Spontaneous seizure and memory loss in mice expressing an epileptic encephalopathy variant in the calmodulin-binding domain of Kv7.2

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Epileptic encephalopathy (EE) is characterized by seizures that respond poorly to antiseizure drugs, psychomotor delay, and cognitive and behavioral impairments. One of the frequently mutated genes in EE is KCNQ2, which encodes the K7.2 subunit of voltage-gated K7 potassium channels. K7 channels composed of K7.2 and K7.3 are enriched at the axonal surface, where they potently suppress neuronal excitability. Previously, we reported that the de novo dominant EE mutation M546V in human K7.2 blocks calmodulin binding to K7.2 and axonal surface expression of K7 channels via their intracellular retention. However, whether these pathogenic mechanisms underlie epileptic seizures and behavioral comorbidities remains unknown. Here, we report conditional transgenic cKcnq2−/M547V mice, in which expression of mouse K7.2-M547V (equivalent to human K7.2-M546V) is induced in forebrain excitatory pyramidal neurons and astrocytes. These mice display early mortality, spontaneous seizures, enhanced seizure susceptibility, memory impairment, and repetitive behaviors. Furthermore, hippocampal pathology shows widespread neurodegeneration and reactive astrocytes. This study demonstrates that the impairment in axonal surface expression of K7.7 channels is associated with epileptic seizures, cognitive and behavioral deficits, and neuronal loss in KCNQ2-related EE.

KCNQ2 | seizures | epilepsy

Epileptic encephalopathies (EEs) are a collection of heterogeneous disorders in which early-onset severe seizures contribute to developmental delay and progressive cognitive and behavioral impairments (1). Current treatments for EEs have limited efficacy in alleviating seizures and comorbidities (2), posing an urgent need to understand the etiology of EEs and find new therapeutic targets. Recent discoveries of epilepsy-related genes in multiple laboratories and through the large Epilepsy, Epilepsy, and EuroEPINOMICS-RES consortia have identified a diverse array of proteins that may contribute to epileptogenesis (3–5). Among them, dominant variants associated with benign familial neonatal epilepsy (BFNE) and EE have been identified in patients with EE (https://www.rikee.org; ClinVar Database, NCBI). EEs are clustered at the functional domains of K7.2 important for voltage-dependent opening of K7.7 channels (13) and typically decrease the function of heterotetrameric channels by 20 to 75% (13–17). EEs variants are also enriched at helices A and B in the intracellular C-terminal tail of K7.2 (13, 14, 16), which mediate calmodulin (CaM) binding critical for axonal enrichment of K7.7 channels (18). Among these variants, a mutation of methionine at amino acid position 546 to valine (M546V) was found in a male patient who displayed drug-resistant neonatal tonic-clonic seizures and later developed profoundintellectual and language disability, spasticity, and autistic behavior (15). While this mutation in helix B abolishes current expression of homomeric but not heteromeric channels in heterologous cells (14, 17), it severely reduces CaM binding and axonal surface expression of heteromeric channels in cultured hippocampal neurons (14). This mutation also induces their critical roles in reducing neuronal excitability. By contrast, activation of Gq-coupled receptors, including muscarinic acetylcholine receptors, inhibits I_M by depleting the lipid cofactor PIP2, resulting in enhanced AP firing (12).

To date, 193 dominant variants in KCNQ2 and 2 variants in KCNQ3 have been identified in patients with EE (https://www.rikee.org; ClinVar Database, NCBI). EEs variants are clustered at the functional domains of K7.2 important for voltage-dependent opening of K7.7 channels (13) and typically decrease the function of heterotetrameric channels by 20 to 75% (13–17). EEs variants are also enriched at helices A and B in the intracellular C-terminal tail of K7.2 (13, 14, 16), which mediate calmodulin (CaM) binding critical for axonal enrichment of K7.7 channels (18). Among these variants, a mutation of methionine at amino acid position 546 to valine (M546V) was found in a male patient who displayed drug-resistant neonatal tonic-clonic seizures and later developed profound intellectual and language disability, spasticity, and autistic behavior (15). While this mutation in helix B abolishes current expression of homomeric but not heteromeric channels in heterologous cells (14, 17), it severely reduces CaM binding and axonal surface expression of heteromeric channels in cultured hippocampal neurons (14). This mutation also induces their critical roles in reducing neuronal excitability. By contrast, activation of Gq-coupled receptors, including muscarinic acetylcholine receptors, inhibits I_M by depleting the lipid cofactor PIP2, resulting in enhanced AP firing (12).

