Retigabine, a \(K_v7.2/K_v7.3\)-Channel Opener, Attenuates Drug-Induced Seizures in Knock-In Mice Harboring \(Kcnq2\) Mutations

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Abstract

The hetero-tetrameric voltage-gated potassium channel \(K_v7.2/K_v7.3\), which is encoded by \(KCNQ2\) and \(KCNQ3\), plays an important role in limiting network excitability in the neonatal brain. \(K_v7.2/K_v7.3\) dysfunction resulting from \(KCNQ2\) mutations predominantly causes self-limited or benign epilepsy in neonates, but also causes early onset epileptic encephalopathy. Retigabine (RTG), a \(K_v7.2/K_v7.3\)-channel opener, seems to be a rational antiepileptic drug for epilepsies caused by \(KCNQ2\) mutations. We therefore evaluated the effects of RTG on seizures in two strains of knock-in mice harboring different \(Kcnq2\) mutations, in comparison to the effects of phenobarbital (PB), which is the first-line antiepileptic drug for seizures in neonates. The subjects were heterozygous knock-in mice (\(Kcnq2^{Y284C/+}\) and \(Kcnq2^{A306T/+}\)) bearing the Y284C or A306T \(Kcnq2\) mutation, respectively, and their wild-type (WT) littermates, at 63–100 days of age. Seizures induced by intraperitoneal injection of kainic acid (KA, 12mg/kg) were recorded using a video-electroencephalography (EEG) monitoring system. Effects of RTG on KA-induced seizures of both strains of knock-in mice were assessed using seizure scores from a modified Racine’s scale and compared with those of PB. The number and total duration of spike bursts on EEG and behaviors monitored by video recording were also used to evaluate the effects of RTG and PB. Both \(Kcnq2^{Y284C/+}\) and \(Kcnq2^{A306T/+}\) mice showed significantly more KA-induced seizures than WT mice. RTG significantly attenuated KA-induced seizure activities in both \(Kcnq2^{Y284C/+}\) and \(Kcnq2^{A306T/+}\) mice, and more markedly than PB. This is the first reported evidence of RTG ameliorating KA-induced seizures in knock-in mice bearing mutations of \(Kcnq2\), with more marked effects than those observed with PB. RTG or other \(K_v7.2\)-channel openers may be considered as first-line antiepileptic treatments for epilepsies resulting from \(KCNQ2\) mutations.
Introduction

Kv7.2/Kv7.3, a hetero-tetrameric voltage-gated potassium channel, consists of two types of subunits, which are encoded by KCNQ2 and KCNQ3. Kv7.2/Kv7.3 is predominantly expressed in the hippocampus, neocortex, and the granular layer of the cerebellum [1–4] and generates the neuronal M-current, which stabilizes the membrane potential and controls neuronal excitability. Kv7.2/Kv7.3 thus plays an important role in limiting network excitability in the neonatal brain, where GABAergic action is depolarizing and excitatory [5].

Mutations in KCNQ2 and KCNQ3 are known to cause predominantly benign familial or non-familial neonatal epilepsy (BFNE or BNE) [2,6,7], both of which remit spontaneously in late infancy and thus are self-limited. However, a recent line of evidence shows that some KCNQ2 mutations also cause early onset epileptic encephalopathies (EOEEs) or early infantile epileptic encephalopathies (EIEE), such as Ohtahara syndrome [8–12], which are associated with intractable seizures followed by profound psychomotor delay. In general, most individuals with BFNE or BNE have a benign course; however, some patients may have varying degrees of developmental delays and epilepsy recurring later in their life [11,12]. The development of rational therapy for epilepsies caused by dysfunctions resulting from mutated Kv7.2/Kv7.3 is thus urgently needed.

Retigabine (RTG), a Kv7.2/Kv7.3 opener, increases open channel probability and leads to hyperpolarization of the membrane potential. Hence, RTG may stabilize the resting membrane potential and suppress repetitive firing caused by KCNQ2 mutations. Several in vitro electrophysiological studies on reconstituted Kv7.2/Kv7.3 have suggested that seizures caused by KCNQ2 mutations might respond to RTG [13]. However, no study has evaluated the effects of RTG on seizures in genetically engineered animals harboring Kcnq2 mutations, or compared these effects with the effects of phenobarbital (PB). At present, PB is the first-line and most commonly used anti-epileptic drug (AED) for neonatal seizures, including BFNE or EOEEs. Therefore, we here used knock-in mice bearing mutations in Kcnq2, the mouse orthologue of KCNQ2, to compare the effects of RTG on drug-induced seizures in the animals with those of PB.

