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OVEREXPRESSING WILD-TYPE $\gamma 2$ SUBUNITS RESCUED THE SEIZURE PHENOTYPE IN *GABRG2*^{+/Q390X} DRAVET SYNDROME MICE

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Summary

Objective—The mutant γ -aminobutyric acid type A (GABA_A) receptor $\gamma 2$ (Q390X) subunit, (Q351X in the mature peptide) has been associated with the epileptic encephalopathy, Dravet syndrome, and the epilepsy syndrome genetic epilepsy with febrile seizures plus (GEFS+). The mutation generates a premature stop codon that results in translation of a stable truncated and misfolded $\gamma 2$ subunit that accumulates in neurons, forms intracellular aggregates, disrupts incorporation of $\gamma 2$ subunits into GABA_A receptors and affects trafficking of partnering α and β subunits. Heterozygous *Gabrg2*^{+/Q390X} knock-in (KI) mice had reduced cortical inhibition, spike wave discharges on EEG, a lower seizure threshold to the convulsant drug pentylenetetrazol (PTZ) and spontaneous generalized tonic-clonic seizures. In this proof of principal study, we attempted to rescue these deficits in KI mice using a $\gamma 2$ subunit gene (*GABRG2*) replacement therapy.

Methods—We introduced the *GABRG2* allele by crossing *Gabrg2*^{+/Q390X} KI mice with bacterial artificial chromosome (BAC) transgenic mice overexpressing HA (hemagglutinin) tagged human $\gamma 2^{\text{HA}}$ subunits and compared GABA_A receptor subunit expression by western blot and immunohistochemical staining, seizure threshold by monitoring mouse behavior after PTZ-injection, and thalamocortical inhibition and network oscillation by slice recording.

Results—Compared to KI mice, adult mice carrying both mutant allele and transgene had increased wild-type $\gamma 2$ and partnering $\alpha 1$ and $\beta 2/3$ subunits, increased miniature inhibitory postsynaptic current (mIPSC) amplitudes recorded from layer VI cortical neurons, reduced thalamocortical network oscillations, and higher PTZ seizure threshold.

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Conception and design of the study: X.H., C.Z., R.L.M.; Data collection: X.H., C.Z., J.Q.K., W.S., M.T., K.V.; Data analysis: X.H., C.Z., A.P., J.Q.K., R.L.M.; Manuscript preparation: X.H., C.Z., J.Q.K., R.L.M.; Contributed equally: X.H., C.Z.

Potential Conflicts of Interest

The authors have no potential conflicts of interest to disclosure.

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Significance—Based on these results we suggest that seizures in a genetic epilepsy syndrome caused by epilepsy mutant $\gamma 2(Q390X)$ subunits with dominant negative effects could be rescued potentially by overexpression of wild-type $\gamma 2$ subunits.

Keywords

GABA_A receptors; genetic epilepsies; *GABRG2(Q390X)* mutation; gene-replacement therapy; Dravet syndrome; epileptic encephalopathy

Introduction

Epilepsy is a common neurological disorder affecting more than 65 million people worldwide ¹. Some of the epilepsies, including the severe epileptic encephalopathy Dravet syndrome, are poorly responsive to conventional treatments ^{2–5}. Many antiepileptic drugs (AEDs) have undesirable side effects, and while some current therapies provide symptomatic treatment, none of them interfere with the epileptogenesis process ⁶. Alternative strategies that target the underlying genetic causes of epilepsy could be promising in the future.

Many epilepsy-associated mutations in individuals and families with epilepsy have been shown to alter or even disrupt function of ion channels such as γ -aminobutyric acid (GABA) type A (GABA_A) receptor channels ^{7, 8}. GABA_A receptors are the major receptor mediating fast inhibitory neurotransmission and controlling network excitability in the CNS. Among currently known epilepsy-associated mutations identified in GABA_A receptor subunit genes (*GABRs*), about one half of them are found in *GABRG2*, which encodes the $\gamma 2$ subunit ⁹. Epilepsy-associated mutant $\gamma 2$ subunits exhibit a wide range of effects on the function and biogenesis of GABA_A receptors *in vitro* and *in vivo*, from subtle alteration in current kinetic properties to loss of function and dominant negative effects ¹⁰. Mice carrying the *GABRG2* mutation (R82Q) have reduced cortical inhibition, spontaneous spike-wave discharges and thermal-induced seizures, mimicking seizure phenotypes identified in patients ^{8; 11–13}.