Significance

Epileptic encephalopathy (EE) is a devastating neurologic disorder characterized by early-onset seizures with severe cognitive and psychomotor impairments. EE is associated with dominant mutations in the KCNQ2 gene which encodes the K7.2 subunit of K7 potassium channels. Previously, we reported that multiple EE mutations in the intracellular calmodulin-binding domain of K7.2 decreased surface expression of axonal K7.7 channels critical for suppressing neuronal excitability. Here, we generated conditional knockin mice carrying one of these mutations, M547V. These mice displayed spontaneous seizures, cognitive impairment, neurodegeneration, and reactive astroglisis, implicating abnormal K7.7 surface expression as a key etiology of KCNQ2-associated EE.


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ubiquitination and proteasomal degradation of K<sub>7.2</sub>, whereas the presence of K<sub>7.3</sub> blocks this degradation and accumulates ubiquitinated K<sub>7.2</sub> (14). However, whether these pathogenic mechanisms underlie epileptic seizures and behavioral deficits in EE remains unknown.

In this study, we investigated the contribution of the EE variant M546V by generating conditional transgenic mice in which heterozygous expression of mouse K<sub>7.2</sub>-M547V was induced in forebrain excitatory pyramidal neurons. M546 in the human K<sub>7.2</sub> is conserved in the mouse K<sub>7.2</sub> at amino acid position 547. These mice showed widespread neurodegeneration and reactive astrogliosis in the hippocampus and cortex, and displayed spontaneous seizures and cognitive deficit, providing a causal link between M546V-mediated disruption of axonal surface expression of K<sub>7.7</sub> channels and K<sub>7</sub>QA-associated continuous jumping and digging in their home cage (Movies S1 and S2). Some showed motionless and rigid postures (Movie S3). Curiously, the high mortality of cKcnq<sup>2+/−M547V</sup> mice was not observed from P21 (Fig. 1G). There were no obvious differences in gross brain appearance or size between genotypes at P30 and P120 (SI Appendix, Fig. S6 A and B). The surviving cKcnq<sup>2+/−M547V</sup> males weighed less than control males at P120, but this genotype difference was not observed in females (SI Appendix, Fig. S6C).

**cKcnq<sup>2+/−M547V</sup> Mice Display Spontaneous Seizures and Extreme Seizure Sensitivity to Kainic Acid.** The patient with the de novo M546V mutation displayed drug-resistant neonatal tonic-clonic seizures (15). To test if spontaneous seizures occur in cKcnq<sup>2+/−M547V</sup> mice, we performed simultaneous video monitoring of behavioral seizures and electroencephalogram (EEG) recording at P60 to P85 (Fig. 2 A–C). All cKcnq<sup>2+/−M547V</sup> males displayed recurrent spontaneous behavioral seizures followed by death during recovery from electrode implantation surgery, precluding EEG recordings. Of six cKcnq<sup>2+/−M547V</sup> females, three survived for EEG recording. Two mice demonstrated spontaneous electrographic seizures that coincided with stage 1 or 2 seizures (Figs. 1B and C and Movies S4 and S5). By contrast, Kcnq<sup>2+/−</sup> females did not show electrographic and behavioral seizures (Fig. 2A).

Although Kcnq<sup>2+/−</sup> mice do not have spontaneous seizures (19, 21), they display enhanced sensitivity to seizures induced by pentylentetrazole (21) and kainic acid (KA) (22). To compare seizure propensity of cKcnq<sup>2+/−M547V</sup> mice with that of Kcnq<sup>2−/−</sup> mice, the (P120 to P180) received systemic injection of KA to induce seizures that arise from the hippocampus (23). A lower dose of KA (15 mg/kg, intraperitoneal [i.p.]) was used to reduce mortality (22). Control mice displayed only transient stage 2 seizures, whereas cKcnq<sup>2+/−M547V</sup> mice showed stage 8 seizures by 40 min post-KA injection (Fig. 2D) and displayed a significantly higher cumulative seizure score and shorter latency to stage 4 seizure and death than control mice regardless of sex (Fig. 2 E–G and SI Appendix, Table S1). Kcnq<sup>2−/−</sup> males and females reached stage 4 and 6 seizures, respectively, by 40 min post-KA injection (Fig. 2D) and displayed a lower cumulative seizure score than cKcnq<sup>2+/−M547V</sup> mice (Fig. 2E). These results indicate that cKcnq<sup>2+/−M547V</sup> mice had significantly higher seizure susceptibility than Kcnq<sup>2−/−</sup> mice.

