, i.p.), and RTG (7 mg/kg, i.p.) in rats. $^{*}P < 0.05$ vs. PTZ group, n = 6–7. (C) A dose-dependent reduction of seizure stages before and after intraperitoneal administrations of SCR2682 (0.4, 0.8, 1.2, or 1.6 mg/kg) or RTG (7 mg/kg) in rat model of PTZ-induced seizures. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. PTZ group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. SCR2682 (1.6 mg/kg) group, n = 10. (D) A schematic timeline for the mouse model of MES and intraperitoneal compound administrations. (E) Dose-dependent reduction of MES by intraperitoneal injections of SCR2682 (0.25, 0.5, 1, or 2 mg/kg) or RTG (10 mg/kg) or saline 30 minute prior to MES in mice. XE991 (3 mg/kg, i.p.) was injected 5 minutes before SCR2682 (2 mg/kg, i.p.). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$ vs. SCR2682 (0.25 mg/kg) group; $^{\#\#}P < 0.01$ vs. SCR2682 (2 mg/kg) group, n = 9.

(Fig. 2B). In contrast, pretreatment of SCR2682 (1.6 mg/kg, i.p.) and RTG (7 mg/kg, i.p.) resulted in a significant reduction of KA-induced spikes in frequency and amplitude as compared with the KA group (Fig. 2B). We analyzed the PSD of EEG recorded in rats within 1 hour after ICV KA or ACSF injection (Fig. 2C). The quantitative analysis of the PSD revealed that the kindled rats induced by KA displayed higher PSD as compared with the control group (Fig. 2D). Preadministration of SCR2682 (0.4, 0.8, or 1.6 mg/kg) significantly reduced the PSD in a dose-dependent manner (Fig. 2D). These results further demonstrated the anticonvulsant effect of SCR2682 on KA-induced acute seizures in rats.

To further explore the anticonvulsant effect of SCR2682 on chronic seizures, we used a chronic rat model of SRSs (Fig. 3A). Administrations of SCR2682 at different doses (0.4, 0.8, or 1.6 mg/kg, i.p.) reduced daily seizure occurrence frequency and duration in a dose-dependent manner, as compared with their baseline or KA alone and RTG groups (Figs. 3B). In the EEG recordings, the chronic SRSs induced by KA were similar to KA-induced acute seizures, displaying abnormal and frequent spikes with increased amplitudes as compared with the baseline of EEG (Fig. 3C). In comparison with the KA group, SCR2682 (1.6 mg/kg) or RTG (7 mg/kg) treatment significantly decreased the spikes and

