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Electrophysiological and pharmacological characterization of a novel and potent neuronal Kv7 channel opener SCR2682 for antiepilepsy

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ABSTRACT: Voltage-gated Kv7/KCNQ/M potassium channels play an essential role in the control of membrane potential and neuronal excitability. Activation of the neuronal Kv7/KCNQ/M-current represents an attractive therapeutic strategy for treatment of hyperexcitability-related neuropsychiatric disorders such as epilepsy, pain, and depression, which is an unmet medical need. In this study, we synthesized and characterized a novel compound, *N*-(4-(2-bromo-6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl)-2,6-dimethylphenyl)-3,3-dimethylbutanamide (SCR2682) 2,6-dimethyl-4-(piperidin-yl) phenyl)-amide derivative, that exhibits selective and potent activation of neuronal Kv7/KCNQ/M-channels. Whole-cell patch-clamp recordings of human embryonic kidney 293 cells expressing Kv7.2/Kv7.3 channels show that SCR2682 selectively activates the channel current in a dose-dependent manner with an EC_{50} of 9.8 ± 0.4 nM, which is ∼100-fold more potent than a U.S. Food and Drug Administration–approved antiepileptic drug (retigabine) for treatment of partial epilepsy. SCR2682 shifts voltage-dependent activation of the Kv7.2/7.3 current toward more negative membrane potential, to about −37 mV ($V_{1/2}$). SCR2682 also activates the native M-current in rat hippocampal or cortical neurons, causing marked hyperpolarization and potent inhibition of neuronal firings. Mechanistically, mutating the tryptophan residue 236 located at the fifth transmembrane segment of Kv7.2 abolishes the chemical activation of the channel by SCR2682. Furthermore, intraperitoneal or intragastric administration of SCR2682 results in a dose-dependent inhibition of seizures by maximal electroshock. Taken together, our findings demonstrate that a novel small molecule, SCR2682, selectively and potently activates neuronal Kv7 channels and reverses epileptic seizures in rodents. Thus, SCR2682 may warrant further evaluation for clinical development of antiepileptic therapy.—Zhang, F., Liu, Y., Tang, F., Liang, B., Chen, H., Zhang, H., Wang, K. Electrophysiological and pharmacological characterization of a novel and potent neuronal Kv7 channel opener SCR2682 for antiepilepsy. FASEB J. 33, 9154–9166 (2019). www.fasebj.org

KEY WORDS: Kv7/KCNQ/M-channel · neurons · seizures

The activation of Kv7/KCNQ/M-channels generates low-threshold, noninactivating voltage-dependent K^+ currents for suppression of neuronal hyperexcitability, which defines the fundamental mechanism of neuropsychiatric disorders such as epilepsy, pain, migraines, anxiety, depression, and schizophrenia (1–4). The Kv7 potassium channel subfamily is composed of 5 members, Kv7.1 through Kv7.5 (or KCNQ1–5) (5). With the exception of Kv7.1, which is predominantly expressed in the heart, the remaining 4 isoforms (Kv7.2 through Kv7.5) are primarily neuronal homo- or heterotetramers. The heteromeric Kv7.2/Kv7.3 channels in particular exhibit biophysical and pharmacological properties reminiscent of native M-currents in neurons (6–9). The native M-current that is inhibited by muscarine plays a critical role in controlling neuronal excitability (6–9). Loss-of-function mutations in either Kv7.2 or Kv7.3 subunit can cause neonatal epilepsy in humans or in rodents (10–13). Therefore, activation of the Kv7/KCNQ/M-channel represents an attractive therapeutic strategy for epilepsy-, migraine-, neuropathic pain-, or hyperexcitability-related neuropsychiatric disorders (14).

ABBREVIATIONS: HEK293, human embryonic kidney 293; hERG, human ether-a-go-go-related gene; IC_{50}, half maximal inhibitory concentration; i.g., intragastric; MES, maximal electroshock; RMP, resting membrane potential; RTG, retigabine; SCR2682, *N*-(4-(2-bromo-6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl)-2,6-dimethylphenyl)-3,3-dimethylbutanamide; ZnPy, zinc pyrithione

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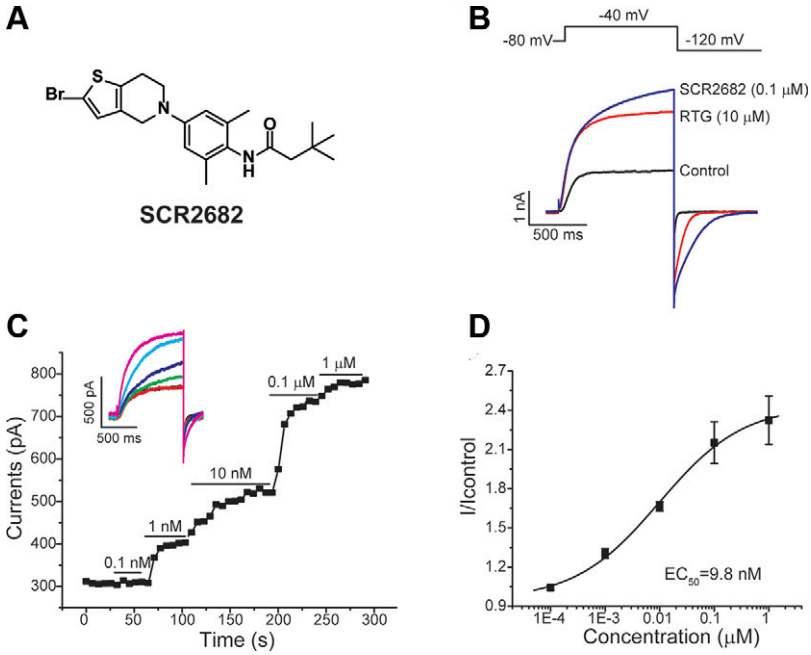

Figure 1. Dose-dependent activation of neuronal Kv7.2/7.3 channels expressed in HEK293 cells by compound SCR2682. Kv7.2/7.3 currents were elicited by depolarizing potential at −40 mV from holding potential at −80 mV before repolarizing to −120 mV. *A*) Chemical structure of SCR2682. *B*) Representative outward Kv7.2/7.3 currents in response to SCR2682 0.1 μM (blue) or retigabine 10 μM (red) or control without compound (black). *C*) Time-dependent activation of Kv7.2/Kv7.3 currents by different concentrations of SCR2682 in response to depolarization potential at −40 mV. Representative current traces from each concentration are shown in the insets. *D*) Logistic fitting for concentration-dependent activation of Kv7.2/Kv7.3 currents by SCR2682 reveals an EC_{50} value of 9.8 ± 0.4 nM (n = 6) and a slope factor of 0.6 ± 0.2 (n = 6).

There are 2 drugs that activate Kv7/M-channels that have been used for treatment of epilepsy and pain. Retigabine (RTG) or ezogabine [*N*-(2-amino-4-[fluorobenzylamino]-phenyl) carbamic acid; D-23129] as a Kv7/M-channel opener was approved by the U.S. Food and Drug Administration in 2011 for treatment of partial epilepsy (15–19). RTG activates neuronal Kv7.2/Kv7.3 currents in a concentration-dependent manner with an EC_{50} of 0.8–2.0 μM (20, 21). RTG can also activate all subtypes of the Kv7 family such as Kv7.2, Kv7.3, Kv7.4, Kv7.5, and Kv7.2/Kv7.3 channels, except Kv7.1, which is slightly inhibited by RTG (22). However, RTG renders adverse effects, including blue skin discoloration, retinal pigment abnormalities, and even cardiac arrhythmias (23–25), suggesting that a more specific Kv7 subtype activator is required for better efficacy and less toxicity.

Flupirtine, a structural analog of RTG, has been used for decades in a number of European countries as a centrally acting, nonopioid analgesic for the treatment of various pain conditions (26). Flupirtine activates Kv7 channels with an EC_{50} of ~5 μM (27, 28), and it also shows efficacy in many seizure models (29, 30). However, flupirtine has recently been recommended to be withdrawn from the market in 2018 by the European Union because of its liver toxicity (31). Therefore, it is necessary to identify selective and potent neuronal Kv7 openers with fewer side effects for therapies such as epilepsy and pain.

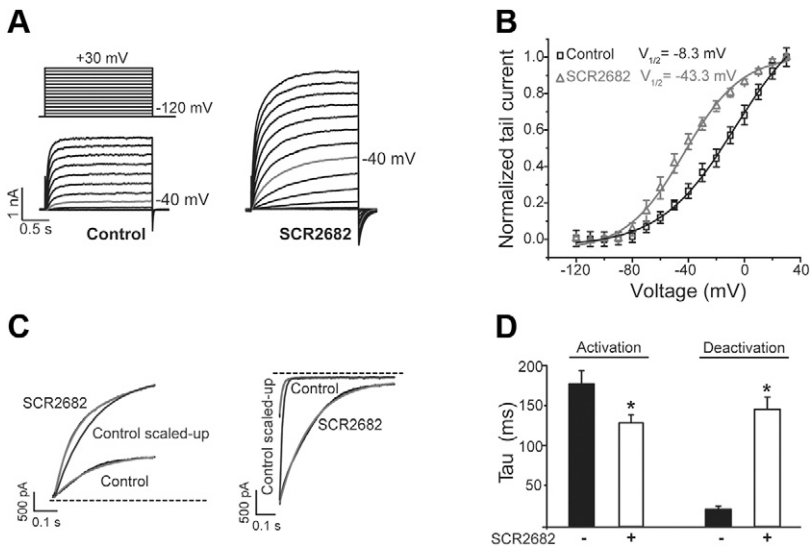

Figure 2. Leftward shift of voltage-dependent activation of neuronal Kv7.2/7.3 channels by compound SCR2682. *A*) A family of representative outward currents elicited by depolarizing voltage steps in 10-mV increments from holding potential at −120 to 30 mV in the presence of SCR2682 (0.1 μM). *B*) Normalized shift of voltage-dependent activation of Kv7.2/Kv7.3 currents generated from tail currents measured at −120 mV before and after SCR2682 (0.1 μM) and fitted with Boltzmann function. *C*) Superimposed activation currents elicited at −40 mV (left panel) and deactivation traces at −120 mV (right panel) in the absence or presence of 0.1 μM SCR2682. The current traces from the control were scaled up to the same current amplitude in response to SCR2682. The dotted lines indicate a current level of 0. *D*) Summary of the effect of 0.1 μM SCR2682 on activation and deactivation time constants (τ) of Kv7.2/7.3 currents. Activation and deactivation kinetics of currents were fitted with a single exponential function. *$P < 0.05$ indicates statistical significance compared with control (n = 5).