Materials and Methods

Animal subjects

Two strains of heterozygous knock-in mice, Kcnq2Y284C/+ and Kcnq2A306T/+ , which harbor heterozygous Y284C or A306T Kcnq2 mutations, respectively, were produced using the "kick-in" system as described elsewhere [14]. Kcnq2Y284C/+ and Kcnq2A306T/+ mice used in this study were congeneric strains produced by more than 10 repeated backcross to C57BL/6J strain mice. Heterozygous mutations of Y284C and A306T have previously been identified in KCNQ2 in individuals with BFNE [15]. Both Y284C and A306T mutations are known to cause the BFNE phenotype, but phenotypic difference in patients has not been reported. Y284C is located in the loop that forms the ion pore of the channel, whereas A306T is located in transmembrane segment 6 (Fig 1A). Y284C and A306T are located at the outer mouth and the inner lining of the channel pore, respectively (Fig 1B). In this study, we used both Kcnq2Y284C/+ and Kcnq2A306T/+ mice to strengthen the results to evaluate potential differences, if any, between the two mutants.

The "kick-in" system that we previously developed is a modified knock-in procedure making use of Cre/mutant lox technology [14]. The system allows rapid production of multiple strains of knock-in mice bearing different mutations in a given portion of target genes. Kcnq2Y284C/+ and Kcnq2A306T/+ mice have been found to exhibit occasional spontaneous
seizures. In contrast to these sparse spontaneous seizures, seizures can be more readily induced by proconvulsants, such as pentylenetetrazol, in both Kcnq2<sub>Y284C/+</sub> and Kcnq2<sub>A306T/+</sub> mice compared to their wild-type (WT) littermates [14].

Mice were housed at 23 ± 2°C with 12 h light–12 h dark cycle (light on 7:00 to 19:00) and were given free access to commercial chow and tap water. Mice used for all experiments were 63–100 days of age (WT mice, n = 13; Kcnq2<sub>Y284C/+</sub> mice, n = 40; Kcnq2<sub>A306T/+</sub> mice, n = 33). To conduct all experiments blindly, genotyping to distinguish knock-in mice from their WT littermates was performed after each experiment, according to methods described elsewhere [14].
Ethics and animal rights protection

The experimental protocols were approved by the Committee for Animal Care and Use of Fukuoka University (Approval number: 294) and all experiments were conducted in compliance with the animal experimentation guidelines "Basic policies on animal experimentation" issued by the Ministry of Education, Sports, Science, and Technology (MEXT) and the Ministry of Health, Labor, and Welfare (MHLW) in Japan. All surgery was performed under sodium pentobarbital anesthesia, and efforts were made to minimize stress to the animals. Because all drug-induced seizures were monitored using a video-electroencephalography (EEG) monitoring system, we were able to observe in real time to determine whether mice were overstressed during seizures. During the course of the study, one Kcnq2^{A306T/+} mouse died after recurrent generalized tonic-clonic seizures and wild jumping approximately 30 minutes after kainic acid (KA) injection. The cause of death is unknown, but may have involved asphyxia or asystole. After the experiments, the mice were euthanized using sodium pentobarbital to minimize suffering.

Structural modeling and docking of the Kv7.2/Kv7.3 channel

The stereoscopic protein structure model of the human Kv7.2/Kv7.3 channel transmembrane domain was remodeled from a PDB file provided by Prof. Bernard Attali (Department of Physiology & Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Israel) [16], which is a modified chimeric version containing the rat Kv2.1 paddle (PDB entry 2R9R). Tetrameric reconstruction and energy minimization were calculated using Molecular Operating Environment (MOE), 2013.08 (Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A2R7, 2014).

EEG with video monitoring

EEGs of mice were recorded with video monitoring as previously described [14]. In brief, mice were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.), and bipolar stainless steel wire electrodes were implanted into the right forehead (2.0 mm anterior to the bregma, 1.5 mm lateral from the midline) and over the right hippocampus (2.0 mm posterior to the bregma, 1.5 mm lateral from the midline). Two electromyogram (EMG) wires were placed in the neck, between the muscle and skin. Digital EEG and video monitoring were performed in the cage for 1 week after the operation (KISSEI COMTEC, Sleep sign version 2, vital recorder, video option, Matsumoto, Japan).

A spike burst is a cluster of high-amplitude and high frequency spikes, each of which lasts a few seconds to several minutes on EEG during KA challenge tests [17] (Fig 1C). The total number and duration of the spike bursts observed for 120 min after KA injection in Kcnq2^{Y284C/+} and Kcnq2^{A306T/+} mice and their WT littermates were compared.