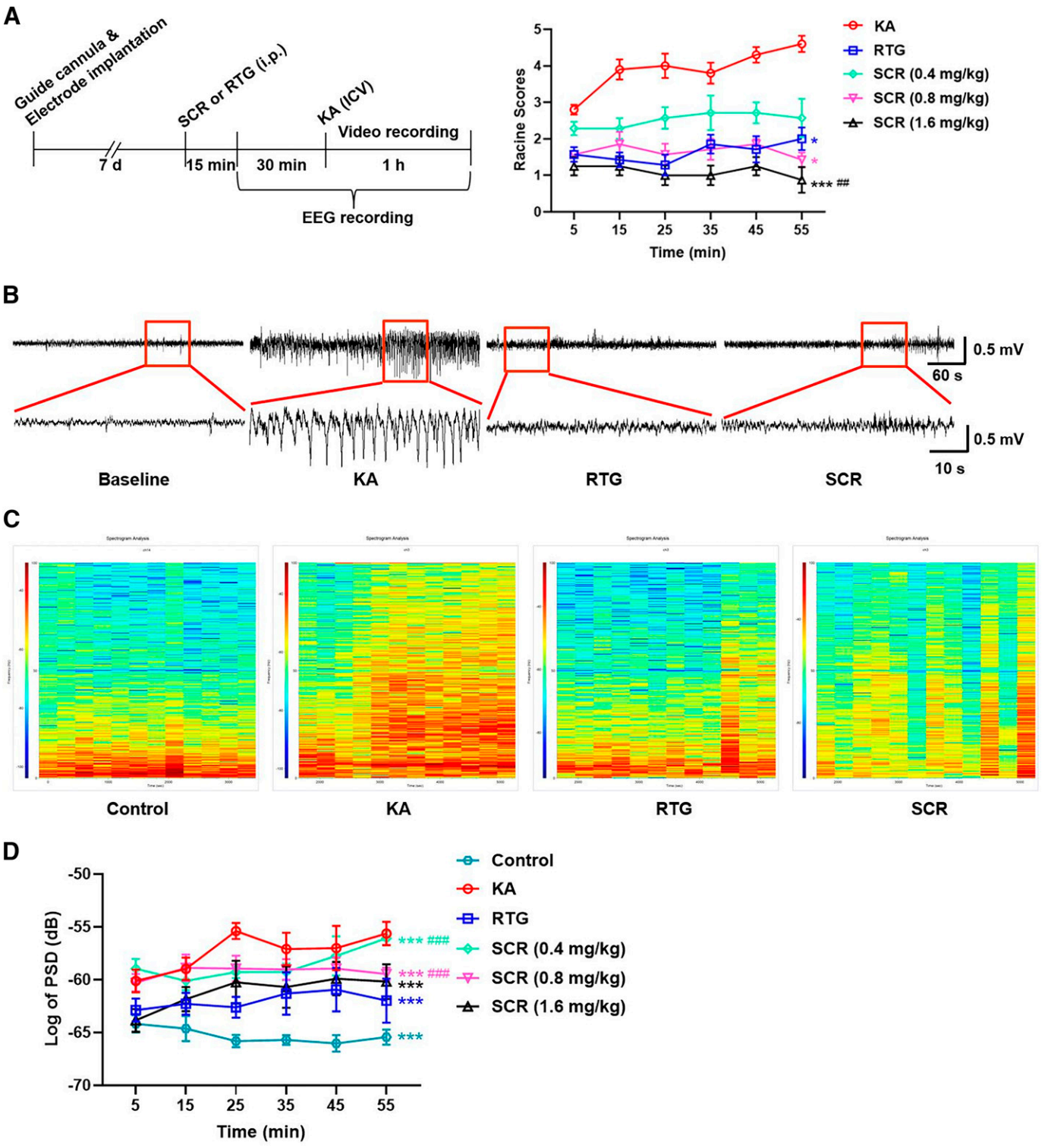

Fig. 2. A dose-dependent antiepileptic effect of SCR2682 in KA-induced acute seizures. (A) Left panel: a schematic timeline for the generation of KA-induced acute seizures by ICV injection (1 μg) in rats before and after intraperitoneal administrations of SCR2682 or RTG. Right panel: a dose-dependent reduction of KA-induced seizures by SCR2682 (0.4, 0.8, or 1.6 mg/kg). Development of seizure behavior after ICV administration of 1 μg KA in rats treated with saline (intraperitoneally), RTG (7 mg/kg, i.p.), or SCR2682 (0.4, 0.8, or 1.6 mg/kg, i.p.). $^{*}P < 0.05$, $^{***}P < 0.001$ vs. KA group; $^{\#\#}P < 0.01$ vs. SCR2682 (0.4 mg/kg) group, n = 7–10. (B) Representative EEG traces recorded from cerebral cortex of rats from the groups of KA, RTG (7 mg/kg), and SCR2682 (1.6 mg/kg). EEG traces of the baseline were recorded from KA group. (C) The representative power spectrum analyses of EEG during 1 hour from the control, KA, RTG (7 mg/kg), and SCR2682 (1.6 mg/kg) groups. (D) Quantitative analysis of PSD during 1 hour from the control, KA, RTG (7 mg/kg), and SCR2682 (0.4, 0.8, or 1.6 mg/kg) groups. $^{***}P < 0.001$ vs. KA group; $^{\#\#\#}P < 0.001$ vs. SCR2682 (1.6 mg/kg) group, n = 5.