In this study, we report the electrophysiological and pharmacological characterization of a newly synthesized compound *N*-(4-(2-bromo-6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl)-2,6-dimethylphenyl)-3,3-dimethylbutanamide (SCR2682) that selectively activates the Kv7/KCNQ/M-current with ~100-fold more potency than RTG. *In vivo* evaluation reveals that SCR2682 is 10 times more efficacious in antiepileptic seizures than RTG. Taken together, our findings demonstrate that the novel small molecule SCR2682 is a selective and potent activator of neuronal Kv7/KCNQ/M-channels. Thus, SCR2682 can be used as a tool for investigation of channel pharmacology, and it also possesses developmental potential for treatment of epilepsy or hyperexcitability-related disorders.

MATERIALS AND METHODS

Chemicals

The synthesis of the 2,6-dimethyl-4-(piperidin-yl)phenyl)-amide derivative SCR2682 compound was accomplished by Simcere Pharmaceuticals (Patent WO2014048165A1; Shanghai, China). The chemical structure of SCR2682 with a molecular mass of

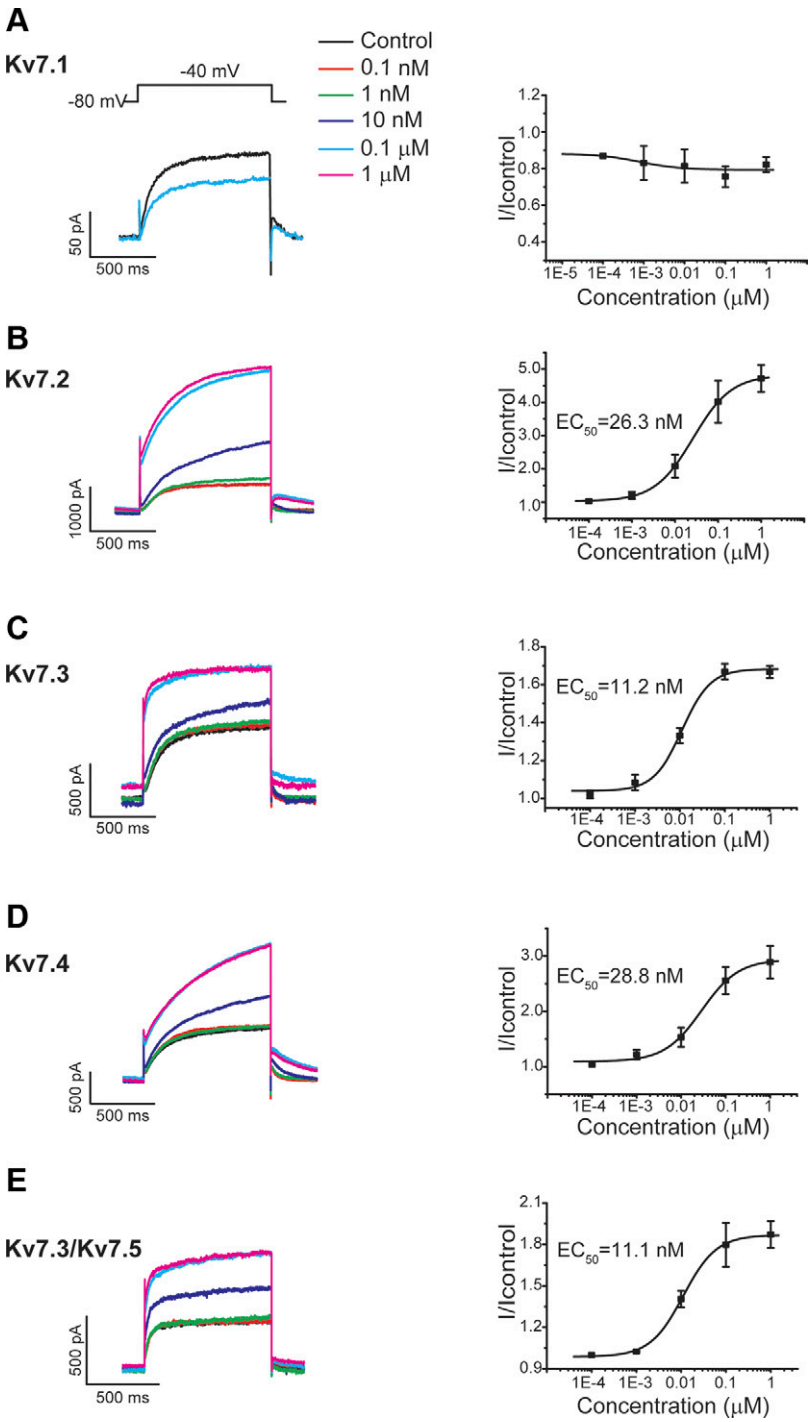

Figure 3. Dose-dependent activation of homotetrameric or heterotetrameric neuronal Kv7 channels by SCR2682. The left panels show representative current traces for dose-dependent activation of Kv7 channels Kv7.1 (*A*), Kv7.2 (*B*), Kv7.3 (*C*), Kv7.4 (*D*), and Kv7.3/Kv7.5 (*E*), expressed in HEK293 cells by SCR2682. The current was recorded using a step protocol consisting of holding potential at −80 mV and a depolarized potential at −40 mV. The right panels are fitted curves for concentration-dependent activation of different Kv7 subtype channels recorded at −40 mV by SCR2682. The EC_{50} values are 26.3 ± 1.3 nM ($n = 5$) for Kv7.2, 11.2 ± 2.3 nM ($n = 6$) for Kv7.3, 28.8 ± 8.7 nM ($n = 5$) for Kv7.4, and 11.1 ± 1.0 nM ($n = 5$) for heteromeric Kv7.3/7.5. SCR2682 at 1.0 μM increased the current by ~4.7 ± 0.4–fold ($n = 5$) for Kv7.2, 1.7 ± 0.03–fold ($n = 6$) for Kv7.3, 2.9 ± 0.3–fold ($n = 5$) for Kv7.4, and 1.9 ± 0.1–fold ($n = 5$) for Kv7.3/Kv7.5 channels. The highest inhibition of Kv7.1 current by SCR2682 was ~24% ($n = 5$).

434.1 is depicted in **Fig. 1*A***. The antiepileptic drug RTG (purity >98% by HPLC–diode-array detector) was synthesized in the Department of New Drugs Development, School of Pharmacy, Hebei Medical University (Shijiazhuang, China), and its chemical structure was verified by mass spectrometry and NMR analysis (32). The Kv7-specific blocker XE991 [10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone] was purchased from MilliporeSigma (Burlington, MA, USA).

DNA constructs

Plasmids in a pcDNA3.1 vector encoding human Kv7.1 [accession number NM000218; GenBank (*https://www.ncbi.nlm.nih.gov/genbank/*)], human Kv7.2 (AF110020), rat Kv7.3 (AF091247), human Kv7.4 (AF105202), and human Kv7.5 (AF249278) were used for transfection and expression in mammalian cells (33). Kv7.2 W236L mutant cDNA in the pcDNA3.1 vector was generated and kindly provided by Dr. Zhaobing Gao (Chinese Academy of Sciences, Shanghai, China).

Cell culture

Human embryonic kidney 293 (HEK293) cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin at 37°C in humidified atmospheric air with 5% CO_2. For transfection of cells, a mixture of 4 μg Kv7 cDNAs in a pcDNA3.1 vector, 4 μg red fluorescent protein pcDNAs, and 6 μl Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) were prepared in 1.2 ml DMEM and added to the cell culture for an incubation of 4–6 h. Electrophysiological recordings were carried out 24 h after transfection, and the transfected cells were used within 48 h.

Culture of rat hippocampal and cortical neurons

Primary hippocampal neurons were isolated from brains and cultured as previously described (34, 35). Briefly, freshly dissociated neurons were obtained from the hippocampus or cortex of embryonic d 18 Sprague-Dawley rat brains before being seeded at a density of $4 \times 10^4/cm^2$ onto coverslips (CS-8R; Warner Instruments, Hamden, CT, USA) that were coated with 50 ng/ml poly-D-lysine (MilliporeSigma). Cells were cultured in Neurobasal Medium supplemented with 2% B-27 and 0.5 mM GlutaMax (Thermo Fisher Scientific) for 14–16 d before use.

Electrophysiology

HEK293 cells transfected with Kv7 channel cDNAs were recorded using a perforated patch-clamp technique with amphotericin B (250 μg/ml; MilliporeSigma) in the pipette solutions. Data were acquired at 10 kHz and filtered at 2.5 kHz using a Heka EPC 10 amplifier (Harvard Bioscience, Holliston, MA, USA). Patch electrodes were pulled with a micropipette puller (Sutter Instrument, Novato, CA, USA) and fire-polished to a final resistance of 1–2 MΩ. Series resistances were compensated by 60–80%. The internal pipette solution for the recording of HEK293 cells was prepared as follows (mM): KCl 150, $MgCl_2$ 5, and HEPES 10 at pH 7.4 adjusted with KOH. The external recording solution contained (mM) NaCl 160, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, and HEPES 20 at pH 7.4 adjusted with NaOH.