Drug-induced seizures

To induce seizures, mice received 12 mg/kg body weight KA (Sigma, St Louis, MO, USA) intraperitoneally 30 min after they had acclimated, as previously reported [18]. KA stocks, dissolved in water, were stored at -20°C; aliquots were diluted in normal saline solution immediately before use. The final concentration of KA used for injection was 1.2 mg/ml.

The seizures and behaviors were recorded continuously for more than 120 min with EEGs and video monitoring after drug injection. Seizures were scored based on behavior and EEG findings using a modified Racine’s scale [19] (Table 1) (Fig 2). Spikes and sharp waves were considered to represent seizure activity only when they were associated with abnormal
behaviors that were confirmed on video monitoring and EMG. We evaluated seizures and the effects of AEDs using this scoring system and also based on the number and total duration of spike bursts on EEG for 120 min after KA injection.

**Anti-epileptic drugs**

RTG dihydrochloride (Toronto Research Chemicals, Toronto, Canada) and PB sodium salt (WAKO, Osaka, Japan) were dissolved in physiological saline. All drugs were administered intraperitoneally 30 min before KA injection. Two doses of each AED (5 mg/kg and 15 mg/kg) were given for Kcnq2<sup>Y284C/+</sup> and Kcnq2<sup>A306T/+</sup> mice. Regardless of the dose, the injection volume was adjusted to 10 ml/kg bodyweight. We evaluated seizure scores and EEG findings as described above.

**Statistical data analyses**

Statistical data analyses were performed using the SAS (Statistical Analysis System) Software Package (Ver. 9.4, SAS Institute Inc., Cary, NC, USA) at Fukuoka University. The association of heterozygous Kcnq2 mutations with the seizure score, which is an ordinal measure, was examined using an exact test for ordered differences [20]. Differences in the distribution of the seizure score among WT, Kcnq2<sup>Y284C/+</sup>, and Kcnq2<sup>A306T/+</sup> mice or among mice administered vehicle, PB, or RTG were examined using the Jonckheere–Terpstra test [20] and the logistic regression analyses [21]. The number of spike bursts and the duration of spike bursts were compared among different types of mice, or mice treated with different types of drugs using Poisson regression [20]. Additional analyses were performed for overdispersion in Poisson regression [22]. Negative binomial model with a linear variance function (p = 1) was used to account for overdispersion when exists [22,23]. The t-statistics of dispersion parameter, labeled as “_Alpha” in the COUNTREG procedure, was used to assess the significance of overdispersion [22,23]. The significance level was considered to be less than 0.05, unless indicated otherwise.

**Results**

**Higher sensitivity to KA-induced seizure in Kcnq2 mutant mice**

Both Kcnq2<sup>Y284C/+</sup> and Kcnq2<sup>A306T/+</sup> mice showed significantly more KA-induced seizures, with a higher seizure score, than WT mice (Fig 3A). In WT mice, no KA-induced seizure with a score of > 3 was observed. In contrast, in Kcnq2<sup>Y284C/+</sup> mice, all scores of KA-induced

| Score | Behavioral stage | EEG findings |
|-------|------------------|--------------|
| 0     | No change in behavior | Baseline |
| 1     | Sudden behavioral arrest, motionless staring (with orofacial automatism) | High amplitude activity/slow waves |
| 2     | Head nodding | Spikes, sharp waves |
| 3     | Forelimb clonus with lordotic posture | Spikes or poly spikes, sharp waves |
| 4     | Forelimb clonus, with rearing and falling | Spike bursts/spike and wave discharges |
| 5     | Generalized tonic-clonic activity with loss of postural tone, often resulting in death, wild jumping | Spike bursts/spike and wave discharges |

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Seizures were scored as described in Methods. Similar to KA-induced seizures in Kcnq2Y284C/+ mice, those in Kcnq2A306T/+ mice had higher scores than those observed in WT mice (Fig 3A). The differences in the distribution of the seizure score between the mutant and WT mice and between the two strains of mutant mice were statistically significant (p = 0.0002, Kcnq2Y284C/+ vs. WT; p = 0.0024, Kcnq2A306T/+ vs. WT; p = 0.0982, Kcnq2Y284C/+ vs. Kcnq2A306T/+), as assessed by the logistic regression analysis. These results indicate that Kcnq2Y284C/+ and Kcnq2A306T/+ mice have significantly higher sensitivity to KA-induced seizures than WT mice, and Kcnq2Y284C/+ mice have higher sensitivity to KA-induced seizures than Kcnq2A306T/+ mice.