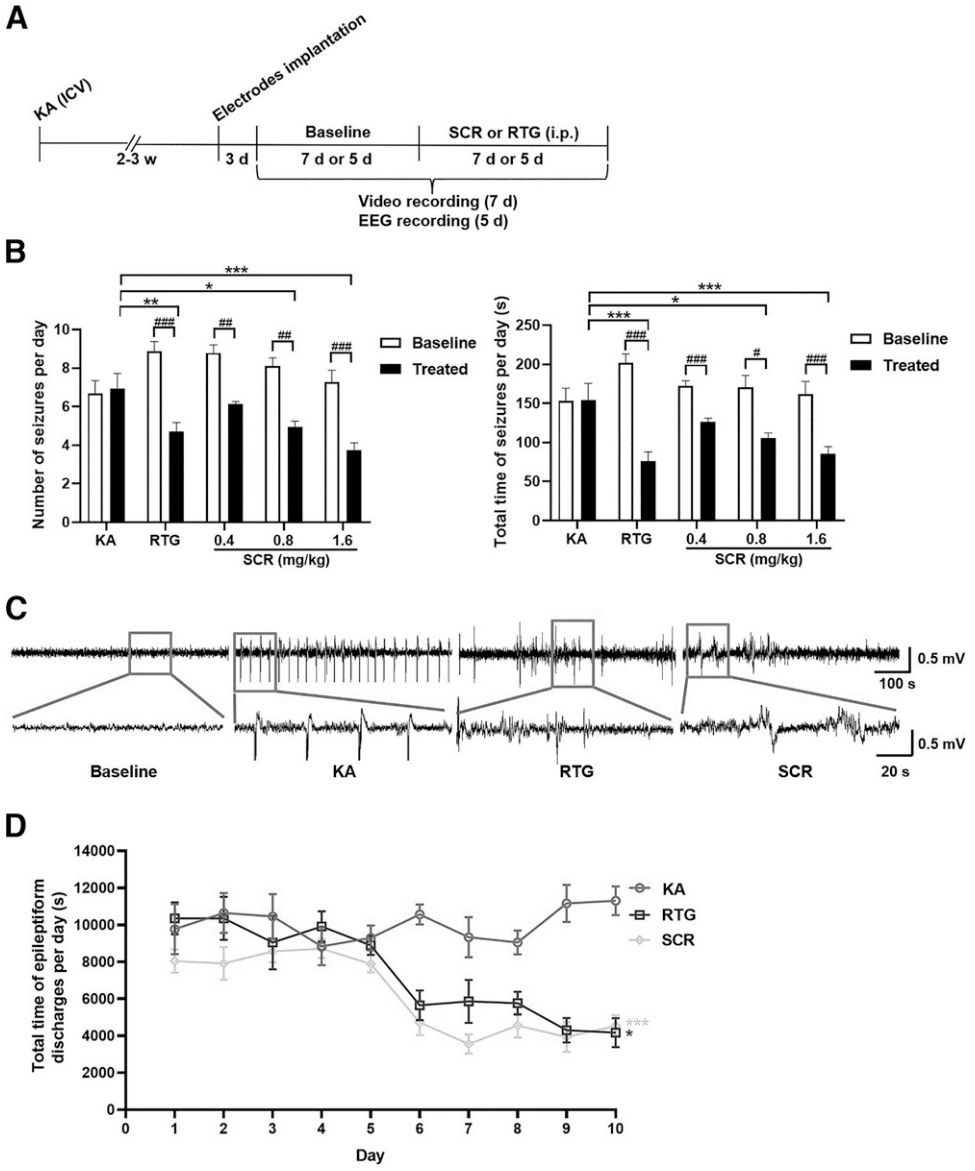

Fig. 3. The antiepileptic activity by SCR2682 in KA-induced chronic SRSs. (A) A schematic timeline for generation of KA-induced chronic SRSs in rats before and after intraperitoneal SCR2682 or RTG. (B) Dose-dependent reduction of the number and time of spontaneous seizures induced by KA per day by SCR2682 (0.4, 0.8, or 1.6 mg/kg) or RTG (7 mg/kg). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. KA group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the baseline, n = 6. (C) Representative EEG traces recorded from rat cerebral cortex from the groups of KA, RTG (7 mg/kg), and SCR2682 (1.6 mg/kg). EEG traces of the baseline were recorded from KA group. (D) Summary for total time of epileptiform discharges induced by KA per day the presence or absence of SCR2682 (1.6 mg/kg) or RTG (7 mg/kg). $^{*}P < 0.05$, $^{***}P < 0.001$ vs. KA group, n = 3.

amplitude in EEG (Fig. 3C). We also recorded the epileptiform discharges that appeared almost every day after the development of SRSs. In comparison with KA group or RTG group, SCR2682 caused a significant reduction of the total time of epileptiform discharges per day (Fig. 3D). These results indicated that SCR2682 exerted the antiepileptic effect on acute and chronic SRSs induced by KA.