**cKcnq<sup>2+/−M547V</sup> Mice Display Cognitive Deficits and Abnormal Behaviors.** The patient with the M546V mutation had profound intellectual disability, spasticity, and autistic behavior (15). To test if these comorbidities occur in cKcnq<sup>2+/−M547V</sup> mice, we performed six behavioral tests at P120 during the dark phase (Fig. 3A). There was no gross abnormality in motor coordination of cKcnq<sup>2+/−M547V</sup> mice as indicated by their similar latency to fall from the rotarod compared with control mice (Fig. 3B and SI Appendix, Table S2). In the open-field test, cKcnq<sup>2+/−M547V</sup> males traveled a longer distance in the entire open-field arena than control males (Fig. 3C and SI Appendix, Table S2), indicative of hyperactivity. Both cKcnq<sup>2+/−M547V</sup> males and females displayed higher numbers of entries into the center of the arena, spent more time, and traveled a longer distance within the center than control mice (Fig. 3C and SI Appendix, Table S2), suggesting enhanced exploratory behavior or reduced anxiety-like behavior.

To test if cKcnq<sup>2+/−M547V</sup> mice display cognitive deficits, we performed the object-location task (OLT) and novel object–recognition task (NORT). The OLT evaluates hippocampus-dependent spatial memory, whereas the NORT tests nonspatial memory of object identity (24). During the OLT, control mice spent significantly more time with the moved object, whereas...
cKcnq2<sup>−/−</sup>/M547V mice did not (Fig. 3D and SI Appendix, Table S2). During the NORT, control but not cKcnq2<sup>−/−</sup>/M547V mice spent significantly more time exploring the novel object (Fig. 3D and SI Appendix, Table S2). These results indicate that cKcnq2<sup>−/−</sup>/M547V mice showed impaired object-recognition memory and hippocampus-dependent spatial memory.

We next tested if cKcnq2<sup>−/−</sup>/M547V mice display autism-like behaviors including social avoidance and repetitive behaviors. In the three-chamber social interaction test (25), both genotypes spent significantly more time on the side of the chamber containing a novel social target mouse as compared with the empty side (Fig. 3E and SI Appendix, Table S3). While there were no genotype differences in males for sniffing duration and frequency, cKcnq2<sup>−/−</sup>/M547V females sniffed the novel mouse for a shorter duration and frequency than control females (Fig. 3E and SI Appendix, Table S3), indicative of reduced sociability. When another novel mouse was placed in the empty side (SI Appendix, Fig. S7), both genotypes spent more time sniffing the new mouse than the familiar mouse (SI Appendix, Fig. S7 and Table S4), suggesting that social preference of the novel mouse is similar between cKcnq2<sup>−/−</sup>/M547V and control mice.
Compared with control mice, cKcnq2\textsuperscript{+/M547V} mice displayed a longer duration of a grooming event and shorter latency of grooming (Fig. 3\textit{F} and \textit{SI Appendix}, Table S2), indicating increased repetitive behaviors (26). In addition, sex differences were observed for latency of grooming (\textit{SI Appendix}, Table S2). In a marble-burying test (27), cKcnq2\textsuperscript{+/M547V} males buried four times as many marbles as control males, but this genotype difference was not observed in females (Fig. 3\textit{G} and \textit{SI Appendix}, Table S2), indicating that cKcnq2\textsuperscript{+/M547V} males displayed enhanced repetitive and compulsive-like behaviors.