In accordance with this higher seizure sensitivity to KA, Kcnq2Y284C/+ mice exhibited frequent and prolonged spike bursts during KA challenges (Fig 3B and 3C). Kcnq2Y284C/+ mice had significantly more and longer spike bursts than WT mice (p = 0.0001, Fig 3B and

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**Fig 2.** Representative electroencephalograms at each score of Modified Racine's scale. Electroencephalogram (EEG) and electromyogram (EMG) were obtained as described in Methods. Each panel shows a representative EEG recording at Score 0 to 5 of a Modified Racine's scale [19]. Spikes and sharp waves were considered part of seizure activities only when they were associated with abnormal behaviors which were confirmed on video monitoring and electromyogram. A spike burst is defined as a cluster of high-amplitude and high frequency spikes, each of which lasts a few seconds to several minutes on EEG during kainic acid challenge tests [17].

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\[ p = 0.0001 \] (Fig 3C), as assessed by using Negative binomial model. Similarly, \( \text{Kcnq}^{-2A306T/+} \) mice showed a tendency to have more and longer spike bursts than WT mice, although the difference was not statistically significant (\( p = 0.3357 \) and \( p = 0.2398 \)). Furthermore, \( \text{Kcnq}^{-2Y284C/+} \) mice had significantly more and longer spike bursts than \( \text{Kcnq}^{-2A306T/+} \) mice (\( p = 0.0102 \), Fig 3B and \( p = 0.0131 \), Fig 3C), as assessed by using Negative binomial model. Thus, compared to
WT and $Kcnq2^{A306T/+}$ mice, $Kcnq2^{Y284C/+}$ mice exhibited more frequent and prolonged spike bursts during KA challenges.

**RTG was superior to PB in ameliorating KA-induced seizures in $Kcnq2^{Y284C/+}$ mice**

Administration of PB or RTG (5 mg/kg and 15 mg/kg) prior to KA challenges reduced the incidence of KA-induced seizures in the $Kcnq2^{Y284C/+}$ mice (Fig 4A). The trend across seizure scores indicated significantly better effects for pretreatment with PB (at doses of 5 and 15 mg/kg) or RTG (at doses of 5 and 15 mg/kg) than with vehicle ($p = 0.0135$, PB vs. vehicle; $p < 0.0001$, RTG vs. vehicle, Fig 4A), as assessed by the Jonckheere–Terpstra test. The preventative effect of RTG was greater than that of PB ($p = 0.0766$), after adjusting for dose by the logistic regression analysis.

Next, we assessed the dose-dependency of the effects of PB and RTG on seizure scores (Fig 4A). The preventative effect of 15 mg/kg PB on KA-induced seizures was statistically significant ($p = 0.0041$, 15 mg/kg PB vs. vehicle), although the effect of 5 mg/kg of PB was not statistically significant ($p = 0.1404$, 5 mg/kg PB vs. vehicle), as assessed by the Jonckheere–Terpstra test. There was no significant difference between the effects of 5 mg/kg and 15 mg/kg PB in $Kcnq2^{Y284C/+}$ mice ($p = 0.2884$, 15 mg/kg vs. 5 mg/kg PB, Fig 4A).

In contrast to PB, RTG demonstrated significant preventative effects for KA-induced seizures at both doses ($p = 0.0012$, 5 mg/kg RTG vs. vehicle; $p = 0.0002$, 15 mg/kg RTG vs. vehicle), as assessed by the Jonckheere–Terpstra test. There was no significant difference in the preventative effects between the two doses of RTG ($p = 0.8829$, 15 mg/kg vs. 5 mg/kg RTG, Fig 4A). Thus, pretreatment with RTG achieved a significant preventative effect for KA-induced seizures even at a low dose (5 mg/kg) in $Kcnq2^{Y284C/+}$ mice.

The difference between the effects of PB and RTG in these mice became evident when they were compared in terms of spike bursts (Fig 4B and 4C). Thus, RTG was superior to PB in ameliorating KA-induced seizures, although both PB and RTG were effective in reducing the number and total duration of spike bursts during KA challenges in $Kcnq2^{Y284C/+}$ mice.