Hippocampal neurons were cultured for 14–16 d before being recorded using a whole-cell patch-clamp configuration. The recording electrodes had a resistance of 2–6 MΩ when filled with the pipette solution containing (mM) K-gluconate 100, KCl 50, EGTA 10, $MgCl_2$ 5, and HEPES 2 at pH 7.3 adjusted with KOH. The extracellular solution was composed of (mM) NaCl 140, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 10, and glucose 10 at pH7.4 adjusted with NaOH. To record action potentials and membrane potentials, the extracellular solution was supplemented with ionotropic glutamate NMDA and non-NMDA receptor inhibitors DL-2-amino-5-phosphonovaleric acid and 6,7-dinitroquinoxaline-2,3-dione (each at 20 μM); and also GABA receptors bicuculline and baclofen (each at 10 μM). For recordings of native M-current in neurons, the extracellular solution was supplemented with tetrodotoxin (1 μM) to block sodium channels. All experiments were carried out in an air-conditioned room with a controlled temperature of 22 ± 1°C.

TABLE 1. *Dose-dependent activation and fold increase of neuronal Kv7 subfamily member currents by SCR2682*

Subfamily member	EC_{50} (nM)	Fold increase (μM)
Kv7.2	26.3 ± 1.3	4.7 ± 0.4
Kv7.3	11.2 ± 2.3	1.7 ± 0.03
Kv7.4	28.8 ± 8.7	2.9 ± 0.3
Kv7.2/7.3	9.8 ± 0.4	2.3 ± 0.2
Kv7.3/7.5	11.1 ± 1.0	1.9 ± 0.1

All values are means ± SEM (n = 5–6). Data were measured at −40 mV, and increase folds were normalized by a control current before SCR2682 (1 μM) was applied.

Rodent models of maximal electroshock tests

A total of 280 rats (180–220 g; male) and 140 mice (18–22 g; male) provided by the Experimental Animal Center of Hebei Province (Shijiazhuang, China) were used for acute tests of antiepileptic activities in maximal electroshock (MES) seizure models. Animal experiments were performed in accordance with the Animal Care and Use Protocols approved by the Ethical Committee of Hebei Medical University (Shijiazhuang, China). At 24 h before tests, rats or mice received electrical stimulations for screening of inducible seizure models. The electrical stimulation parameters were set as follows: single stimulation, frequency at 1.0 HZ, time of 0.25 s, and voltage at 160 V (rat) and 100 V (mouse). Animals with hind limb extension indicative of epileptic onset after electrical stimulation were selected for further tests. Seizure-inducible rats were divided into 7 groups with 10 rats in each group: solvent (5% Tween80) control group, 100 $mg \cdot kg^{-1}$ SCR2682 group, 30 $mg \cdot kg^{-1}$ SCR2682 group, 10 $mg \cdot kg^{-1}$ SCR2682 group, 3 $mg \cdot kg^{-1}$ SCR2682 group, 1 $mg \cdot kg^{-1}$ SCR2682 group, and 0.3 $mg \cdot kg^{-1}$ SCR2682 group. The Kv7 opener RTG was used as a positive control. Seizure-inducible mice were also divided into 7 groups with 10 mice in each group: solvent (5% Tween80) control group, 30 $mg \cdot kg^{-1}$ SCR2682 group [for intragastric (i.g.) administration], 15 $mg \cdot kg^{-1}$ SCR2682 group (for intraperitoneal administration), 10 $mg \cdot kg^{-1}$ SCR2682 group, 3 $mg \cdot kg^{-1}$ SCR2682 group, 1 $mg \cdot kg^{-1}$ SCR2682 group, and 0.3 $mg \cdot kg^{-1}$ SCR2682 group. The compound was administered intraperitoneally and i.g. at a volume of 10 $ml \cdot kg^{-1}$. Animals were given electrical stimulations, and epileptic seizure behaviors were observed 30 min after intraperitoneal injection of different concentrations of compounds or 1 and 3.6 h after intragastric administration in mice or rats.

Atomic absorption Rb^+ efflux assay

The atomic absorption Rb^+ efflux assay was carried out as previously described by Wang *et al*. (36). Briefly, cells with a density of 2×10^5 cells per well were cultured in a 96-well plate overnight,

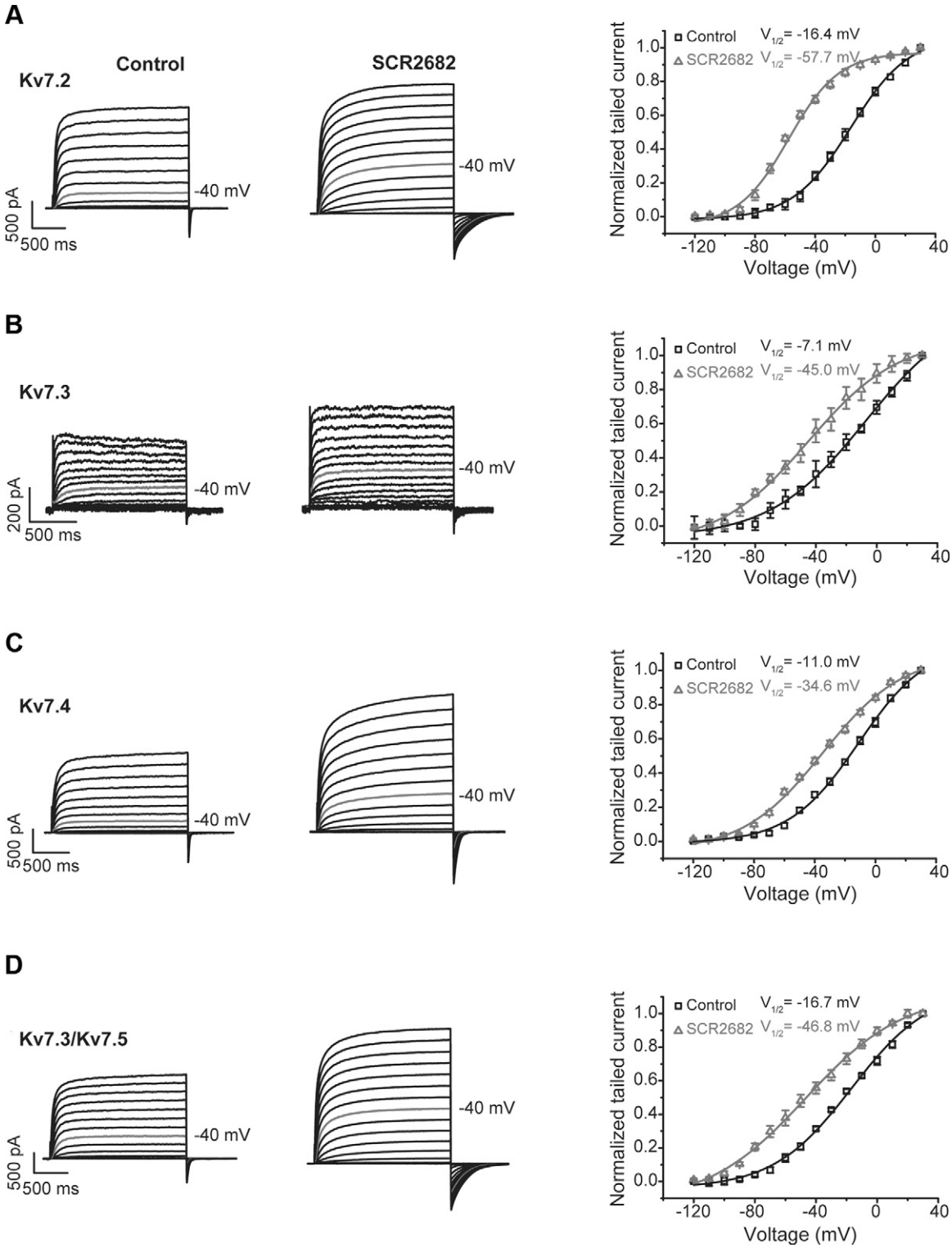

Figure 4. Leftward shift of voltage-dependent activation of homotetrameric or heterotetrameric Kv7 channels expressed in HEK293 cells by SCR2682. Representative current traces of Kv7.2 (*A*), Kv7.3 (*B*), Kv7.4 (*C*), and Kv7.3/Kv7.5 (*D*) channels in the absence (control, left panels) or in the presence of SCR2682 (0.1 μM) (middle panels). The currents were recorded using the voltage steps from a holding potential at −120 mV to voltages between −120 and 30 mV in 10-mV increments. Right panels show the voltage-dependent activation curves for Kv7.2, Kv7.3, Kv7.4, and Kv7.3/Kv7.5 channels generated from tail currents without or with SCR2682 (0.1 μM). Tail currents were measured at −120 mV (n = 5–7). The threshold for activation of Kv7.2, Kv7.3, Kv7.4, and heterotetrameric Kv7.3/Kv7.5 channels by SCR2682 was significantly shifted to a more hyperpolarized potential at −41.3 ± 1.4 mV (n = 6), −37.8 ± 1.7 mV (n = 7), −23.7 ± 1.9 mV (n = 6), and −30.2 ± 2.4 mV (n = 5), respectively.

TABLE 2. *Leftward shift of activation curves of neuronal Kv7 subfamily members by SCR2682 (0.1 μM)*

Subfamily member	$V_{1/2}$ control (mV)	$V_{1/2}$ SCR2682 (mV)	$\Delta V_{1/2}$ (mV)
Kv7.2	−16.4 ± 1.2	−57.7 ± 1.4*	41.3 ± 1.4
Kv7.3	−7.1 ± 5.0	−45.0 ± 1.8*	37.8 ± 1.7
Kv7.4	−11.0 ± 1.9	−34.6 ± 1.7*	23.7 ± 1.9
Kv7.2/7.3	−8.3 ± 2.7	−43.3 ± 2.1*	35.0 ± 2.7
Kv7.3/7.5	−16.7 ± 2.4	−46.8 ± 1.9*	30.2 ± 2.4

All values are presented as means ± SEM (n = 5–7). *$P < 0.05$ indicates statistical significance in comparison with the control.

and the monolayer cells were loaded with Rb^+ loading buffer for 3 h at 37°C before being washed 3 times gently with wash buffer. The cells were incubated with depolarization buffer containing different compounds for 10 min. Finally, the supernatant was collected and measured using an atomic absorption automated Ion Channel Reader 8000 (ICR8000; Aurora Biomed, Vancouver, Canada).