\textbf{cKcnq2\textsuperscript{+/M547V} Mice Display Neurodegeneration and Reactive Astrogliosis.} To understand the pathologic basis for spontaneous seizures and behavioral deficits of cKcnq2\textsuperscript{+/M547V} mice, we next performed immunostaining with verified anti-K\textsubscript{7.2} antibodies (\textit{SI Appendix}, Fig. S8). The hippocampi and cortices of cKcnq2\textsuperscript{+/M547V} mice showed GFP expression at P30, P60, and P120, whereas those of control mice did not (\textit{SI Appendix}, Fig. S1), indicating Cre-dependent expression of the \textit{Kcnq2-M547V-ires-EGFP} transgene in forebrain pyramidal neurons (Fig. 1\textit{B}). In the hippocampi of control mice at P120, we observed strong expression at the AIS was weaker in the cortical pyramidal neurons (Fig. 4\textit{A}) and neuronal marker NeuN (Fig. 4\textit{B}), which colocalize with GFP but not neuronal marker NeuN (\textit{SI Appendix}, Fig. S9\textit{A}). Interestingly, the baseline activity of this mouse showed a larger peak amplitude (31.5 ± 0.5 μV, \(P < 0.05\)) and a higher frequency (2.73 ± 0.36 Hz, \(P < 0.005\)) than the Kcnq2\textsuperscript{+/M547V} female in B. The behavioral seizures of the cKcnq2\textsuperscript{+/M547V} female mice in B and C are shown in Movies S4 and S5. (D–G) The control (Kcnq2\textsuperscript{+/+}), Kcnq2\textsuperscript{-/-}, and cKcnq2\textsuperscript{+/M547V} mice (P120 to P180) were injected with a low dose of KA (15 mg/kg, i.p.) and their behavioral seizures were rated with a modified Racine, Pinal, and Rovner scale at each 10-min interval. (D and E) Average seizure scores (D) and cumulative seizure scores (E) per mouse over the first 2 h after KA injection. (F) Latency to stage 4 seizure. (G) Latency to death. Data are shown as mean ± SEM. The number of male mice used: control (\(n = 10\)), Kcnq2\textsuperscript{+/+} (\(n = 7\)), and cKcnq2\textsuperscript{+/M547V} (\(n = 8\)). The number of female mice used: control (\(n = 10\)), Kcnq2\textsuperscript{+/+} (\(n = 8\)), and cKcnq2\textsuperscript{+/M547V} (\(n = 7\)). Post hoc Tukey test results are shown here (* \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.005\)). SI Appendix, Table S1 shows two-way ANOVA test results for control and with genotype as one factor and sex as the other.
We have previously shown that the analogous M546V mutation in human Kv7.2 induces not only ubiquitination but also severe impairments in axonal surface expression of Kv7.2/Kv7.3 channels and their intracellular retention (14). Intracellular accumulation of polyubiquitinated proteins in the endoplasmic reticulum (ER) has been shown to induce cell death (28).

To test if expression of Kv7.2-M547V induces neuronal death in vivo, brain cryosections were subjected to staining with Fluoro-Jade C, a fluorescein-derived fluorochrome which specifically binds to degenerating neurons (29). Fluoro-Jade C staining revealed a larger number of degenerating hippocampal neurons in cKcnq2+/M547V mice than in control mice in P30, P60, and P120 (Fig. 4B and SI Appendix, Fig. S14).

Reactive astrocytes mediate neuroinflammation by secreting inflammatory factors and function as phagocytes for degenerated axons and apoptotic neurons in brain injury and neurodegenerative diseases (30). Compared with control mice, cKcnq2+/M547V hippocampi showed significant age-dependent increases in the number of astrocytes and GFAP expression per astrocyte (Fig. 4C–E and SI Appendix, Figs. S10–S12), indicative of the activation and significant presence of reactive astrocytes (30).

Discussion

Contribution of the Forebrain Expression of KcNq2-M547V to Epileptic Seizures in Mice. Most children with BFNE and EE mutations in KCNQ2 and KCNQ3 have mild epileptic seizures that are remitted later in life or managed by antiseizure drugs (31). A subset of children has a more severe disease phenotype including refractory seizures (31). One such mutation is M546V, located in the CaM-binding helix B of Kv7.2 (15). We show that cKcnq2+/M547V mice displayed spontaneous generalized tonic-clonic seizures as early as P60 (Fig. 2A–C) and continuous jumping and prolonged rigidity by P21 (Movies S1–S3).
Since jumping and rigidity often occur with generalized tonic-clonic convulsions in mouse models of status epilepticus (32), these mice might have displayed subtle behavioral seizures that were not scored by our analyses.