Both PB and RTG reduced the number of spike bursts (Fig 4B). PB at a dose of 5 mg/kg produced a significant reduction of the number of spike bursts ($p = 0.0256$, 5 mg/kg PB vs. vehicle, Fig 4B), whereas 15 mg/kg PB did not have a statistically significant effect ($p = 0.2171$, 15 mg/kg PB vs. vehicle), as assessed by using Negative binomial model. There was no significant difference between these two doses of PB in terms of the number of spike bursts. RTG, in contrast, reduced the number of spike bursts significantly at both 5 mg/kg and 15 mg/kg doses ($p = 0.0049$, 5 mg/kg RTG vs. vehicle; $p = 0.0012$, 15 mg/kg RTG vs. vehicle, Fig 4B), as assessed by using Negative binomial model. This effect of RTG was not significantly dose-dependent ($p = 0.5358$, 15 mg/kg vs. 5 mg/kg RTG). The effects of RTG on the number of spike bursts were significantly better than those of PB in $Kcnq2^{Y284C/+}$ after adjusting for dose ($p = 0.0026$).

In accordance with the effects on the number of spike bursts, PB and RTG were effective in shortening the total duration of spike bursts, and the effects of RTG were more marked than that of PB (Fig 4C). PB, at a dose of 5 mg/kg, reduced the total duration of spike bursts ($p = 0.0851$, 5 mg/kg PB vs. vehicle, Fig 4C), as assessed by using Negative binomial model. In contrast, RTG shortened the total duration of spike bursts significantly at both 5 mg/kg and 15 mg/kg doses ($p = 0.0011$, 5 mg/kg RTG vs. vehicle; and $p = 0.0003$, 15 mg/kg RTG vs. vehicle, Fig 4C), as assessed by using Negative binomial model. This effect of RTG was not significantly dose-dependent ($p = 0.5815$, 15 mg/kg vs. 5 mg/kg RTG). The effects of RTG on shortening the total duration of spike bursts were significantly better than those of PB after adjusting for dose ($p < 0.0001$).
Fig 4. Effects of phenobarbital (PB) and retigabine (RTG) at low and high doses on seizures in Kcnq2Y284C/+ mice. A. Distribution of seizure scores in Kcnq2Y284C/+ mice administered vehicle (n = 6) (upper panel, in gray color), PB at doses of 5 mg/kg (n = 7) and 15 mg/kg (n = 7) (middle panel, in light gray and gray color, respectively), or RTG at doses of 5 mg/kg (n = 10) and 15 mg/kg (n = 10) (lower panel, in light gray and gray color, respectively). Between and within drug differences in the distribution of seizure scores were examined by the Jonckheere–Terpstra test, as described in the Methods. B and C. Box-and-whisker plots showing the mean (●), median (middle bar in the rectangle), and 10th (bottom bar), 25th (bottom of rectangle), 75th (top of rectangle), and 90th (top bar) percentiles of the number (B) and duration (C) of spike bursts in Kcnq2Y284C/+ mice administered vehicle (n = 6), PB at doses of 5 mg/kg (n = 7) and 15 mg/kg (n = 7), or RTG at doses of 5 mg/kg (n = 10) and 15 mg/kg (n = 10). PB and RTG at doses of 5 mg/kg are shown in light gray color. Between and within drug differences in terms of the number and duration of spike bursts were examined using Negative binomial model, as described in the Methods. Raw data for bar and box plots are included in S1 File.

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RTG was superior to PB in ameliorating KA-induced seizures in Kcnq2<sup>A306T/+</sup> mice

The effects of PB and RTG were also evaluated and compared in heterozygous Kcnq2<sup>A306T/+</sup> mice (Fig 5). Administration of PB and RTG (5 mg/kg and 15 mg/kg) prior to KA challenges reduced the incidence of KA-induced seizures in Kcnq2<sup>A306T/+</sup> mice (Fig 5A). The trend across seizure scores indicated significantly better effects of pretreatment with RTG (at doses of 5 and 15 mg/kg) than with vehicle (p = 0.0015, RTG vs. vehicle, Fig 5A), although the preventive effects of PB (at doses of 5 and 15 mg/kg) on KA-induced seizures was not statistically significant (p = 0.1144, PB vs. vehicle), as assessed by the Jonckheere–Terpstra test. Furthermore, RTG had better preventive effects on KA-induced seizures than PB in Kcnq2<sup>A306T/+</sup> mice, although the difference did not reach statistical significance (p = 0.1857), after adjusting for dose by the logistic regression analysis.

Next, we assessed the dose-dependency of the effects of RTG on KA-induced seizure score in Kcnq2<sup>A306T/+</sup> mice (Fig 5A). Both 5 mg/kg RTG and 15 mg/kg RTG demonstrated significant preventative effects on KA-induced seizures (p = 0.0029, 5 mg/kg RTG vs. vehicle; p = 0.0165, 15 mg/kg RTG vs. vehicle), but the effect of RTG was not significantly dose-dependent (p = 0.6084, 15 mg/kg vs. 5 mg/kg RTG, Fig 5A), as assessed by the Jonckheere–Terpstra test. Thus, similar to Kcnq2<sup>Y284C/+</sup> mice, pretreatment with RTG achieved a significant preventative effect even at a low dose in Kcnq2<sup>A306T/+</sup> mice.