Attenuation of KA-Induced Neuronal Loss by SCR2682

We examined any neuroprotective effect of SCR2682 on KA-induced neuronal loss using the Nissl-staining assay. As shown in Fig. 4A, there was an extensive loss of neurons in the hippocampal CA3 area of rats treated with ICV injection of 1 μg KA in the left lateral ventricle of the brain. In contrast, pretreatment of SCR2682 (1.6 mg/kg) showed a significant attenuation of neuronal loss (Fig. 4A). Similarly, in the RTG (7 mg/kg) group of rats, there was also a significant attenuation of neuronal loss in the CA3 region.

We also carried out immunostaining of mature neurons in hippocampal tissues using antibodies against neuronal nuclei (NeuN) as a marker. As shown in Fig. 4B, NeuN immunostaining revealed that the number of NeuN-positive neurons in hippocampal CA3 areas was significantly increased in rats treated with either SCR2682 (1.6 mg/kg, i.p.) or RTG (7 mg/kg, i.p.), as compared with KA alone. These results showed that SCR2682 attenuated the neuronal loss induced by KA.

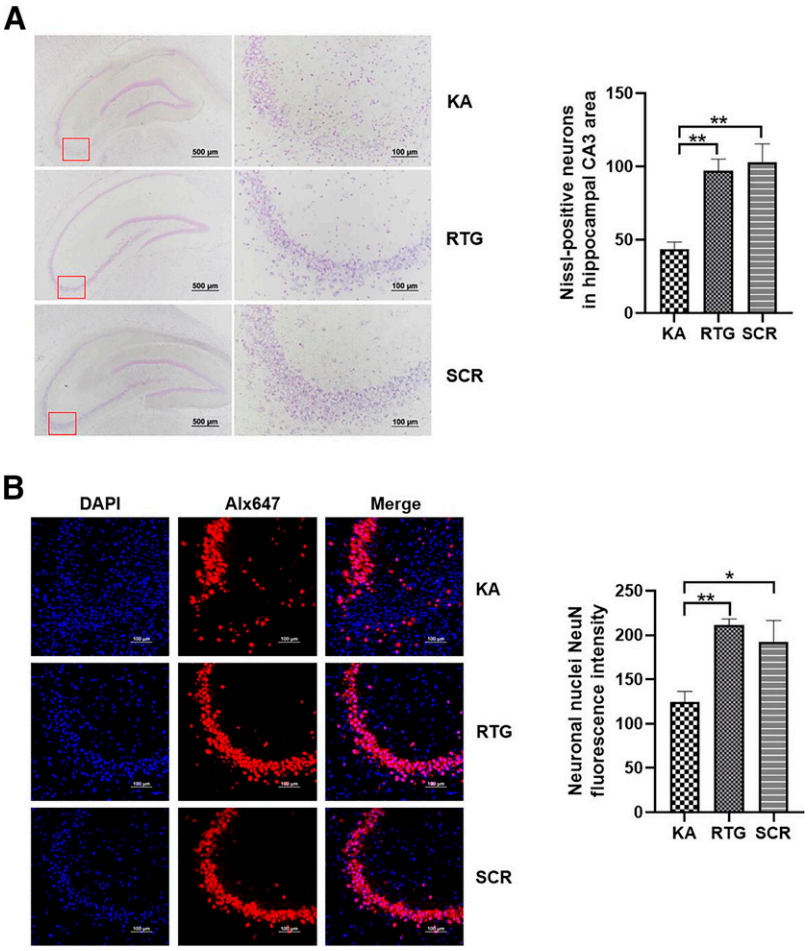

Fig. 4. Neuroprotective effect of SCR2682 on hippocampal neurons from KA-induced chronic SRSs in Nissl-staining and fluorescence immunostaining. (A) Left panel: representative Nissl-stained hippocampal sections from rats treated with KA, RTG (7 mg/kg), and SCR2682 (1.6 mg/kg). Magnification images of CA3 are shown in the right panel, respectively. Scale bar indicates 500 μm for low magnification or 100 μm for high magnification. Right panel: quantitative analysis for a number of neurons in hippocampal CA3 region from rats treated with saline, RTG (7 mg/kg), and SCR2682 (1.6 mg/kg). $^{**}P < 0.01$ vs. KA group, n = 3–4. (B) Left panel: representative NeuN immunostaining of hippocampal CA3 sections from the groups of KA, RTG (7 mg/kg), and SCR2682 (1.6 mg/kg). The cell nucleuses were stained with DAPI. The NeuN were immunohistochemically marked with Alexa fluor 647 Goat anti-rabbit IgG. Scale bar: 100 μm. Right panel: quantitative analysis of the fluorescence intensity in hippocampal CA3 region from the groups of KA, RTG (7 mg/kg), and SCR2682 (1.6 mg/kg). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. KA group, n = 3–4.