Due to a mild 25 to 40% reduction in current density of heteromeric Kv7 channels, haploinsufficiency is proposed to mediate BFNE caused by $KCNQ2$ variants such as A306T and Y284C (31). However, heterozygous KI mice expressing these variants and $Kcnq2^{+/+}$/C0 mice do not show spontaneous seizures (19, 21, 33–35). Furthermore, $cKcnq2^{+/M547V}$ mice are more susceptible to KA-induced seizures than $Kcnq2^{+/+}$/C0 mice (Fig. 2 D–G). These differences suggest that the seizure phenotypes in $cKcnq2^{+/M547V}$ mice do not occur as a simple consequence of the loss of one $KCNQ2$ allele.

Although the M546V variant does not reduce current density of heteromeric K7.2/K7.3 channels in Xenopus oocytes (14, 17), it severely decreases axonal surface expression in cultured hippocampal neurons by >70% (14), suggesting that a dominant-negative suppression of axonal K v7 channels may underlie EE. In support of this idea, a recurrent EE mutation, T274M, in the pore loop exerts a dominant-negative effect by decreasing current density of homomeric channels by 70 to 80% (17) and its heterozygous KI mice develop transient generalized spontaneous seizures from P20 (36). Furthermore,
conditional homozygous deletion of the Kcnq2 gene from forebrain excitatory pyramidal neurons or genetic suppression of K7 current in the first postnatal weeks by overexpressing the K7.2-containing dominant-negative pore mutation G279S induces spontaneous seizure in mice (11, 37, 38). Thus, we speculate that a significant loss of K7 current early in development may contribute to the development of spontaneous seizures in EE.

In cKcnq2+/M547V mice, the expression of the Kcnq2-M547V-ires-EGFP transgene is driven by the CAG promoter from the Rosa locus in Cre-expressing forebrain glutamatergic neurons, whereas the expression of WT Kcnq2 is driven by the native promoter (Fig. 1 B and C), and Kcnq2 transcript levels are 1.94-fold higher than those in control mice (SI Appendix, Fig. S2). This is a different genotype from the human patient containing a heterozygous M546V mutation. The cKcnq2+/M547V mice also show the unexpected off-target expression of the transgene in some astrocytes (SI Appendix, Fig. S12), although its contribution to seizure and behavioral phenotypes is unclear. However, hippocampal K7.2 protein levels were comparable between control and cKcnq2+/M547V mice at P60 and P120 (Fig. 1E and SI Appendix, Fig. S4), when seizures and behaviors were examined (Figs. 2 and 3), suggesting that heightened seizure susceptibility and abnormal behaviors are likely due to impaired axonal expression of K7.2 channels.

In cKcnq2+/M547V mice born at the expected Mendelian ratio (SI Appendix, Fig. S5C), we observed postnatal death (Fig. 1F), which is considerably earlier than homozygous K1 mice of BFNE variants (34) and conditional homozygous K7.2 KO mice (11). This is relevant to KCNQ2-associated EE because most patients appear normal at birth but develop severe seizures and clinical encephalopathy within the first days of life (31). We speculate that neonatal seizures may likely cause the early postnatal death in cKcnq2+/M547V mice, as the higher expression of K7.2-M547V at birth (SI Appendix, Fig. S5) is expected to induce forebrain hyperexcitability via its dominant-negative suppression of K7 channels. In KCNQ2-associated EE, seizure frequency diminishes with age (31). Curiously, cKcnq2+/M547V mice that had survived by P21 showed a low risk of mortality (Fig. 1G), and displayed reduced K7.2 expression at P30 (SI Appendix, Fig. S5). Although the mechanism underlying this transient decrease in K7.2 is unknown, our findings suggest that the extent of K7.2-M547V expression in the early postnatal period may contribute to early mortality.

Effects of the Forebrain Expression of K7.2-M547V on Cognition and Behavior of Mice. The patient with the M546V mutation had profound intellectual disability and autistic behavior (15). Similarly, cKcnq2+/M547V mice display cognitive impairments (Fig. 3). They performed poorly on the OLT (Fig. 3D), which tests hippocampus-dependent spatial memory (24). Similar hippocampus-dependent spatial memory deficits were reported in the heterozygous T274M KI mice (36) and transgenic mice overexpressing dominant-negative K7.2-G279S (37). The cKcnq2+/M547V mice also showed deficits in nonspatial memory of object identity in NORT (Fig. 3D), which relies on the peri-rhinal cortex and to a lesser extent the hippocampus (24, 39). Neuronal hyperexcitability and degeneration in the hippocampus and cortex (Figs. 2 and 4 and SI Appendix, Figs. S9–S13) may underlie these memory deficits in cKcnq2+/M547V mice.