Data analysis and statistics

The concentration-response curve was fitted by the logistic equation of $y = A_2 + [(A_1 - A_2)/(1 + (x/x_0)^p]$, where x is the drug concentration and p is the Hill coefficient. Channel current activation curves were generated by plotting the normalized tail current amplitude against step potentials and were fitted with a

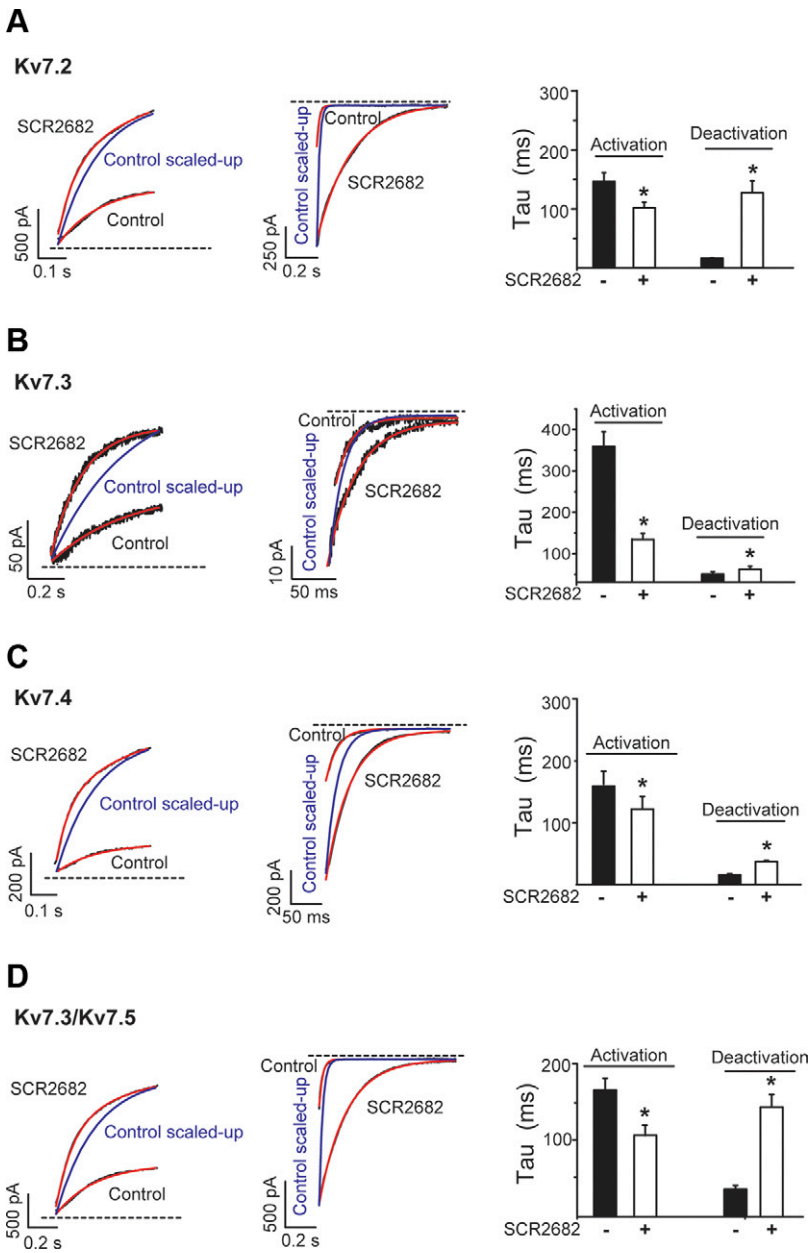

Figure 5. Effects of SCR2682 on activation and deactivation kinetics of neuronal Kv7 currents. Activation and deactivation of Kv7 currents Kv7.2 (*A*), Kv7.3 (*B*), Kv7.4 (*C*), and Kv7.3/Kv7.5 (*D*) were recorded by a depolarization step at −40 mV and a hyperpolarization step at −120 mV, respectively. The amplitudes of control current traces were scaled up to normalize them to the amplitudes of Kv7 currents in the presence of 0.1 μM SCR2682. Left panels: activation current traces; middle panels: deactivation current traces. Activation of Kv7 currents and deactivation of Kv7 currents were fitted with a single exponential function. The time constants from the fittings are shown in the right panels (n = 5–7). *$P <$ 0.05.

Boltzmann equation of $y = A/1 + \exp[(V_h - V_m)/k]$, where A is the amplitude of relationship, V_h is the voltage for half-maximal activation, V_m is the test potential, and k is the slope factor of the curve. The activation and deactivation traces were fitted to a single exponential function: $I = A \times [1 - \exp(-t/\tau)]$, where I is the current, A is amplitudes, t is time, and τ is the time constant. Data were expressed as means ± SEM. Statistical analysis for differences between groups was carried out using paired or unpaired Student's t tests, and a value of $P \leq 0.05$ was considered to be statistically significant.

RESULTS

A dose-dependent activation of neuronal Kv7.2/Kv7.3 channels expressed in HEK293 cells by compound SCR2682

The compound SCR2682 was first synthesized by Shanghai Simcere Pharmaceuticals and identified as a potent Kv7.2 channel opener with an EC_{50} of 0.26 μM among its derivatives using an atomic absorption Rb^+ efflux assay as previously described (36, 37). The chemical structure of representative SCR2682 is depicted in Fig. 1*A* under the published Patent Cooperation Treaty (WO2014048165A1). Whole-cell patch-clamp recordings of Kv7.2/7.3 channels expressed in HEK293 cells revealed that perfusing SCR2682 (0.1 μM) or RTG (10 μM) as a positive control increased the steady-state current (−40 mV) and deactivation tail current (−120 mV) amplitude of Kv7.2/7.3 channels, and the effects could be washed out (Fig. 1*A, B*). Further administration of different concentrations of SCR2682 resulted in a dose-dependent activation of Kv7.2/7.3 currents elicited by depolarization potential at −40 mV (Fig. 1*C*). As shown in Fig. 1*D*, fitting the concentration-response curve gave rise to an EC_{50} value of 9.8 ± 0.4 nM with a Hill coefficient at 0.6 ± 0.1 (Fig. 1*D*) (n = 6), which is ~100-fold more potent than RTG (20, 38).

We further evaluated the effect of SCR2682 on voltage-dependent activation of Kv7.2/7.3 currents. The tail current amplitude at −120 mV obtained from different test potentials was normalized and fitted by Boltzmann function. Perfusion of 0.1 μM SCR2682 caused a significant leftward shift of Kv7.2/7.3 channel activation to more hyperpolarized membrane potential at −43.3 from −8.3 mV (**Fig. 2*A, B***). The analysis of channel-gating kinetics fitted by a single exponential function revealed the time constant of the SCR2682-induced current to be 136.3 ± 7.0 ms (n = 5) as compared with that of the control at 179.2 ± 16.8 ms (Fig. 2*C, D*). SCR2682 (0.1 μM) also significantly slowed down the channel deactivation kinetics ~7-fold to 147.4 ± 15.4 from 21.8 ± 4.3 ms (n = 5; Fig. 2*C, D*). These results indicate that compound SCR2682 can activate Kv7.2/7.3 channels expressed in HEK293 cells in both dose- and voltage-dependent manners.

Selective activation of Kv7.2–7.5 members by SCR2682

The Kv7/KCNQ/M-channel subfamily is composed of 5 members, including Kv7.1 to Kv7.5 (9). To investigate the effect of SCR2682 on Kv7 subfamily members, we recorded homo- or heterotetrameric Kv7 channel currents activated at −40 mV. As shown in **Fig. 3** and **Table 1**, SCR2682 increased neuronal Kv7.2, Kv7.3, Kv7.4, and Kv7.3/Kv7.5 channel currents in a dose-dependent manner, with EC_{50} values at 26.3 ± 1.3, 11.2 ± 2.3, 28.8 ± 8.7, and 11.1 ± 1.0 nM, respectively. At a concentration of 1.0 μM, SCR2682 increased the amplitude of Kv7.2, Kv7.3, Kv7.4, and Kv7.3/Kv7.5 currents ~4.7-fold (n = 5), 1.7-fold (n = 6), 2.9-fold (n = 5), and 1.9-fold (n = 5), respectively, as measured at −40 mV when the current amplitude was stable. In contrast, SCR2682 only had weak inhibition of the cardiac Kv7.1 current, with a maximum inhibition of ~24% at 0.1 μM (n = 5; Fig. 3).

We also tested the effect of SCR2682 on the shift of channel activation. Perfusion of SCR2682 (0.1 μM) significantly shifted the threshold for activation of Kv7.2, Kv7.3, Kv7.4, and heterotetrameric Kv7.3/Kv7.5 channels to more hyperpolarized membrane potentials at −41.3 ± 1.4 mV (n = 6), −37.8 ± 1.7 mV (n = 7), −23.7 ± 1.9 mV (n = 6), and −30.2 ± 2.4 mV (n = 5), respectively (**Fig. 4** and **Table 2**), which is in a similar order as their EC_{50} values. SCR2682 (0.1 μM) also significantly accelerated the activation and slowed down the deactivation kinetics of Kv7.2, Kv7.3, Kv7.4, and Kv7.3/Kv7.5 channel currents (n = 5–7; **Fig. 5** and **Table 3**). These results indicate that SCR2682 is a selective channel opener for neuronal Kv7 channels but not the cardiac Kv7.1 channel.