Improvement of PTZ- or KA-Induced Spatial Memory Deficits by SCR2682

We adopted the MWM test and further evaluated the effect of SCR2682 on spatial learning and memory in PTZ-kindled rats by recoding of escape latency and total times of crossing the original platform area within 60 seconds (Fig. 5A). The representative motion trajectories from the groups of KA, RTG (7 mg/kg), and SCR2682 (1.6 mg/kg) were presented on a 5-day MWM task consisting of a place navigation test of 4 consecutive days from day 1 to day 4 and an SPT at day 5 (Fig. 5B). As shown in Fig. 5C, the escape latency was significantly longer in PTZ-kindling rats as compared with the control group at day 4 of training phase, suggesting that the spatial cognitive impairment was induced by PTZ kindling. In contrast, the average escape latency of kindled rats was reduced after intraperitoneal administrations of SCR2682 at doses of 0.4, 0.8 and 1.6 mg/kg or RTG (7 mg/kg) (Fig. 5C). As shown in Fig. 5D, in the SPT, the total times of PTZ kindled rats crossing the original platform area within 60 seconds were significantly lower than that of the control group. In contrast, rats pretreated with different doses of SCR2682 (0.4, 0.8, and 1.6 mg/kg, i.p.) or RTG (7 mg/kg, i.p.) and increased the platform crossings, as compared with PTZ-kindled rats (Fig. 5D). These results showed that the Kv7 channel opener SCR2682 improved the spatial learning and memory of PTZ-kindled rats.

The Y-maze test was also used to further confirm the effect of SCR2682 on spatial working memory in rat model of chronic SRSs induced by ICV administration of 1 μg KA. After 3 weeks, rats received treatments of SCR2682 (1.6 mg/kg, i.p.) or RTG (7 mg/kg, i.p.) or saline once a day for 2 weeks and their time spent in the novel, familiar, and start arms was recorded in Y-maze test (Fig. 6A) and also motion trajectories from all groups (Fig. 6B). As shown in Fig. 6C, rats in the KA group showed a significant decrease in the time spent in the novel arm as compared with the control group without ICV KA. In contrast, intraperitoneal administrations of SCR2682 (1.6 mg/kg) or RTG (7 mg/kg) for 2 weeks significantly