The *GABRG2(Q390X)* mutation, one of the most severe epilepsy-associated *GABRG2* mutations, is associated with genetic epilepsy with febrile seizures plus (GEFS+) and was identified in a patient with Dravet syndrome ¹⁴. *In vitro* mutant $\gamma 2(Q390X)$ subunits severely disrupted the trafficking and function of mutant $\gamma 2(Q390X)$ subunits and also affected the trafficking of partnering α and β subunits and wild-type $\gamma 2$ subunits ¹⁵. Mutant $\gamma 2(Q390X)$ subunits were slowly degraded and formed intracellular aggregates ¹⁶. Recently we found that heterozygous *Gabrg2^{+/Q390X}* KI mice showed decreased expression of wild-type $\gamma 2$ subunits, aggregation of mutant subunits, reduced cortical inhibition, and behavioral and electrographic seizures, which were much more severe than in heterozygous *Gabrg2^{+/-}* knock-out mice^{17,18}. Thus, it is interesting to ask whether the epilepsy phenotype in *Gabrg2^{+/Q390X}* mice expressing the mutant subunits could be improved/rescued by CNS expression of functional wild-type $\gamma 2$ subunits.

Here we explored whether gene-replacement could rescue the seizure phenotype of *Gabrg2^{+/Q390X}* KI mice. To compensate for the reduced inhibition caused by mutant $\gamma 2(Q390X)$ subunits, we introduced hemagglutinin (HA) tagged $\gamma 2^{\text{HA}}$ subunits into

Gabrg2^{+/Q390X} KI mice using Tg(*hGABRG2^{HA}*) bacterial artificial chromosome (BAC) transgenic mice¹⁹. We found that expressing the BAC transgene in the KI mice not only restored the total amount of wild-type $\gamma 2$ subunits, but also increased the expression of partnering α and β subunits in the somatosensory cortex, reversed the reduction of miniature inhibitory postsynaptic currents (mIPSCs) and pentylenetetrazol (PTZ) seizure threshold, and reduced the intensified thalamocortical oscillations, demonstrating a rescue of the seizure phenotype.

Materials and Methods

Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University. Tg(*hGABRG2^{HA}*) transgenic mice containing the *GABRG2^{HA}* gene and endogenous *hGABRG2* promoter were generated in C57BL/6J mice by pronucleus injection in the Vanderbilt Transgenic/ES Cell Shared Resource Facility¹⁹. Four different transgenic mouse lines were generated, but only one of them expressed transgene protein. Thus, we used this mouse line in our study. *Gabrg2^{+/Q390X}* KI mice were generated in a C57BL/6J;129svJ mixed background and were backcrossed into the C57BL/6J background for more than 8 generations. Tg(*hGABRG2^{HA}*) transgenic mice were bred with *Gabrg2^{+/Q390X}* KI mice, generating offspring with four different genotypes, as described in Figure 1A.

Mouse tail samples collected at P14 – P21 were extracted using REDExtract-N-Amp tissue PCR kit (Sigma-Aldrich) according to the manufacturer's manual. Mice were genotyped with Tg(*hGABRG2^{HA}*) forward (5'-TACCCCTACGACGTGCCCCGACTACGCC-3') and reverse (5'-CACCTCTCCCACTCATAGGCCTGAATG-3') (324 bp) primers for the transgene; and *Gabrg2* forward (5'-ATGGCGATGGAAGTTGACA-3') and reverse (5'-TGATGTTGCTCATGCCTCTC-3') primers (323 bp for the wild-type allele and 405 bp for the mutant allele) for the endogenous alleles.