TABLE 3. *Effects of SCR2682 on channel activation and deactivation kinetics of neuronal Kv7 channels*

	Time constant (ms) activation (−40 mV)		Time constant (ms) deactivation (−120 mV)	
Kv7 channel	Control	SCR2682	Control	SCR2682
Kv7.2	148.3 ± 10.7	102.0 ± 8.4*	16.4 ± 0.8	127.3 ± 20.6*
Kv7.3	357.7 ± 35.7	139.5 ± 17.6*	19.6 ± 5.3	31.7 ± 7.0*
Kv7.4	165.3 ± 17.4	136.3 ± 13.9*	15.3 ± 2.0	36.7 ± 1.8*
Kv7.2/7.3	179.2 ± 16.8	136.3 ± 7.0*	21.8 ± 4.3	147.4 ± 15.4*
Kv7.3/7.5	167.0 ± 8.0	109.0 ± 6.4*	36.0 ± 5.1	143.6 ± 16.3*

SCR2682 was 0.1 μM, and all values are presented as the means ± SEM (n = 5–7). *$P < 0.05$ indicates statistical significance in comparison with the control.

SCR2682 enhances the native M-current and dampens firing in rat hippocampal and cortical neurons

To examine whether SCR2682 could also activate native M-current, we recorded cultured neurons acutely isolated from rat hippocampus and cortex. The M-current of hippocampal neurons was activated by a depolarizing voltage of −20 mV, and the tail current was elicited at −60 mV (**Fig. 6*A***). Applications of SCR2682 (1 μM) or RTG (10 μM) increased the M-current that was blocked by specific inhibitor XE991 (3 μM) (Fig. 6*A*). SCR2682 also gave rise to a

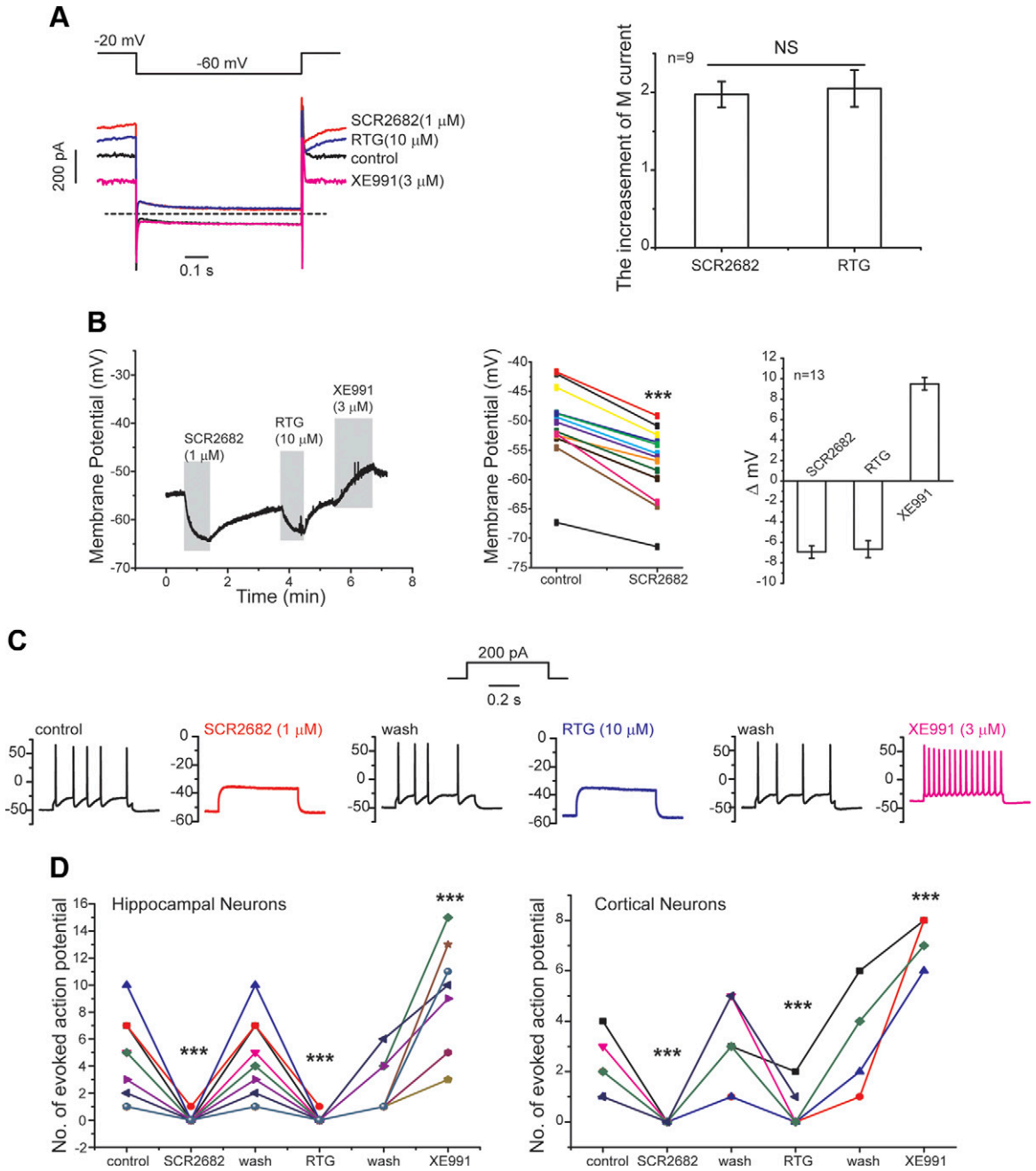

Figure 6. Enhancement of native M-current and attenuation of neuronal firings in hippocampal or cortical neurons by SCR2682. *A*) Left, representative M-current traces were recorded in hippocampal neurons using the indicated protocol. SCR2682 (1.0 μM) or RTG (10 μM) as positive control enhanced the amplitude of M-current that was blocked by specific blocker XE991 (3.0 μM). Right, effect of SCR2682 (1 μM) or RTG (10 μM) on XE991-sensitive M-current measured at −20 mV; $n = 9$. *B*) Left, representative RMP before and after application of SCR2682, RTG, or XE991. Middle, hyperpolarization shift of RMP by SCR2682 (1 μM) to −57.5 ± 1.7 mV ($n = 13$) from −50.5 ± 1.8 mV ($n = 13$) recorded in hippocampal neurons. Right, the change of membrane potentials in hippocampal neurons in response to SCR2682 at 1.0 μM for hyperpolarization (−6.95 ± 0.6 mV), RTG at 10 μM for hyperpolarization (−6.66 ± 0.8 mV), or XE991 at 3 μM for depolarization (9.49 ± 0.6 mV). *C*) Representative traces for action potentials in the absence or presence of SCR2682 (1.0 μM), RTG (10 μM), or XE991 (3.0 μM). The action potential was evoked by injection of 200 pA depolarizing current. *D, E*) Summary of the evoked action potential recorded in cultured hippocampal and cortical neurons. NS, not significant. ***$P < 0.001$ indicates statistical significance in comparison with the control.

significant hyperpolarization of resting membrane potential (RMP) of neurons ~7 mV from −50.5 ± 1.8 to −57.5 ± 1.7 mV (Fig. 6*B*). Similarly, firing spikes induced by injecting current into hippocampal or cortical neurons were abolished by perfusion of either SCR2682 (1 μM) or RTG (10 μM) (Fig. 6*C–E*). These results demonstrate that SCR2682 activates native M-current and significantly reduces the excitability of hippocampal or cortical neurons, suggesting that SCR2682 may have antiepileptic activity.

Antiepileptic effects of SCR2682 on MES-induced seizures in rodents

To test the effect of SCR2682 on epileptic seizures, we utilized both rat and mouse models of MES that induce seizures and investigated the ability of SCR2682 to protect against hind limb extension induced by MES. Intraperitoneal injections of SCR2682 in rats resulted in a concentration-dependent reduction of seizure incidence in rats, with an ED_{50} value of 0.4 mg/kg, as compared with an ED_{50} of 2.0 mg/kg for RTG (**Fig. 7*A***). Further i.g. administration of SCR2682 in different doses also gave rise to a concentration-dependent reduction of seizure incidence with an ED_{50} value of 1.7 mg/kg, which is ~10-fold more efficacious than RTG, which had an ED_{50} of 12.5 mg/kg (Fig. 7*B*). Interestingly, the anticonvulsant activity of SCR2682 was still maintained 24 h after intragastric administration, with an ED_{50} of 5.5 mg/kg, whereas no antiepileptic activity was observed for RTG 24 h after i.g. administration (Fig. 7*C*). We also investigated the anticonvulsant activity of SCR2682 in a mouse seizure model, and a similar antiepileptic effect was observed, with an ED_{50} of 1.6 mg/kg (i.p.) and 2.7 mg/kg (i.g.) (Fig. 7*D*). These results demonstrate that SCR2682 is more efficacious in antiepileptic activity than RTG in rodent seizure models.

Identification of a residue critical for activation of Kv7 currents by SCR2682

The tryptophan residue 236 located at the fifth transmembrane segment of Kv7.2 has been shown to be a critical amino acid in RTG-mediated channel activation because mutating tryptophan to leucine in Kv7.2 (W236L) renders the channel insensitive to activation by RTG (39, 40). To explore the underlying molecular mechanism by which SCR2682 activates Kv7 channels, we tested the effect of SCR2682 on a Kv7.2 W236L mutant. As shown in **Fig. 8**, the Kv7.2 W236L mutant channel was insensitive to current activation by RTG (10 μM). Similarly, SCR2682 (0.1 μM) also had no obvious effect on activation of the Kv7.2 W236L mutant, whereas another opener, QO-58 {pyrazolo[1,5-a] pyrimidin-7(4*H*)-one, 10 μM}, markedly enhanced Kv7.2 (W236L) currents and caused a significant leftward shift ($\Delta V_{1/2}$ = 61.2 mV) of the channel activation curve (n = 5; $P < 0.05$) (39). These data indicate that SCR2682 acts on Kv7 W236 and shares a similar activation mechanism with RTG but not QO-58 (32, 40), although SCR2682 is ~100 times more potent than RTG. Our results also confirm that the residue W236 is critical for Kv7.2 channel activation.