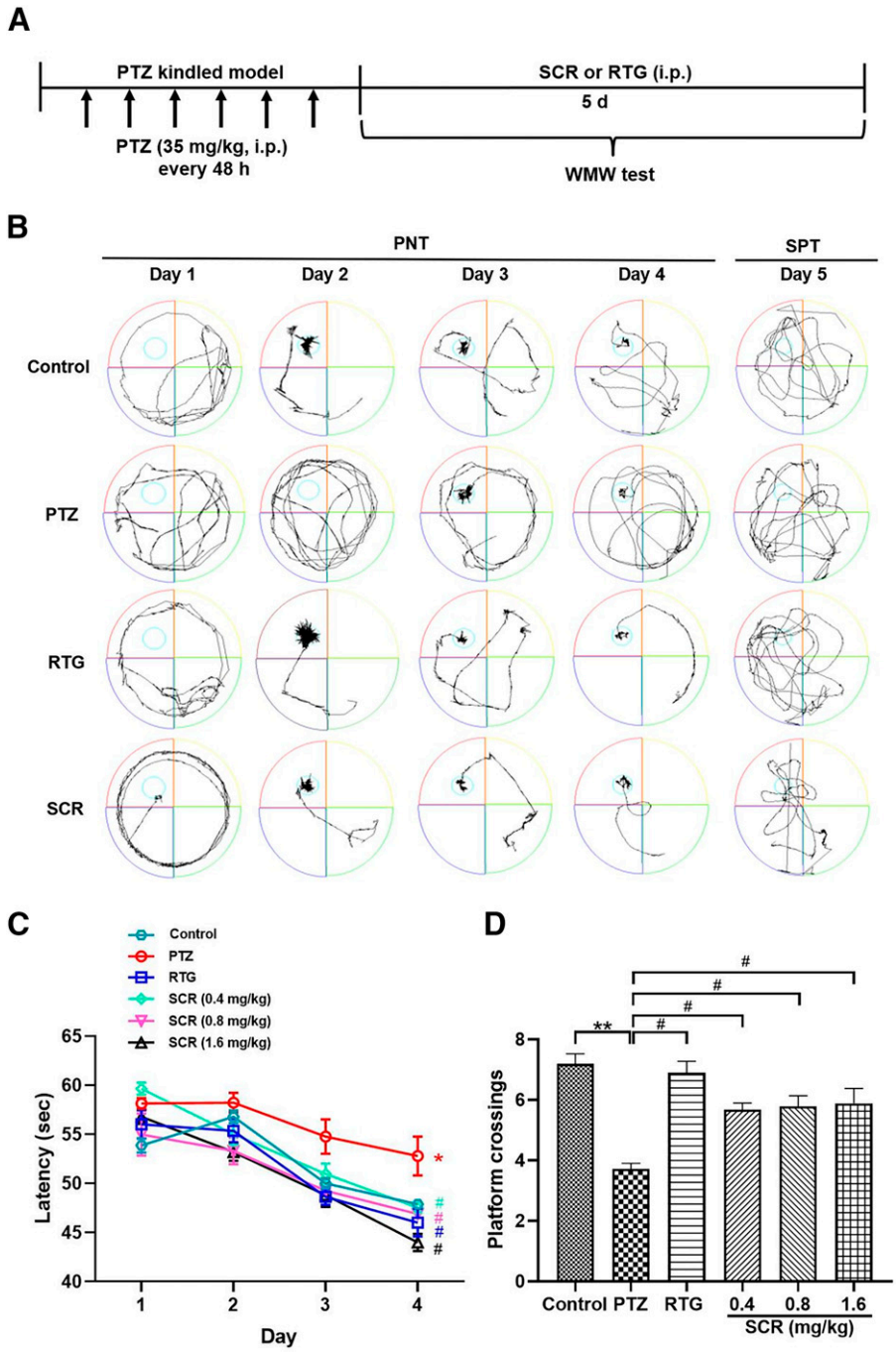

Fig. 5. Attenuation of PTZ-induced memory impairment by SCR2682 in rat MWM tests. (A) A schematic diagram for evaluation of memory deficits in MWM tests. (B), Representative motion trajectories from the control, PTZ, RTG (7 mg/kg), and SCR2682 (1.6 mg/kg) groups in the MWM test. (C) The escape latency during four consecutive days from the control, PTZ, RTG (7 mg/kg), and SCR2682 (0.4, 0.8, and 1.6 mg/kg) groups in place navigation test. $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. PTZ group, n = 6. (D) Platform crossings from the control, PTZ, RTG (7 mg/kg), and SCR2682 (0.4, 0.8, and 1.6 mg/kg) groups in SPT. $^{**}P < 0.01$ vs. control group; $^{\#}P < 0.05$ vs. PTZ group, n = 6.

increased the time spent in the novel arm, as compared with the KA group (Fig. 6C). These results indicated that Kv7 channel activator SCR2682 attenuated the spatial working memory deficiency for KA-induced chronic SRSs.

Discussion

Pharmacological activation of neuronal Kv7 currents by the channel openers as the mechanism of action represents an attractive strategy for therapy of epilepsy. RTG (or ezogabine) was the first opener approved for the treatment of partial epilepsy although it was recently discontinued due to its metabolites for side effects of discoloration in various tissues (Stafstrom et al., 2011). Nevertheless, it is necessary to identify novel specific Kv7 openers for effective treatment of epilepsy.

SCR2682 is a structurally distinct lead compound with better potency and efficacy in comparison with RTG (Zhang et al.,