We also observed locomotor hyperactivity of cKcnq2+/M547V males (Fig. 3C) similar to the transgenic males overexpressing dominant-negative K7.2-G279S (37). Hippocampal sclerosis in these mice (Fig. 4C) (37) may underlie their locomotor hyperactivity given that hippocampal lesion and damage result in heightened locomotion in mice (40). Although the open-field test performed under aversive bright light is widely used to examine anxiety in mice (41), increased entry and duration of cKcnq2+/M547V mice to the exposed center in the dark (Fig. 3C) suggests their enhanced exploratory behavior in novel environments. Interestingly, the heterozygous T274M KI mice do not exhibit changes in anxiety, exploration, and repetitive behaviors (36). These behavioral differences suggest that the M546V mutation may have a different pathophysiology from the T274M mutation.

Comparison between cKcnq2+/M547V and Kcnq2+/− mice revealed that both genotypes show enhanced repetitive behaviors (Fig. 3 F and G) (22), which is one of the core clinical symptoms of autism (42). Since cKcnq2+/M547V mice have heterozygous forebrain expression of K7.2-M547V in Kcnq2+/− mice (Fig. 1B) and repetitive grooming behavior is mediated by GABAergic output from the striatum (43), we speculate that enhanced repetitive behaviors in cKcnq2+/M547V mice may likely be due to the heterozygous loss of Kcnq2 in the striatum. In the three-chamber social interaction test, only cKcnq2+/+M547V females showed a mild reduction in sociability (Fig. 3E), which might be contributed by the hyperactivity and damage in the hippocampus and cortex (Figs. 2 and 4 and SI Appendix, Figs. S9–S13), important for social approach and cognition (44, 45).

Neuropathology in Mouse Forebrain Induced by K7.2-M547V. The present study provides evidence that the expression of EE mutant K7.2-M547V induces neurodegeneration and reactive astroglisis in the hippocampus and cortex of mice (Fig. 4). These findings are consistent with the MRI of a patient with the M546V mutation, which showed substantial brain lesions and neuronal loss indicated by small frontal lobes, thinned splenium of the corpus callosum, ventriculomegaly, and increased cerebral spinal fluid space (15). This patient also showed intractable seizures (15). In mesial temporal lobe epilepsy, hippocampal sclerosis is closely associated with drug-resistant seizures, progressive cognitive decline, high risk of mortality, and sudden unexpected death in epilepsy (46). Although epileptic seizures are thought to cause cognitive and behavioral impairments in EE (1, 47), our findings suggest that severe seizure phenotypes, reduced viability, and cognitive deficits seen in cKcnq2+/M547V mice may be due to neurodegeneration and neuroinflammation in addition to hyperexcitability in the hippocampus and cortex (Figs. 2–4 and SI Appendix, Figs. S9–S14).

It is noteworthy that neurodegeneration and neuroinflammation were observed in cKcnq2+/M547V mice and mice over-expressing K7.2-G279S but not in mice heterozygous for K7.2-T274M, even though all three mutations cause dominant-negative suppression of K7 channels (36–38). These differences may be related to transgene promoter effects in vivo (exogenous promoter vs. native promoter) and different “mutant over WT” expression ratios, which dictate the extent of dominant-negative suppression of tetrameric K+ channels (48). However, these differences also highlight the complexity and heterogeneity of the pathophysiology associated with each mutation. Whereas the T274M mutation induces dominant-negative current suppression of human K7.2 channels (17), the analogous mutation to M547V in the human K7.2 short isoform (M518V) not only blocks their current and protein expression by increasing ubiquitination but also induces dominant-negative suppression of axonal K7 channels by retaining them intracellularly (14). These unique effects on K7.2 expression may contribute to neurodegeneration.