The difference between PB and RTG became more evident when they were compared in terms of their effects on both the number and total duration of spike bursts during KA challenges. Thus, RTG was superior to PB in ameliorating KA-induced seizures in terms of reducing the number and total duration of spike bursts during KA challenges (Fig 5B and 5C).

RTG demonstrated the ability to significantly reduce the number of spike bursts during KA challenges at both 5 mg/kg and 15 mg/kg doses (p < 0.0001, 5 mg/kg RTG vs. vehicle; p = 0.0224, 15 mg/kg RTG vs. vehicle; Fig 5B), whereas the effects of PB at 5 mg/kg and 15 mg/kg doses on the number of spike bursts were not statistically significant (p = 0.7222, 5 mg/kg PB vs. vehicle; p = 0.1174, 15 mg/kg PB vs. vehicle), as assessed by using Negative binomial model. The effects of RTG in terms of the number of spike bursts were significantly better than those of PB in Kcnq2<sup>A306T/+</sup> mice after adjusting for dose (p = 0.0603).

In accordance with the effects on the number of spike bursts, PB and RTG were effective in shortening the total duration of spike bursts, and the effect of RTG was more marked than that of PB (Fig 5C). The effect of PB on shortening the total duration of spike bursts was significant at a dose of 15 mg/kg but not at 5 mg/kg (p = 0.0413, 15 mg/kg PB vs. vehicle; p = 0.5787, 5 mg/kg PB vs. vehicle, Fig 5C), as assessed by using Negative binomial model. In contrast, RTG demonstrated a significant effect at both 5 mg/kg and 15 mg/kg doses (p < 0.0001, 5 mg/kg RTG vs. vehicle; p = 0.0117, 15 mg/kg RTG vs. vehicle, Fig 5C) on shortening the total duration of spike bursts, as assessed by using Negative binomial model. The difference between RTG and PB in terms of the total duration of spike bursts in Kcnq2<sup>A306T/+</sup> mice was also statistically significant after adjusting for doses (p = 0.0740).

**Discussion**

In the present study, both Kcnq2<sup>Y284C/+</sup> and Kcnq2<sup>A306T/+</sup> heterozygous mutant mice showed significantly higher sensitivity to KA-induced seizures than WT mice. Accordingly, both mutant mice exhibited more frequent and prolonged spike bursts during KA challenges. RTG, a pan K<sub>7.2-K<sub>7.5</sub> channel opener [24–28], suppressed these KA-induced seizure activities more effectively than PB, which is currently the first-line AED for neonatal seizures, including BFNE, resulting from KCNQ2 mutations.
Kcnq2<sup>Y284C/+</sup> and Kcnq2<sup>A306T/+</sup> mice are prone to KA induced seizures

We previously showed high sensitivity to proconvulsive treatment in Kcnq2<sup>Y284C/+</sup> and Kcnq2<sup>A306T/+</sup> mice, using pentylenetetrazole (PTZ) [14]. Similar findings were reported in

Fig 5. The effects of phenobarbital (PB) and retigabine (RTG) at low and high doses on seizure in Kcnq2<sup>A306T/+</sup> mice. A. Distribution of seizure scores in Kcnq2<sup>A306T/+</sup> mice administered vehicle (n = 7) (upper panel, in gray color), PB at doses of 5 mg/kg (n = 5) and 15 mg/kg (n = 7) (middle panel, in light gray and gray color, respectively), or RTG at doses of 5 mg/kg (n = 6) and 15 mg/kg (n = 8) (lower panel, in light gray and gray color, respectively). Between and within drug differences in the distribution of seizure scores were examined by the Jonckheere–Terpstra test, as described in the Methods. B and C. Box-and-whisker plots showing the mean (●), median (middle bar in the rectangle), and 10th (bottom bar), 25th (bottom of rectangle), 75th (top of rectangle), and 90th (top bar) percentiles of the number (B) and duration (C) of spike bursts in Kcnq2<sup>A306T/+</sup> mice administered vehicle (n = 7), PB at doses of 5 mg/kg (n = 5) and 15 mg/kg (n = 6), or RTG at doses of 5 mg/kg (n = 6) and 15 mg/kg (n = 8). PB and RTG at doses of 5 mg/kg are shown in light gray color. Between and within drug differences in terms of the number and duration of spike bursts were examined using Negative binomial model, as described in the Methods. Raw data for bar and box plots are included in S1 File.