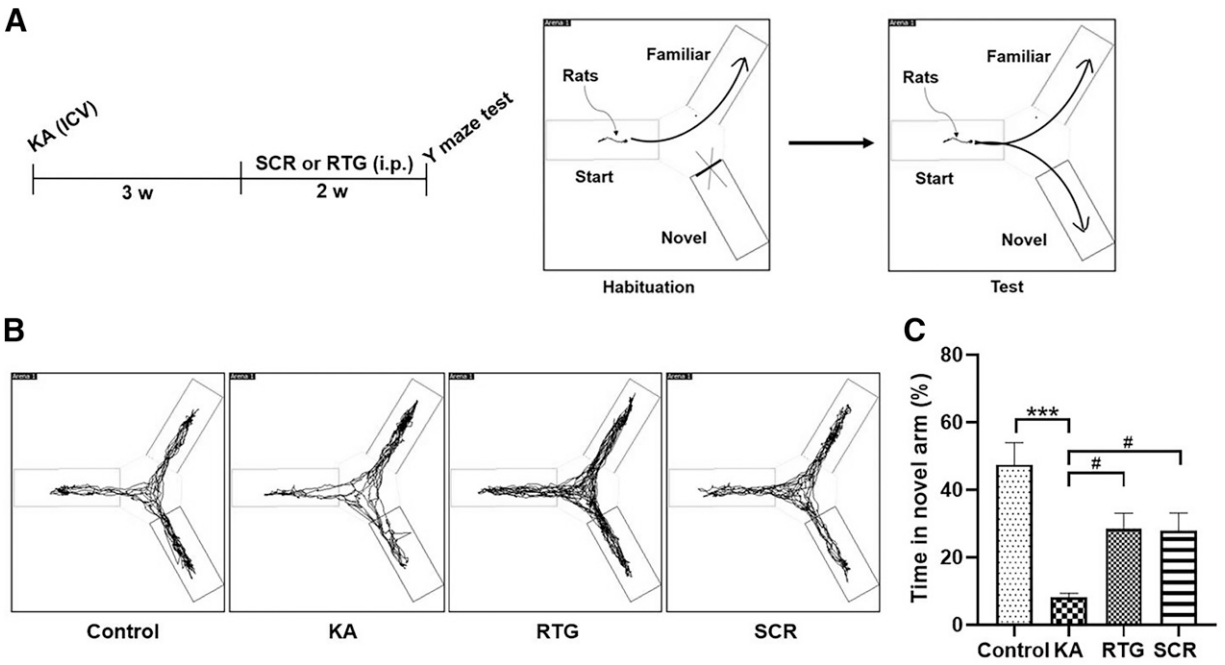

Fig. 6. Attenuation of KA-induced memory impairment by SCR2682 in Y-maze test. (A) Left panel: a schematic diagram for KA-induced chronic SRSs before intraperitoneal injections of SCR2682 or RTG. Right panel: a schematic diagram for a Y-maze test. (B) Representative motion trajectories from the control, KA, RTG (7 mg/kg), and SCR2682 (1.6 mg/kg) groups in Y-maze test. (C) A summary for the spent time proportion in the novel arm from the control, KA, RTG (7 mg/kg), and SCR2682 (1.6 mg/kg) groups. $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$ vs. KA group, n = 6.

2019; Liu et al., 2021). Electrophysiological recordings show that SCR2682 activates Kv7 currents in concentration-dependent manner with an EC50 of 9.8 ± 0.4 nM, which is about 100-fold more potent than EC50 of 0.86 ± 0.04 μM of RTG (Jia et al., 2011; Gunthorpe et al., 2012). In vivo seizure models, SCR2682 reduces seizure incidence with an ED50 of 0.4 mg/kg, as compared with an ED50 of 2.0 mg/kg for RTG. In addition, SCR2682 may possess less risk on urinary retention and also may not impose a significant risk of cardiac arrhythmia (Zhang et al., 2019). In this study, we show that SCR2682 protects from seizures and alleviates learning and memory impairment in rodent models of epilepsy.

The MES seizure model, one of the most widely used seizure models, is relevant to human generalized tonic–clonic and myoclonic seizures (White et al., 1995). The MES test is also considered as one of the gold standards in screening new anticonvulsant substances (Holmes, 2007). In this study, we examined the antiepileptic activity of SCR2682 against the convulsions induced by MES and demonstrated that SCR2682 can dose-dependently reduce the incidence of seizures. The anticonvulsant effect of SCR2682 was antagonized by co-administration of a specific Kv7 blocker XE991, indicating that the anticonvulsant activity of SCR2682 is mediated by the activation of Kv7 channels.

PTZ, a noncompetitive $GABA_A$ receptor antagonist, is commonly used to replicate seizures in animals (Ptácek, 1997). The kindled rats can slowly develop chronic seizures over a period of time at a subconvulsive dose of repeated administrations (Hansen et al., 2004). In the time-dependent antiepileptic experiments, SCR2682 produces the maximum anticonvulsive effect for 45 minute after administration and exhibits a significant inhibition of PTZ-induced seizures in a dose-dependent manner.