It is unclear how expression of K7.2-M547V results in neurodegeneration in vivo. The M547V-induced severe reduction in axonal K7 channels in the forebrain excitatory neurons and subsequent increase in their excitability may lead to excitotoxicity induced by excessive release of glutamate. The same mutation is also expected to induce ubiquitination and proteasome degradation of K7.2, whereas the presence of K7.3 blocks this degradation and induces intracellular accumulation of ubiquitinated K7.2 (14). Since impairment of the ubiquitin-
proteasome system and ER stress response can activate programmed cell death (28), we speculate that intracellular retention of Kcnn2-M547V proteins in the ER and their continual clearance could saturate the capacity of the ER and proteasome, leading to apoptosis. Future studies shall test these hypotheses to identify the mechanisms underlying neurodegeneration and its contribution to refractory seizures associated with this EE variant.

Materials and Methods

Generation of Conditional Forebrain Heterozygous Kl of Kcnn2-M547V in Mice. The transgenic mice for conditional expression of the mouse Kcnn2 gene containing the M547V mutation (ATG to GTG) (designated as Kcnn2-M547Vfl/fl) on the C57BL/6J genetic background were generated at Cyagen Biosciences by CRISPR-Cas-mediated genome engineering (49). In these mice, the cassette CAG-LoxP-Stop-LoxP-Kcnn2-M547V-IRESGFP-polycya was cloned into intron 1 of the Gt(ROSA26) locus on mouse chromosome 6 (GenBank accession no. NR_027008.1) in a reverse direction. This cassette was flanked by 2.5- and 4.5-kb homology arms, which were generated by PCR using a bacterial artificial chromosome (BAC) clone from the C57BL/6j library as template. To induce heterozygous expression of Kcnn2-M547V-IRESGFP in the forebrain pyramidal neurons via Cre-mediated removal of an upstream floxed-stop cassette (designated as cKcnq2-M547V mice), Kcnn2-M547V mice were crossed to heterozygous Kcnn2 KO Kcnn2+/- mice (designated as Kcnn2+-/-); Jax gene stock 005830 and Emx1tm1Dgen mice (Jax.org stock number 005628). Both Kcm2DmIgen+ and Emx1tm1Dgen mice were in the C57BL/6J background (19, 20). The control mice used in these studies were Kcnn2+/-, Kcnn2-M547Vfl/fl, Kcnn2-M547Vfl/fl, Emx1tm1Dgen, and Emx1tm1Dgen mice.

Video-ECoG Monitoring in Freely Moving Mice. To examine spontaneous seizures, mice at P51 to P108 were subjected to a video EEG monitoring system (Pinnacle Technology) from 2:00 to 4:30 PM as described (50). The electrical signal was band-pass-filtered from 1 to 100 Hz and digitized at 200 Hz. EEG epileptiform activity was identified by repetitive occurrence, large amplitude, and sharp morphology compared with baseline EEG activity (50).


Kainate-Induced Seizures. Behavioral seizures were induced in mice (P120 to P180) with KA (15 mg/kg, i.p.; Abcam) and monitored using a modified Racine, Pinal, and Rovner scale as described (22).

Behavioral Studies. Behavioral tests with a test interval of >2 d were started at P120 as described (22) from least to most invasive assays in the following order: object location and novel object recognition, self-grooming, marble burying, open field, rotarod, and three-chamber social interaction tests.

Immunohistochemistry of Mouse Brain Cryosections. Coronal brain cryosections (20-μm-thick) were immunostained with antibodies for Kcnn2, (Synaptic Systems), ankyrin-G, GFAP (NeuroMab), and Gfap (Abcam) or stained for Fluoro-Jade C (Biosensis) and DAPI (Invitrogen) after permeabilization. Confocal fluorescent images (1-μm optical distance) were analyzed by the ImageJ program (NIH) and our algorithm “ANIMA.”

Statistical Analysis. Data are reported as mean ± SEM. OriginPro v9.5 (OriginLab) was used to perform statistical analyses. Comparisons between two groups were conducted with the Student’s two-tailed t test. Behavioral data were analyzed using two-way ANOVA. Tukey tests were used to establish post hoc pairwise differences between means. A P value < 0.05 was considered statistically significant.

A detailed description of each method is provided in SI Appendix.

Data Availability. All study data are included in the article and/or supporting information. The source data sets that were generated and analyzed during the current study and presented as main figures and supplementary figures are available in the Figraph repository (DOI: 10.6084/m9.figshare.17124350).

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Spontaneous seizure and memory loss in mice expressing an epileptic encephalopathy variant in the calmodulin-binding domain of Kcnn2.