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early studies with Szt1 mice, a spontaneous mutant mouse strain that harbors a microdeletion affecting the C-terminus of mouse Kcnq2 and also its adjacent two genes, Chnra4 and Arfgap-1 [29–33]. Heterozygous Kcnq2 knock-out mice have also demonstrated hypersensitivity to PTZ [34]. Furthermore, a high sensitivity to proconvulsive treatments has been observed in BFNE-mutation knock-in mice that were independently generated by another group. These mutations included the A306T mutation in Kcnq2 and the G311V mutation in Kcnq3, which is an ortholog of a KCNQ3 mutation found in human BFNE [35,36]. Similar to our Kcnq2^{Y284C/+} and Kcnq2^{A306T/+} mice, these heterozygous mutant mice did not show spontaneous seizures, although their homozygotes did. The Kcnq2^{A306T/+} and Kcnq3^{G311V/+} mice had significantly higher sensitivity to electroconvulsive seizure and kindling acquisition [35,36]. Interestingly, they demonstrated genotype-related differences in sensitivity. In agreement with this indication, our present study found that Kcnq2^{Y284C/+} mice were more prone to KA-induced seizures than Kcnq2^{A306T/+} mice in terms of the frequency and duration of seizure bursts upon EEG, suggesting that the BFNE mutations vary in sensitivity.

Thus, the hypersensitivity to proconvulsants observed in Kcnq2-deficient mice and the resulting impairment of M-currents hamper the stabilization of the resting membrane potential and subthreshold levels of membrane excitability [37]. Therefore, these findings in mouse models provide compelling evidence for the crucial role of M-currents in controlling neuronal excitability [5], supporting findings from electrophysiological studies on reconstituted channels in vitro [38–44].

RTG significantly attenuates KA-induces seizures in Kcnq2^{Y284C/+} and Kcnq2^{A306T/+} mice

In both Kcnq2^{Y284C/+} and Kcnq2^{A306T/+} mice, RTG, an opener of K,7 channels, was more effective in preventing KA-induced seizures than PB, not only in terms of the incidence of seizures, but also in terms of suppressing the spike bursts on EEG.

To our knowledge, this is the first application and comparison of RTG with other AEDs in genetically engineered animal models bearing Kcnq2 BFNE mutations. The effects of another potassium channel opener, flupirtine, which is an analogue of RTG, were previously evaluated and compared with PB, albeit in experimental seizure models involving WT rodents. Flupirtine has a better effect on experimental seizures than PB [45].

In addition, RTG has been used in Szt1 mice, but was found less effective in reducing electroconvulsions in Szt1 mice than in their WT controls [30]. However, its effect was not compared with those of other AEDs in this model [30]. Therefore, the present study, for the first time, has provided evidence that RTG would provide better prevention of seizure activity in the presence of genetically impaired K,7.2.

Our findings also support in vitro electrophysiological findings that RTG ameliorates the dysfunction of K,7.2 caused by KCNQ2 mutations, obtained either from reconstituted channels in Xenopus laevis oocytes [44,46] or brain slice patch clamping in mice bearing a KCNQ2 microdeletion [37,47–49]. Interestingly, a report of a voltage clamping assay assessing the effect of RTG on reconstituted K,7.2 in Xenopus laevis oocytes revealed that the effect of RTG on lowering action potential in WT K,7.2 was significantly greater than that on K,7.2 harboring EOEE mutations [44]. BFNE-related KCNQ2 mutations cause haploinsufficiency, while EOEE mutations cause a dominant-negative effect. Hence, given that K,7.2/K,7.3 is a hetero-tetramer, RTG may exert its function in both Kcnq2^{Y284C/+} and Kcnq2^{A306T/+} mice by ameliorating the pore region, which consists of two KCNQ3 subunits and one normal KCNQ2 subunit derived from WT alleles (Fig 1B).
RTG is more effective than PB against KA-induced seizures in $\text{Kcnq2}^{Y284C/+}$ and $\text{Kcnq2}^{A306T/+}$ mice

PB is widely used as the first-line AED for neonatal seizures, including BFNE resulting from KCNQ2 mutations. PB exerts its pharmacological function mainly as a GABA$_A$ receptor agonist, but also as an antagonist of the AMPA/kainate–glutamate receptor, inhibiting glutamate release, which is controlled by the P/Q-type calcium channel [50,51]. The NMDA and AMPA subtypes of glutamate receptors are highly expressed between the first and second postnatal weeks in rats and in the neonatal period in humans [52,53]. It is therefore believed that PB is useful for treating neonatal seizures because the additional reduction of glutamate receptors may reduce the severity of neonatal seizures [51].