Immunohistochemistry

Adult mice were anesthetized using isoflurane, followed by transcardial perfusion of 20 ml ice-cold 4% paraformaldehyde. Brains were removed and postfixed at 4 °C overnight, then cryoprotected in 30% sucrose at 4 °C for 48 hours. Brain sections of 50 μ m thickness were cut using a microtome and stored at -20 °C prior to immunostaining. Slices were incubated in rabbit monoclonal anti-HA antibody (1:500; Cell Signaling, #3724) in PBS with 0.3% Triton X and 4% horse serum at 4 °C for 48 h, followed by 2 h incubation in IRDye800-conjugated donkey anti-rabbit IgG secondary antibody (1:1000; Li-COR). Immunolabeled slices were mounted using glycerol/propyl gallate and scanned with a resolution of 42 μ m using the Odyssey imaging system (LI-COR). To study the cellular expression of GABA_A receptor subunits, the brains were fixed in 4% paraformaldehyde for 30 min, and maintained in 30% sucrose before sectioning on a cryostat at 30 μ m before staining. The rabbit anti- $\alpha 1$ antibody (1:100; Abcam, #ab33299), the mouse anti- $\beta 2/3$ (1:200; Millipore, #MAB341) antibody, and the nuclei staining reagent To-pro-3 (1:500) were used²⁰. The images were acquired under confocal microscopy with objective of 63X and analyzed by ImageJ.

Western blot

Brains were removed from CO₂-euthanized adult mice followed by dissection out of the cortices and thalami. Cortical and thalamic samples were collected in RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with 1% protease inhibitor (Sigma) and homogenized by sonication. Collected samples were subjected to gel electrophoresis using NuPAGE® (Invitrogen) precast gel and then transferred to PVDF-FL membranes (Millipore). Monoclonal anti-HA antibody (mouse; 1:1000; Covance, # MMS-101P; or rabbit; 1:1000, Cell Signaling, #3724) and polyclonal anti- γ 2 antibodies (rabbit, 1:500, Millipore, AB5559) were used to detect the HA-tagged and untagged γ 2 subunits, respectively. Anti-sodium potassium ATPase antibody (Abcam) was used as a loading control. After incubation with primary antibodies, IRDye® (LI-COR Biosciences) conjugated secondary antibody was used, and the signals were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences). The integrated intensity value of each specific band was calculated using the Odyssey 3.0 software (LI-COR Biosciences).

PTZ seizure threshold

Male adult mice (2 – 4 month old) received intraperitoneal injection of 55 mg/kg of PTZ, and then were video-monitored for 20 min. The latency to generalized tonic clonic seizures and death were recorded.

Whole cell slice recording

Brain slices were prepared according to published methods^{21; 22}. Briefly, adult mice (2–6 month old, either gender) were anesthetized with isoflurane and perfused transcardially with ice-cooled dissection solution (4 °C) (mM: 2.5 KCl, 0.5 CaCl₂, 10 MgSO₄, 1.25 NaH₂PO₄, 24 NaHCO₃, 11 Glucose, 214 Sucrose). Mice were then decapitated, and their brains were removed. Coronal slices (300 μ m) were prepared using a LEICA VT-1200S vibratome (Leica Inc) with oxygenated (bubbling with 95% O₂/5% CO₂) dissection solution. The slices were transferred to an incubation chamber containing oxygenated ACSF (mM: 126 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgCl₂, 26 NaHCO₃, 10 Glucose, pH 7.4). After a 40 min incubation at 35 – 36 °C, the slices were allowed to recover at room temperature for at least 1 hour before experiments.

Slices were viewed with an upright Eclipse FN-1IR-DIC microscope (Nikon) and recordings were obtained and digitized out using a MultiClamp 700B amplifier and Digidata 1440A (Molecular devices Inc.), respectively. Whole cell recordings were made from layer VI pyramidal neurons in the somatosensory cortex. The neurons were selected based on their apical dendrites and location above the white matter, and mIPSCs were isolated pharmacologically by including 10–20 μ M NBQX and 1 μ M tetrodotoxin in the ACSF (flow rate: 1–1.5 ml per min). The internal pipette solution for recordings²¹ contained (mM): 135 CsCl, 10 HEPES, 10 EGTA, 5 QX-314, 5 ATP-Mg (290–295 mOsm, pH = 7.3), and filled glass electrodes had 3–5 M Ω resistances. Access resistances during recording were continuously monitored and confirmed to be less than 20–25 M Ω . The access resistances were compensated by 70% and cell capacitances were also compensated. Recordings with access resistance variations >20% or magnitudes larger than 25 M Ω were discarded. Junction potentials were compensated when electrodes were in ACSF. The chloride ion

reversal potential was calculated to be close to 0 mV, and cells were clamped at -60 mV. Data were collected using the Clampex program 10.2 (Molecular devices Inc.), and synaptic currents were filtered at 2 kHz and digitized at 10 KHz. All recordings were performed at room temperature (24°C) and were made continuously for 20–30 min after membrane rupture.