Lack of human ether-a-go-go-related gene channel inhibition by SCR2682

Activation of human ether-a-go-go-related gene (hERG) potassium channels generates rapid component of delayed rectifier potassium current crucial for repolarization of cardiac action potentials, and inhibition of rapid component of delayed rectifier potassium current increases the risk of arrhythmia or sudden death (41). To evaluate the potential risk for cardiac liability, we tested the

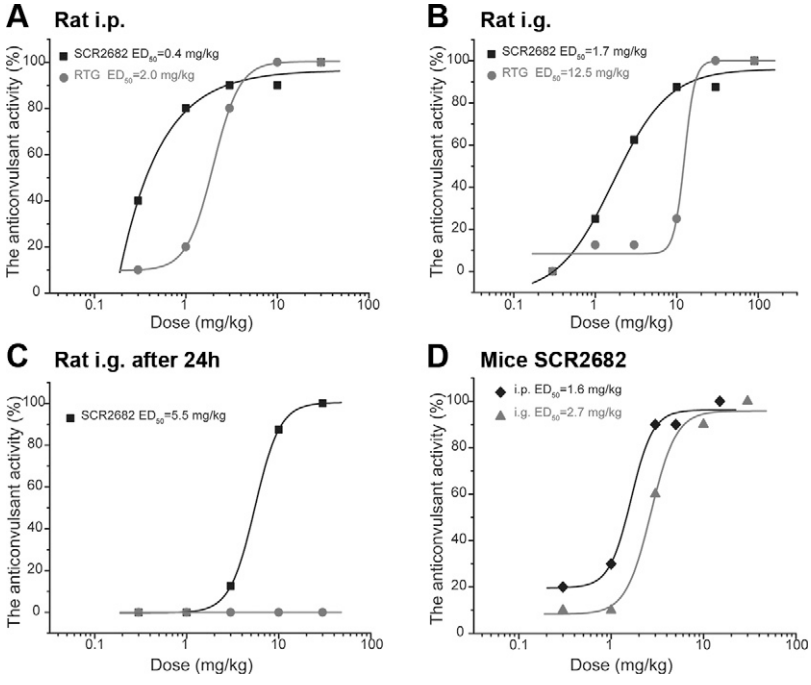

Figure 7. Antiepileptic activity of SCR2682 in rodent models of MES-induced seizures. *A*) In rats, dose-dependent protection of seizures by intraperitoneal injection of SCR2682 or RTG 30 min before electroshock stimulation, with an ED_{50} value of 0.4 mg/kg for SCR2682 and an ED_{50} value of 2.0 mg/kg for RTG. *B*) Dose-dependent protection of seizures by i.g. administration of SCR2682 with an ED_{50} value of 1.7 mg/kg or RTG at 12.5 mg/kg 3.6 h before the electroshock stimulation in rats. *C*) Dose-dependent protection of mouse seizures by i.g. administration of SCR2682 with an ED_{50} value of 5.5 mg/kg or RTG without efficacy 24 h before the electroshock stimulation. *D*) In mice, dose-dependent protection of seizures by intraperitional and i.g. administration of SCR2682 30 min before the electroshock stimuli, with ED_{50} values of 1.6 and 2.7 mg/kg, respectively.

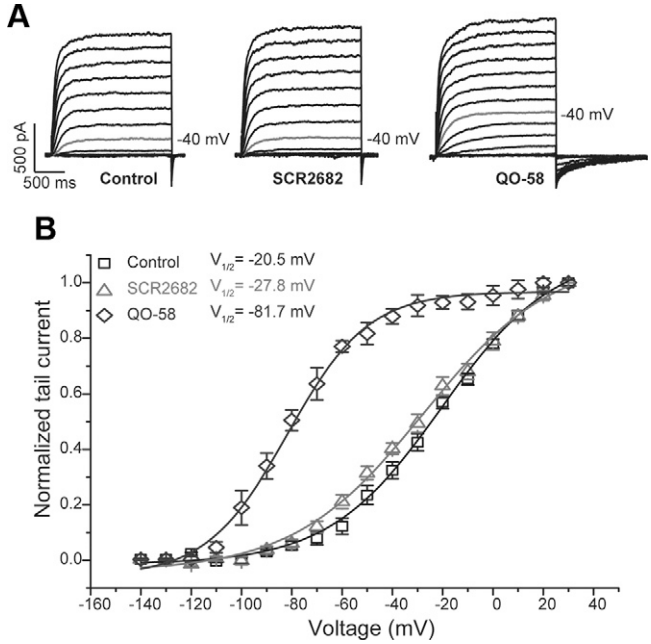

Figure 8. Mutation (W236L) of the Kv7.2 channel abolishes SCR2682-mediated voltage-dependent activation of the channel current. *A*) The current of Kv7.2 W236L mutant was recorded using a voltage protocol with holding potential at −120 mV before depolarization from −120 to 30 mV in 10-mV steps in the absence and presence of 0.1 μM SCR2682 or 10 μM QO-58. *B*) Voltage-dependent activation curves of Kv7.2 W236L currents were generated from the tail currents (at −120 mV) and fitted with Boltzmann function. $V_{1/2}$ was −20.5 ± 1.8 mV (n = 8) for control, −27.8 ± 2.6 mV (n = 8), and −81.7 ± 1.3 mV (n = 8) for 0.1 μM SCR2682 and 10 μM QO-58, respectively.

effect of SCR2682 on cardiac voltage-gated hERG channels (Kv11.1) stably expressed in HEK293 cells. As shown in **Fig. 9**, SCR2682 at 1 μM had no obvious inhibitory effect on hERG channels, and increasing the SCR2682 concentration to 100 μM had a weak inhibition of the hERG current. A further increase of SCR2682 to 300 μM only resulted in slight inhibition, with a half maximal inhibitory concentration (IC_{50}) value of 202 ± 71.5 μM and a Hill coefficient of 0.9 ± 0.3 (n = 4), indicating that SCR2682 has little inhibition on hERG current and may not impose a significant risk of cardiac arrhythmia.

DISCUSSION

Chemical activation of neuronal Kv7 channels by small molecules has emerged as a therapeutic strategy for treatment of hyperexcitability-related disorders such as seizures and pain. In this study, we synthesized and characterized, to the best of our knowledge, the most potent neuronal Kv7/KCNQ/M-channel opener, SCR2682, which selectively activates the channels. Several outstanding features of SCR2682 can be summarized based on its effects on Kv7 currents. First, SCR2682 potently activates neuronal homotetrameric and heterotetrameric Kv7 channel currents. The EC_{50} value for SCR2682 activation of Kv7.2/Kv7.3 currents is 9.8 nM, whereas RTG, approved by the U.S. Food and Drug Administration for treatment of partial epilepsy, has an EC_{50} value of 0.8–2.0 μM (20, 21, 42). The potency of SCR2682 in activating the Kv7.2/Kv7.3 channel is ~100 times more than RTG and >200 times more than other Kv7 openers such as *N*-(6-chloro-pyridin-3-yl)-3,4-difluoro-benzamide (5 μM), *N*-(6-chloropyridin-3-yl)-4-fluorobenzamide (6 μM), and QO-58 (2.3 μM) (39, 43). Second, SCR2682 causes a remarkable leftward shift of voltage-dependent activation of neuronal Kv7 channels toward more negative potential without activation of cardiac Kv7.1 channel. The leftward shift of voltage-dependent activation of neuronal Kv7 channels by SCR2682 lowers the membrane potential threshold and hyperpolarizes neurons, thus greatly reducing firings of hippocampal and cortical neurons. Third, SCR2682 increases Kv7.2 channel current amplitude by ~5-fold as compared with a ~2-fold increase for other homotetrameric or hetrotetrameric members of Kv7 subfamily. Fourth, SCR2682 exhibits a weak inhibition on the cardiac hERG channel with an IC_{50} value of 202 ± 71.5 μM, which has ~4 times less cardiac liability compared with RTG, which inhibits hERG with an IC_{50} of 59 μM (38). All these characteristics of SCR2682 are well manifested in its antiepileptic activity in rodent models, indicating that it possesses therapeutic potential for epilepsy, and it can be used as a potent tool molecule for further understanding of pharmacological functions of neuronal Kv7 channels.

Recent investigations indicate that the tryptophan residue (W236) in Kv7.2 or W265 in Kv7.3 functions as a key determinant of the RTG-mediated activation of neuronal Kv7.2/Kv7.3 channels. When tryptophan 236 is mutated

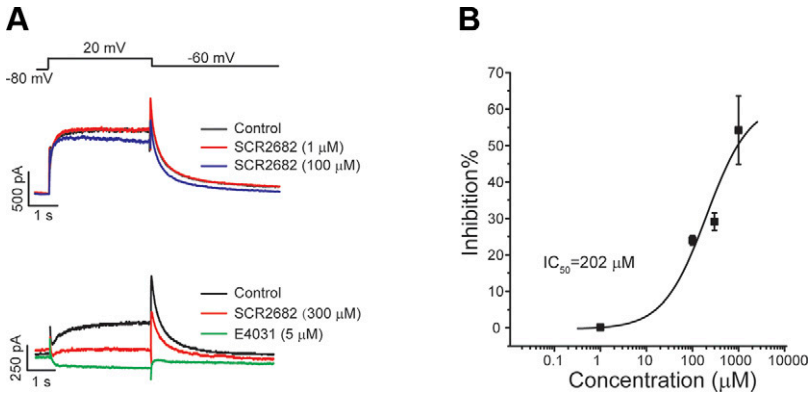

Figure 9. Weak inhibition of hERG channel current stably expressed in HEK293 cells by compound SCR2682. *A*) Representative outward currents elicited by step depolarization to 20 mV from a holding potential of −80 mV before repolarization to −60 mV. hERG currents were recorded in the presence of different concentrations (1–300 μM) of SCR2682 or 5 μM E4031. *B*) The analysis for concentration-dependent inhibition of hERG current by SCR2682 with an IC_{50} value of 202 ± 71.5 μM (n = 6).