Both MES and PTZ tests are similar in that they evoke seizures, but they do not simulate the recurrent epileptic state in humans receiving epilepsy treatment (Barker-Haliski and White, 2020). The KA-induced models of epilepsy are developed to successfully mimic SRSs (Rusina et al., 2021). KA, as an analog of glutamate, activates glutamate ion receptors and causes cation influx and membrane depolarization (Nadler, 1981; Wang et al., 2005). After a nanomolar KA injected unilaterally into the lateral ventricle, rats exhibit sustained motor seizure activities such as bilateral forelimb clonus and rearing, and over time they develop chronic TLE (Zhu et al., 2012). At a single dose tested in this study, RTG is nearly as efficacious as SCR2682 in the models of seizures induced by PTZ or KA. Nevertheless, SCR2682 has been shown to be more efficacious in MES-induced seizures than RTG (Zhang et al., 2019). In our behavioral tests and EEG recordings, data show that SCR2682 exerts anticonvulsant effects on KA-induced acute and chronic SRSs in a dose-dependent manner.

Frequent seizures exacerbate neurocognitive dysfunction, and effective anticonvulsive therapy is essential to limit and alleviate cognitive comorbidities (Breuer et al., 2016; Nickels et al., 2016). PTZ-induced seizures or KA-induced spontaneous recurrent chronic seizures can occur with a slowly developing cognitive disorder (Csernansky et al., 1998; Williams et al., 2009; Aniol et al., 2013; Casillas-Espinosa et al., 2019). In this study, our observations for PTZ-induced seizure causing cognitive deficits in rats are also consistent with the previous reports (Sayin et al., 2004; Xie et al., 2012). SCR2682 significantly attenuates spatial learning and memory deficits by shortening the escape latencies and increasing times crossing the original platform area during the probe trial. We also find in this study that chronic SRSs induced by the KA disrupt the short-term spatial learning in Y-maze tests. In

contrast, the groups treated with SCR2682 or RTG show the better performance with more time spent in the novel arm. These observations indicate that attenuation of seizures by SCR2682 improves cognitive function in chronic seizure models.

The prolonged KA-induced seizure activity results in neuronal degeneration in the hippocampus, which is similar to patients with TLE (Sutula et al., 1989; Du et al., 1995). Hippocampal sclerosis is one of the classic pathologic characteristics in TLE, and hippocampal neuron loss leads to cognitive impairment caused by chronic epilepsy (Fernandes et al., 2015). Our results in Nissl staining and immunofluorescence show that neuronal loss is obvious in the KA group, supporting the notion that SRSs are deleterious to neurons and highly neurodegenerative. The CA3 region of the hippocampus shows a less loss of neurons in either SCR2682 or RTG group. These results demonstrate that SCR2682 or RTG can attenuate neuronal damage in the CA3 region of the hippocampus from KA-induced chronic SRSs. The anticonvulsant agent RTG and its structurally related flupirtine, a centrally acting nonopioid analgesic, have been suggested to exert neuroprotective activity in rat organotypic hippocampal slices likely through antioxidant action, but not an involvement of Kv7 activation (Boscia et al., 2006). SCR2682 (or its metabolite) is structurally distinct from RTG or flupirtine whose toxic metabolites cause the retinal pigmentation and skin discoloration (Groseclose and Castellino, 2019; Wurm et al., 2022), suggesting a mechanistic difference between SCR2682 and RTG in neuroprotection.

In conclusion, activation of neuronal Kv7 currents by a novel channel opener SCR2682 protects from seizures in rodent models of epilepsy. SCR2682 is also capable of ameliorating cognitive deficits resulted from seizures. Therefore, pharmacological activation of Kv7 by SCR2682 may hold a promise for effective therapy of epilepsy with cognitive impairment.

Authorship Contributions

Participated in research design: Zhuang, L. Wang, Ju.

Conducted experiments: Zhuang, Liu, Yang, Gao.

Performed data analysis: Zhuang, Ju.

Wrote or contributed to the writing of the manuscript: Zhuang, Ju, K. Wang.

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Address correspondence to: Chuanxia Ju, Department of Pharmacology, School of Pharmacy, Qingdao University Medical College, #1 Ningde Road, Qingdao 266071, China. E-mail: jucx@qdu.edu.cn; or KeWei Wang, Department of Pharmacology, School of Pharmacy, Qingdao University Medical College, #1 Ningde Road, Qingdao 266071, China. E-mail: wangkw@qdu.edu.cn