Thalamocortical oscillations

Horizontal slices ($350\text{--}400\text{ }\mu\text{m}$) containing the thalamocortical circuitry were prepared²³ and placed on top of a self-made nylon mesh interface (only one side of the slice contacted the ACSF solution). The network oscillations were recorded using one tungsten electrode (MultiClamp 700B, current-clamp mode) in the ventrobasal nucleus (VBn) of the thalamus and were evoked using one concentric bipolar stimulating electrode in the internal capsule. The multiunit recordings were band-filtered (between 100 Hz and 3 kHz)²⁴. Each stimulus lasted for 0.1 or 0.3 ms. The experiments were performed at $31\text{--}32^{\circ}\text{C}$.

Data analysis

The whole cell slice recordings were analyzed with Clampfit (Molecular Devices Inc.) using threshold detection (at least 2.5x baseline RMS with no clear synaptic events)^{22; 24}. Histogram and cumulative graphs were constructed. The network oscillation data were analyzed with both Clampfit (for spike histogram and autocorrelation function) and Matlab to obtain autocorrelograms. Oscillation indices were calculated to compare different groups (both littermates and congenic mice were used)^{23; 25}. Numerical data were reported as mean $\pm$ S.E for all experiments, and statistical differences were determined by the pair wise Student's t-test/One-Way ANOVA or by the Mantel-Cox method.

Results

Reduced total levels of wild-type $\gamma 2$ subunits were restored in *Gabrg2*^{+/^{Q390X}} KI mice by introducing the human *GABRG2*^{HA} BAC transgene

Previously we generated BAC transgenic mice expressing HA-tagged human $\gamma 2$ subunits under the control of the endogenous *hGABRG2* promoter (*Tg(hGABRG2*^{HA}*)* mice) that express $\gamma 2^{\text{HA}}$ subunits in neurons¹⁹. The human $\gamma 2^{\text{HA}}$ subunits were expressed in mouse brain in a pattern that was similar to that of endogenous $\gamma 2$ subunits, and the behavior of transgenic mice was indistinguishable from their wild-type littermates. To introduce the transgene to the *Gabrg2*^{+/^{Q390X}} mice that carried the Dravet syndrome and GEFS+-associated Q390X mutation¹⁷, we crossed the heterozygous KI mice with the transgenic mice. Offspring with four different genotypes were generated: wt;0 (*Gabrg2*^{+/⁺}), het;0 (*Gabrg2*^{+/^{Q390X}}), wt;Tg (*Gabrg2*^{+/⁺};Tg(*hGABRG2*^{HA})), and het;Tg (*Gabrg2*^{+/^{Q390X}};Tg(*hGABRG2*^{HA})) (Figure 1A). Similar numbers of mice from each genotype were born, consistent with Mendelian inheritance (data not shown). Although a reduced birthrate of heterozygous knock-in mice was reported in a mixed C57BL/6J/129Svj background, this was not found in the pure C57BL/6J background¹⁷, suggesting different susceptibility of different strains. Overexpressing wild-type $\gamma 2$ subunits did not affect the birth rate of any of the mice with different genotypes, and thus expression of the transgene could be a potential experimental method for treatment of epilepsy.