TABLE 4. *Kinetic parameters after i.g. administration of SCR2682 in rats and intraperitoneal or i.g. administration in mice*

	Rats	Mice	
Parameter	i.g. 7.3 mg/kg	i.p. 22.5 mg/kg	i.g. 22.5 mg/kg
$T_{1/2}$z (h)	6.37	12.10	5.13
T_{max} (h)	3.667	2.00	4.00
C_{max} (ng/ml)	1223.82	1231.83	180.94
AUC_{last} (h × ng/ml)	16,723.00	13,330.85	1637.11
$AUC_{inf\text{-}obs}$ (h × ng/ml)		13,458.47	1730.64
Vz_obs (ml/kg)	3730.63	29,187.03	96,204.76
Cl_obs (ml/h/kg)	403.29	1671.81	13,000.94
MRT_{last} (h)	7.96	14.03	7.11

$T_{1/2}$, half-time; T_{max}, time to reach C_{max}; C_{max}, maximum concentration; AUC, area under the drug concentration-time curve; Vz, apparent volume of distribution; Cl, clearance; MRT, mean residence time

to leucine in Kv7.2, Kv7.3, Kv7.4, or Kv7.3/Kv7.5, these channels are no longer potentiated by RTG (44). Interestingly, Trp236 is also a site activated by many other Kv7 channel openers, such as BMS-204352, acrylamide (S)-1, acrylamide (S)-2, *N*-(6-chloropyridin-3-yl)-4-fluorobenzamide, and celecoxib (43, 45, 46). However, different Kv7 channel openers exhibit differential mechanisms underlying Kv7 activation. Zinc pyrithione (ZnPy) has been reported to strongly potentiate all Kv7 channels except Kv7.3 (47). The action of ZnPy is independent of W236, and residues Leu249 and Leu275 contribute to ZnPy binding or affect the rate of ZnPy access to the binding site (47). NH29 likely operates *via* a voltage sensor trapping mechanism (48). In the helix S4, R198A and R207W mutant channels show very weak stimulation by NH29 (48). Our previous studies also show that 3 residues, Val224, Val225, and Tyr226 in Kv7.2, are important for opener QO-58–mediated activation of the channel (32). RTG requires only 1 drug-sensitive subunit to generate its full effect on activating Kv7 tetramers, whereas another selective Kv7.2/7.3 opener, *N*-(2-chloro-5-pyrimidinyl)-3,4-difluorobenzamide, requires 4 drug-sensitive subunits for channel activation (49, 50). Consistent with these findings, our data also show that SCR2682 no longer enhances the current amplitude or shifts the voltage-dependent activation of Kv7.2 W236L mutant, whereas our previously identified Kv7.2/Kv7.3 opener QO-58 still causes a significant leftward shift of the voltage-dependent activation of the Kv7.2 W236L mutant channel (39). These findings demonstrate that although SCR2682 shares a similar molecular mechanism for Kv7 activation with RTG, SCR2682 is ~100-fold more potent and 7-fold more effective than RTG. Such a distinct pharmacological difference between SCR2682 and RTG is likely related to their chemical structures, which require further investigation.

SCR2682 shows greater antiepileptic efficacy in both mouse and rat models. Interestingly, SCR2682 still exhibits antiepileptic activity 24 h post-i.g. administration, when RTG has no antiepileptic effect. We also further tested the blood concentration and kinetics of SCR2682 in both rodent models (**Table 4**). The half-life of SCR2682 in the blood is ~6.5 h in rats, which may partially explain the long-term effect after oral administration.

The clinical use of RTG has been restricted to adjunctive treatment for drug-resistant partial epilepsy because of the significant risk of skin discoloration, retinal pigmentation, urinary retention, sedation, and corrected QT interval prolongation (51). The Kv7.4 channel is expressed in the brain and also in the bladder (52). The effect of SCR2682 on activation of Kv7.4 channel is much weaker (2.9-fold) with an EC_{50} of 28.9 nM when compared with its activation of the Kv7.2/7.3 channel with an EC_{50} of 9.8 nM, which suggests less risk on urinary retention, although we cannot completely rule out the potential risk that SCR2682 may cause by activating Kv7.4 channels in the bladder.

In summary, a 2,6-dimethyl-4-(piperidin-yl)phenyl)-amide derivative, SCR2682, was identified as a lead compound with superior potency and efficacy compared with the commercially available antiepileptic drug, RTG (ezogabine). SCR2682 activates heterotetrameric Kv7.2/Kv7.3 channels with an EC_{50} of 9.8 nM, whereas its IC_{50} value for inhibition of hERG current is ~202 μM, accounting for a ~20,000-fold difference window in potency. Therefore, SCR2682 might be more beneficial, with less side effects than RTG, and thus possessing developmental potential for treatment of epilepsy or hyperexcitability-related disorders. FJ

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AUTHOR CONTRIBUTIONS

F. Zhang and Y. Liu performed the electrophysiological and behavioral experiment and data analyses; F. Tang, B. Liang, and H. Chen synthesized the compound and

performed the pharmacokinetic experiment; F. Zhang, Y. Liu, H. Chen, H. Zhang, and K. Wang designed the research; F. Zhang, Y. Liu, and K. Wang wrote the manuscript; and all authors have read, commented on, and approved the content of the manuscript.