In the neonatal brain, however, the environment and function of neurotransmitters are different from those in the mature brain. For example, GABAergic action is depolarizing and excitatory in the neonatal brain because of the higher intracellular chloride concentration caused by the predominant expression of NKCC1 over that of KCC2 [5,54–56]. The composition of the subunits of GABA$_A$ receptors also changes during brain development [57,58]. Thus, the α4 subunits of GABA receptors, which render the receptor less sensitive to benzodiazepines, are relatively overexpressed at developmental stages compared with GABA$_A$ receptors in the adult brains that consist predominantly of the α1 subunit [57,59,60]. Some AEDs, such as PB and benzodiazepines, function as agonists to GABA$_A$ receptors and may be less effective in neonates than in adults because of the characteristics of the GABA$_A$ receptor in the neonatal brain [61,62]. Consequently, intractable neonatal seizures, for which PB does not work well, are clinically encountered.

In contrast, RTG exerts its antiepileptic effect by opening K$_v$7 channels, including K$_v$7.2 and K$_v$7.3. RTG binds to a hydrophobic pocket in the ion pore between transmembrane segments S5 and S6 (Fig 1A and 1B), opening the pore and increasing the membrane potassium conductance of neurons [63]. The increment of potassium currents reduces the generation of action potentials. Therefore, RTG can both suppress the hyperexcitability of neurons via potassium channels and function as an AED [63]. In addition, K$_v$7.2/K$_v$7.3 channels are predominantly expressed in the axon initial segment of neurons, which is a region crucial for controlling neuronal excitability [37,64,65]. Furthermore, K$_v$7.2 and K$_v$7.3 appear to be particularly important for the neonatal brain, as they are highly expressed from late fetal life to early infancy [66,67]. Although RTG is known to cause positive allosteric modulation of GABA$_A$ receptors, this effect is observed only at high concentrations, and no significant interaction with glutamate receptors has been observed [24,68]. Therefore, RTG is considered a rational AED for use in neonatal seizures, specifically for those resulting from K$_v$7.2 or K$_v$7.3 dysfunction.

Potassium channel openers in neonatal seizures

RTG has been approved by the FDA as an add-on therapy for partial-onset seizures [69]. However, clinical application of RTG has recently been limited because of rising concerns regarding its adverse effects, such as blue skin discoloration and eye abnormalities resulting from pigment changes in the retina [70,71]. Nevertheless, several lines of evidence, including our findings on the efficacy of RTG for reducing seizures in Kcnq2 mutant mice, warrant further investigation of the therapeutic potential of RTG and related potassium-channel openers [37,64,65]. In addition, mutations of KCNQ2 are known to cause not only benign epilepsy, but also malignant phenotypes, e.g., EOEE and Ohtahara syndrome, for which treatments based on an understanding of the underlying pathomechanisms are urgently required [8–11]. RTG has been shown to be effective for seizures resulting from KCNQ2 mutations with a dominant-negative effect [44,72,73], which is suspected to be an underlying mechanism of some cases of EOEE.
addition, a new potassium-channel opener with fewer side effects was recently developed and has been shown to be effective for epilepsy [74].

Taken together, these previous findings and our present study suggest high potential of RTG in treating epilepsy resulting from KCNQ2 mutations. However, there are limitations in applying the results of the present study directly to human BFNE or EOEE. Seizures in the mice were induced by KA and not spontaneous, and the age of the mice used does not necessarily correspond to neonates. Therefore, further study of potassium-channel openers should provide insights into the treatment of neonatal seizures in the near future.

Conclusions

We have here provided the first evidence that RTG, a K₇.7 potassium-channel opener, ameliorates KA-induced seizures in knock-in mice bearing mutations of Kcnq2, the orthologue of KCNQ2 that encodes K₇.7.2 channels in humans. Furthermore, the effect of RTG is superior to that of PB, the accepted first-line AED for neonatal seizures. Given the efficacy of RTG in animal models of neonatal epilepsy caused by KCNQ2 mutations, potassium channel openers should be considered as a therapeutic option for BFNE/BNE and perhaps even for EOEE.

Supporting Information

S1 File. Dataset for bar and box plots.
(XLSX)

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Author Contributions

Conceived and designed the experiments: YI YT SH. Performed the experiments: YI YT MD. Analyzed the data: YI YT BZ SH. Contributed reagents/materials/analysis tools: MD BZ. Wrote the paper: YI YT MD BZ TU AI SH.

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