We collected brain sections (50 μ m) from wt;0 and het;Tg littermate mice and stained the transgenic human $\gamma 2^{\text{HA}}$ subunits using an anti-HA antibody. As reported in the transgenic mice, exogenous human $\gamma 2^{\text{HA}}$ subunits were expressed across the whole brain of het;Tg mice, including cortex, hippocampus, thalamus, and cerebellum (Figure 1B). To determine whether the total amount of wild-type $\gamma 2$ subunits was up-regulated by the transgene, we prepared cortical lysates from adult mice and performed western blotting for full length $\gamma 2$ subunits using an antibody recognizing the M3-M4 loop of both human and mouse $\gamma 2$ subunits but not recognizing truncated mutant $\gamma 2(\text{Q390X})$ subunits (Figure 1C1). While the total amount of wild-type $\gamma 2$ subunits was reduced in the cortex of het;0 mice (0.68 ± 0.04 , $n = 7$ for each genotype, $p = 0.0001$, two-tailed t test), it was restored to control levels in het;Tg mice (1.27 ± 0.18 , $n = 7$ for each genotype, $p = 0.01$, two-tailed t test) (Figure 1C2, left). A similar restoration was also observed in thalamic lysates (het;0: 0.68 ± 0.04 , het;Tg: 0.96 ± 0.06 , $n = 4$ for each genotype) (wt;0 vs. het;0, $p = 0.0019$; het;0 vs. het;Tg, $p = 0.0262$) (Figure 1C2, right). Thus, the total amount of wild-type $\gamma 2$ subunits in the somatosensory cortex and thalamus, which were reduced in the het;0 mice, were restored to the level in wt;0 mice by introducing the transgene.

The reduction of partnering subunits in the somatosensory cortex of *Gabrg2^{+/Q390X}* KI mice was reversed by introducing the human *GABRG2^{HA}* BAC transgene

Mutant $\gamma 2(\text{Q390X})$ subunits severely affected the trafficking of partnering α and β subunits¹⁵ *in vitro* and reduced $\gamma 2$ subunit puncta in somatosensory cortex of *Gabrg2^{+/Q390X}* KI mice¹⁸. Here we further compared the expression pattern of partnering $\alpha 1$ and $\beta 2/3$ subunits *ex vivo* among wt;0, het;0, and het;Tg littermates by immunohistochemistry (Figure 2A). We found that $\alpha 1$ and $\beta 2/3$ subunit expression was greatly reduced in the somatosensory cortex of KI mice reflecting the dominant negative effects of mutant $\gamma 2(\text{Q390X})$ subunits. The mean raw fluorescence value of $\alpha 1$ subunits in wild-type mice was 58.8 ± 9.2 while the mean value of $\alpha 1$ subunits in mutant mice was 31 ± 9.63 ($p = 0.021$ het;0 vs wt;0, Figure 2B). Similarly, the mean raw fluorescence value of $\beta 2/3$ subunits in the wild-type mice was 61 ± 6.5 while the mean value of the $\beta 2/3$ subunits in the mutant mice was 35.3 ± 4.3 ($p = 0.003$ het;0 vs wt;0, Figure 2B) ($n = 12$ different brain sections from 3 different mice for each genotype). In the presence of the transgene, the levels of $\alpha 1$ and $\beta 2/3$ subunits were increased in the somatosensory cortex (50.6 ± 8.4 ($p = 0.075$ het; Tg vs het;0) for $\alpha 1$ subunits and 49.0 ± 3.1 for $\beta 2/3$ subunits ($p = 0.017$ het;Tg vs het;0, Figure 2B), suggesting an alleviation of the dominant negative effects imposed by the mutant γ subunits.

Reduced GABAergic synaptic transmission in *Gabrg2^{+/Q390X}* KI mice was partially rescued by overexpression of wild-type $\gamma 2$ subunits

Mutant $\gamma 2(\text{Q390X})$ subunits were stable, but were trapped in the ER with partnering subunits, abolishing receptor function. We wondered whether the overexpressed wild-type $\gamma 2$ subunits would alleviate or aggravate the defects. To study whether the wild-type transgene could restore the neuronal inhibition mediated by functional GABA_A receptors, we characterized inhibitory synaptic transmission in the thalamocortical circuitry, which is involved in the pathogenesis of generalized epilepsy^{26; 27}. GABA_A receptor-mediated mIPSCs were recorded from pyramidal neurons in cortical layer VI (Figure 3A, see methods). All mIPSCs recorded from slices from wild-type littermates exhibited fast rising