REFERENCES

1. Korsgaard, M. P., Hartz, B. P., Brown, W. D., Ahring, P. K., Strøbaek, D., and Mirza, N. R. (2005) Anxiolytic effects of Maxipost (BMS-204352) and retigabine via activation of neuronal Kv7 channels. *J. Pharmacol. Exp. Ther.* **314**, 282–292
2. Mora, G., and Tapia, R. (2005) Effects of retigabine on the neurodegeneration and extracellular glutamate changes induced by 4-aminopyridine in rat hippocampus in vivo. *Neurochem. Res.* **30**, 1557–1565
3. Wu, Y. J., Boissard, C. G., Greco, C., Gribkoff, V. K., Harden, D. G., He, H., L'Heureux, A., Kang, S. H., Kinney, G. G., Knox, R. J., Natale, J., Newton, A. E., Lehtinen-Oboma, S., Sinz, M. W., Sivarao, D. V., Starrett, J. E., Jr., Sun, L. Q., Tertyshnikova, S., Thompson, M. W., Weaver, D., Wong, H. S., Zhang, L., and Dworetzky, S. I. (2003) (S)-N-[1-(3-morpholin-4-ylphenyl)ethyl]-3-phenylacrylamide: an orally bioavailable KCNQ2 opener with significant activity in a cortical spreading depression model of migraine. *J. Med. Chem.* **46**, 3197–3200
4. Yeung, S., Schwake, M., Pucovský, V., and Greenwood, Ia. (2008) Bimodal effects of the Kv7 channel activator retigabine on vascular K+ currents. *Br. J. Pharmacol.* **155**, 62–72
5. Robbins, J. (2001) KCNQ potassium channels: physiology, pathophysiology, and pharmacology. *Pharmacol. Ther.* **90**, 1–19
6. Maljevic, S., Wuttke, T. V., and Lerche, H. (2008) Nervous system KV7 disorders: breakdown of a subthreshold brake. *J. Physiol.* **586**, 1791–1801
7. Wang, H. S., Pan, Z., Shi, W., Brown, B. S., Wymore, R. S., Cohen, I. S., Dixon, J. E., and McKinnon, D. (1998) KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel. *Science* **282**, 1890–1893
8. Cooper, E. C., and Jan, L. Y. (2003) M-channels: neurological diseases, neuromodulation, and drug development. *Arch. Neurol.* **60**, 496–500
9. Brown, D. A., and Passmore, G. M. (2009) Neural KCNQ (Kv7) channels. *Br. J. Pharmacol.* **156**, 1185–1195
10. Schroeder, B. C., Kubisch, C., Stein, V., and Jentsch, T. J. (1998) Moderate loss of function of cyclic-AMP-modulated KCNQ2/KCNQ3 K+ channels causes epilepsy. *Nature* **396**, 687–690
11. Singh, N. A., Charlier, C., Stauffer, D., DuPont, B. R., Leach, R. J., Melis, R., Ronen, G. M., Bjerre, I., Quattlebaum, T., Murphy, J. V., McHarg, M. L., Gagnon, D., Rosales, T. O., Peiffer, A., Anderson, V. E., and Leppert, M. (1998) A novel potassium channel gene, KCNQ2, is mutated in an inherited epilepsy of newborns. *Nat. Genet.* **18**, 25–29
12. Peters, H. C., Hu, H., Pongs, O., Storm, J. F., and Isbrandt, D. (2005) Conditional transgenic suppression of M channels in mouse brain reveals functions in neuronal excitability, resonance and behavior. *Nat. Neurosci.* **8**, 51–60
13. Goldberg-Stern, H., Kaufmann, R., Kivity, S., Afawi, Z., and Heron, S. E. (2009) Novel mutation in KCNQ2 causing benign familial neonatal seizures. *Pediatr. Neurol.* **41**, 367–370
14. Wulff, H., Castle, N. A., and Pardo, L. A. (2009) Voltage-gated potassium channels as therapeutic targets. *Nat. Rev. Drug Discov.* **8**, 982–1001
15. Fattore, C., and Perucca, E. (2011) Novel medications for epilepsy. *Drugs* **71**, 2151–2178
16. Weisenberg, J. L., and Wong, M. (2011) Profile of ezogabine (retigabine) and its potential as an adjunctive treatment for patients with partial-onset seizures. *Neuropsychiatr. Dis. Treat.* **7**, 409–414
17. Main, M. J., Cryan, J. E., Dupere, J. R., Cox, B., Clare, J. J., and Burbidge, S. A. (2000) Modulation of KCNQ2/3 potassium channels by the novel anticonvulsant retigabine. *Mol. Pharmacol.* **58**, 253–262
18. Kapetanovic, I. M., Yonekawa, W. D., and Kupferberg, H. J. (1995) The effects of D-23129, a new experimental anticonvulsant drug, on neurotransmitter amino acids in the rat hippocampus in vitro. *Epilepsy Res.* **22**, 167–173
19. Hopkins, C. R. (2012) ACS chemical neuroscience molecule spotlight on Potiga (Ezogabine). *ACS Chem. Neurosci.* **3**, 632
20. Jia, C., Qi, J., Zhang, F., Mi, Y., Zhang, X., Chen, X., Liu, L., Du, X., and Zhang, H. (2011) Activation of KCNQ2/3 potassium channels by novel pyrazolo[1,5-a]pyrimidin-7(4H)-one derivatives. *Pharmacology* **87**, 297–310
21. Blackburn-Munro, G., Dalby-Brown, W., Mirza, N. R., Mikkelsen, J. D., and Blackburn-Munro, R. E. (2005) Retigabine: chemical synthesis to clinical application. *CNS Drug Rev.* **11**, 1–20
22. Tatulian, L., Delmas, P., Abogadie, F. C., and Brown, D. A. (2001) Activation of expressed KCNQ potassium currents and native neuronal M-type potassium currents by the anti-convulsant drug retigabine. *J. Neurosci.* **21**, 5535–5545
23. Rubi, L., Kovar, M., Zebedin-Brandl, E., Koenig, X., Dominguez-Rodriguez, M., Todt, H., Kubista, H., Boehm, S., and Hilber, K. (2017) Modulation of the heart's electrical properties by the anticonvulsant drug retigabine. *Toxicol. Appl. Pharmacol.* **329**, 309–317
24. Garin Shkolnik, T., Feuerman, H., Didkovsky, E., Kaplan, I., Bergman, R., Pavlovsky, L., and Hodak, E. (2014) Blue-gray mucocutaneous discoloration: a new adverse effect of ezogabine. *JAMA Dermatol.* **150**, 984–989
25. Zaugg, B. E., Bell, J. E., Taylor, K. Y., and Bernstein, P. S. (2017) Ezogabine (Potiga) maculopathy. *Retin. Cases Brief Rep.* **11**, 38–43
26. Devulder, J. (2010) Flupirtine in pain management: pharmacological properties and clinical use. *CNS Drugs* **24**, 867–881
27. Wladyka, C. L., and Kunze, D. L. (2006) KCNQ/M-currents contribute to the resting membrane potential in rat visceral sensory neurons. *J. Physiol.* **575**, 175–189
28. Martire, M., Castaldo, P., D'Amico, M., Preziosi, P., Annunziato, L., and Taglialatela, M. (2004) M channels containing KCNQ2 subunits modulate norepinephrine, aspartate, and GABA release from hippocampal nerve terminals. *J. Neurosci.* **24**, 592–597
29. Raol, Y. H., Lapides, D. A., Keating, J. G., Brooks-Kayal, A. R., and Cooper, E. C. (2009) A KCNQ channel opener for experimental neonatal seizures and status epilepticus. *Ann. Neurol.* **65**, 326–336
30. Yu, F., Liu, Y., Wang, Y., Yin, J., Wang, H., Liu, W., Peng, B., and He, X. (2011) Protective effect of the KCNQ activator flupirtine on a model of repetitive febrile seizures. *Epilepsy Res.* **97**, 64–72
31. Szelenyi, I. (2013) Flupirtine, a re-discovered drug, revisited. *Inflamm. Res.* **62**, 251–258
32. Qi, J., Zhang, F., Mi, Y., Fu, Y., Xu, W., Zhang, D., Wu, Y., Du, X., Jia, Q., Wang, K., and Zhang, H. (2011) Design, synthesis and biological activity of pyrazolo[1,5-a]pyrimidin-7(4H)-ones as novel Kv7/KCNQ potassium channel activators. *Eur. J. Med. Chem.* **46**, 934–943
33. Jow, F., and Wang, K. (2000) Cloning and functional expression of rKCNQ2 K(+) channel from rat brain. *Brain Res. Mol. Brain Res.* **80**, 269–278
34. Brewer, G. J., Torricelli, J. R., Evege, E. K., and Price, P. J. (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* **35**, 567–576
35. Feng, S., Ma, S., Jia, C., Su, Y., Yang, S., Zhou, K., Liu, Y., Cheng, J., Lu, D., Fan, L., and Wang, Y. (2016) Sonic hedgehog is a regulator of extracellular glutamate levels and epilepsy. *EMBO Rep.* **17**, 682–694
36. Wang, K., McIlvain, B., Tseng, E., Kowal, D., Jow, F., Shen, R., Zhang, H., Shan, Q. J., He, L., Chen, D., Lu, Q., and Dunlop, J. (2004) Validation of an atomic absorption rubidium ion efflux assay for KCNQ/M-channels using the ion Channel Reader 8000. *Assay Drug Dev. Technol.* **2**, 525–534
37. Jow, F., Shen, R., Chanda, P., Tseng, E., Zhang, H., Kennedy, J., Dunlop, J., and Bowlby, M. R. (2007) Validation of a medium-throughput electrophysiological assay for KCNQ2/3 channel enhancers using IonWorks HT. *J. Biomol. Screen.* **12**, 1059–1067
38. Gunthorpe, M. J., Large, C. H., and Sankar, R. (2012) The mechanism of action of retigabine (ezogabine), a first-in-class K+ channel opener for the treatment of epilepsy. *Epilepsia* **53**, 412–424
39. Zhang, F., Mi, Y., Qi, J. L., Li, J. W., Si, M., Guan, B. C., Du, X. N., An, H. L., and Zhang, H. L. (2013) Modulation of K(v)7 potassium channels by a novel opener pyrazolo[1,5-a]pyrimidin-7(4H)-one compound QO-58. *Br. J. Pharmacol.* **168**, 1030–1042

40. Wuttke, T. V., Seebohm, G., Bail, S., Maljevic, S., and Lerche, H. (2005) The new anticonvulsant retigabine favors voltage-dependent opening of the Kv7.2 (KCNQ2) channel by binding to its activation gate. *Mol. Pharmacol.* **67**, 1009–1017
41. Roden, D. M. (2004) Drug-induced prolongation of the QT interval. *N. Engl. J. Med.* **350**, 1013–1022
42. Wickenden, A. D., Yu, W., Zou, A., Jegla, T., and Wagoner, P. K. (2000) Retigabine, a novel anti-convulsant, enhances activation of KCNQ2/Q3 potassium channels. *Mol. Pharmacol.* **58**, 591–600
43. Gao, Z., Zhang, T., Wu, M., Xiong, Q., Sun, H., Zhang, Y., Zu, L., Wang, W., and Li, M. (2010) Isoform-specific prolongation of Kv7 (KCNQ) potassium channel opening mediated by new molecular determinants for drug-channel interactions. *J. Biol. Chem.* **285**, 28322–28332
44. Schenzer, A., Friedrich, T., Pusch, M., Saftig, P., Jentsch, T. J., Grötzinger, J., and Schwake, M. (2005) Molecular determinants of KCNQ (Kv7) K+ channel sensitivity to the anticonvulsant retigabine. *J. Neurosci.* **25**, 5051–5060
45. Du, X., and Gamper, N. (2013) Potassium channels in peripheral pain pathways: expression, function and therapeutic potential. *Curr. Neuropharmacol.* **11**, 621–640
46. Du, X. N., Zhang, X., Qi, J. L., An, H. L., Li, J. W., Wan, Y. M., Fu, Y., Gao, H. X., Gao, Z. B., Zhan, Y., and Zhang, H. L. (2011) Characteristics and molecular basis of celecoxib modulation on K(v) 7 potassium channels. *Br. J. Pharmacol.* **164**, 1722–1737
47. Xiong, Q., Sun, H., and Li, M. (2007) Zinc pyrithione-mediated activation of voltage-gated KCNQ potassium channels rescues epileptogenic mutants. *Nat. Chem. Biol.* **3**, 287–296
48. Peretz, A., Pell, L., Gofman, Y., Haitin, Y., Shamgar, L., Patrich, E., Kornilov, P., Gourgy-Hacohen, O., Ben-Tal, N., and Attali, B. (2010) Targeting the voltage sensor of Kv7.2 voltage-gated K+ channels with a new gating-modifier. *Proc. Natl. Acad. Sci. USA* **107**, 15637–15642
49. Wang, A. W., Yau, M. C., Wang, C. K., Sharmin, N., Yang, R. Y., Pless, S. A., and Kurata, H. T. (2018) Four drug-sensitive subunits are required for maximal effect of a voltage sensor-targeted KCNQ opener. *J. Gen. Physiol.* **150**, 1432–1443
50. Yau, M. C., Kim, R. Y., Wang, C. K., Li, J., Ammar, T., Yang, R. Y., Pless, S. A., and Kurata, H. T. (2018) One drug-sensitive subunit is sufficient for a near-maximal retigabine effect in KCNQ channels. *J. Gen. Physiol.* **150**, 1421–1431
51. Daniluk, J., Cooper, J. A., Stender, M., and Kowalczyk, A. (2016) Survey of Physicians' understanding of specific risks associated with retigabine. *Drugs Real World Outcomes* **3**, 155–163
52. Svaløe, J., Hansen, H. H., Rønn, L. C., Sheykhzade, M., Munro, G., and Rode, F. (2012) Kv 7 positive modulators reduce detrusor overactivity and increase bladder capacity in rats. *Basic Clin. Pharmacol. Toxicol.* **110**, 145–153

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