and slow decaying phases, which were similar to previous reports²⁸ Compared to those from wt;0 mice (39.65 ± 2.69 pA, 4.96 ± 0.58 Hz, $n = 11$ cells), mIPSCs recorded in het;0 mice had significantly smaller amplitudes (31.02 ± 1.51 pA, $n = 7$ cells, t-test $p = 0.018$) and lower frequencies (2.45 ± 0.34 Hz, $n = 7$ cells, t-test; $p = 0.003$) (Figure 3B, C), showing impaired GABAergic neurotransmission. With het;Tg mice expressing exogenous wild-type $\gamma 2^{\text{HA}}$ subunits, layer VI pyramidal neurons exhibited mIPSC amplitudes (41.23 ± 3.70 pA, $n = 11$ cells) that were similar to those in wt;0 mice (t-test $p = 0.7$) and were larger than those in het;0 mice (t-test, $p = 0.02$), suggesting increased postsynaptic GABA_A receptors. However, mIPSC frequency recorded from het;Tg mice only showed a trend for recovery (3.59 ± 0.64 Hz, $n = 11$ cells; t-test with het, $p = 0.315$), compared with those in het;0 mice. Together these data suggested that overexpressing wild-type $\gamma 2^{\text{HA}}$ subunits in *Gabrg2^{+/Q390X}* KI mice could fully rescue the reduced mIPSC amplitude, but not the frequency, in cortical neurons. The partial rescue of mIPSC frequency (Figure 3B) and the incomplete restoration of partnering subunits (Figure 2B) suggested that deficits caused by mutant $\gamma 2(\text{Q390X})$ subunits could not be completely reversed by an addition of wild-type subunits, consistent with our previous findings that this mutation is more than loss-of-function¹⁷ (see discussion below).

Reduction of PTZ seizure threshold was reversed by overexpressing wild-type $\gamma 2^{\text{HA}}$ subunits in *Gabrg2^{+/Q390X}* KI mice

To determine whether the restoration of GABAergic neurotransmission through overexpression of wild-type $\gamma 2^{\text{HA}}$ subunits could rescue the seizure phenotype in *Gabrg2^{+/Q390X}* KI mice, we compared PTZ seizure threshold among the four genotypes, wt;0 (*Gabrg2^{+/+}*), het;0 (*Gabrg2^{+/Q390X}*), wt;Tg (*Gabrg2^{+/+}*; Tg(*hGABRG2^{HA}*)), and het;Tg (*Gabrg2^{+/Q390X}*; Tg(*hGABRG2^{HA}*)). At high doses, PTZ induced generalized tonic-clonic seizures (GTCSs) in rodents²⁹, one of the seizure phenotypes observed in *Gabrg2^{+/Q390X}* KI mice. We injected 2–4 month-old mice with 55 mg/kg PTZ i.p. and video monitored the mice after injection to assess development of seizures. Het;0 mice developed seizures soon after injection, showing limb extension, tail-jerks, rearing or jumping, and many of the mice also died during seizures. We plotted the survival curve for PTZ-induced GTCSs (Figure 4A) and death (Figure 4B) among the four genotypes and found that het;0 mice had a lower seizure threshold compared to mice with the other three genotypes. The het;0 mice developed characteristic GTCSs, including clonic jerks and limb extension, and died much sooner and more often than wt;0 littermates. Seizure threshold and PTZ-induced death of het;Tg mice were not different from littermate wt;0 mice (GTCS: wt;0 ($n = 5$) vs. het;0 ($n = 7$): $p = 0.0009$; het;0 ($n = 7$) vs. het;Tg ($n = 9$): $p = 0.0001$; wt;0 ($n = 5$) vs. het;Tg ($n = 9$): $p = 0.7713$. Death: wt;0 ($n = 5$) vs. het;0 ($n = 7$): $p = 0.0034$; het;0 ($n = 7$) vs. het;Tg ($n = 9$): $p = 0.0004$; wt;0 ($n = 5$) vs. het;Tg ($n = 9$): $p = 0.5050$), indicating restored expression of full length $\gamma 2$ subunits by expression of the transgene restored the seizure threshold of *Gabrg2^{+/Q390X}* KI mice to that of wild-type mice.

The intensity of thalamocortical network oscillation was reduced by overexpression of wild-type $\gamma 2^{\text{HA}}$ subunits in *Gabrg2^{+/Q390X}* KI mice

Network oscillation in the thalamocortical circuitry plays a significant role in the pathogenesis of generalized epilepsies and strengthened thalamocortical oscillation is a

hallmark of generalized epilepsy^{21; 23; 27; 30; 31}. Although still controversial, it was suggested that hyperexcited cortical neurons can convert thalamocortical synchronous firing into generalized seizures²⁶. To determine whether the network oscillation or neuronal synchrony in *Gabrg2^{+/Q390X}* KI mice was increased, we obtained extracellular multiunit recordings from the VBN of thalamus. Both spontaneous (Figure 5A1) and evoked (Figure 5A2) neuronal firing were observed, and rhythmic synchronous firing occasionally occurred spontaneously in horizontal slices from het:0 mice. Spikes with different amplitudes (based on spike-sorting) were identified in het:0 and het:Tg mice (not wt:0 mice), indicating involvement of multiple neurons and the presence of network oscillations. We studied the oscillatory characteristics of network activity of wt:0, het:0 and het:Tg mice by studying autocorrelation of neuron firing and comparing oscillatory indices derived from autocorrelograms of both spontaneous (Figure 5B) and evoked oscillations (autocorrelograms not shown). We found that neuronal synchrony/oscillation was infrequently observed in wt:0 mice, and when it occurred as bursts, its duration was short (oscillatory index: 0.49 ± 0.10 ; oscillatory duration: 0.72 ± 0.32 s; $n = 8$ slices). In contrast, we found that neuronal synchrony/oscillation was frequently observed in het:0 mice. The neuronal oscillation index in het:0 mice was larger (0.70 ± 0.07 , $n = 8$ slices, t -test $p = 0.03$) (Figure 5C1), and the duration was longer (2.46 ± 0.98 s, $n = 10$ slices, t -test $p = 0.04$) (Figure 5C2) than in wt:0 mice. With wild-type $\gamma 2^{\text{HA}}$ subunits introduced into KI mice (het:Tg), spontaneous and evoked oscillations were less frequent (oscillation index 0.52 ± 0.02 , $n = 10$ slices) compared with het:0 mice and were very similar to the firing pattern observed in wt:0 mice (Figure 5C1). Meanwhile, the oscillation duration of het:Tg mice was also shorter (0.94 ± 0.26 s, $n = 8$ slices) than in het:0 mice (Figure 5C2), suggesting that introducing wild-type $\gamma 2^{\text{HA}}$ subunits into het:0 mice rescued mutation-enhanced neuronal synchrony and network oscillation, which can lead to the elevation of seizure threshold.

Discussion

Target-specific gene therapy has been explored in a variety of disease models including the genetic neurological disorder Rett syndrome^{32–35}. Although different genes, including those encoding the Kv1.1 potassium channel³⁶ and GABA_A receptor $\alpha 1$ subunit³⁷, have been delivered into brain to inhibit the hyperexcitability and seizure development in several epilepsy models, little has been reported for genetic epilepsies, especially those caused by dominant negative mutations. Here we found that target-specific gene replacement therapy could become a future direction to treat severe genetic epilepsies caused by *GABRG2* and by extension to other ion channel gene mutations.

In this study we report rescue of reduced PTZ seizure threshold and reduced thalamocortical oscillations in a *Gabrg2^{+/Q390X}* KI mouse model of Dravet syndrome/GEFS+ by overexpression of wild-type $\gamma 2^{\text{HA}}$ subunits. The *GABRG2(Q390X)* mutation is a severe epilepsy-associated nonsense mutation with loss-of-function and dominant-negative-effects. *In vitro*, mutant $\gamma 2(Q390X)$ subunits were not only trapped in the endoplasmic reticulum but also prevented the trafficking of wild-type α , β and $\gamma 2$ subunits¹⁵. *In vivo* *Gabrg2^{+/Q390X}* KI mice displayed spontaneous myoclonic jerks and GTCS¹⁷. Compared to heterozygous *Gabrg2^{+/-}* KO mice, KI mice had a larger reduction of wild-type $\gamma 2$ subunits, a larger decrease of mIPSC amplitude/frequency and more frequent and severe seizures^{17; 18}, likely

caused by the dominant negative effects of the mutant subunits. Our study explored the effects of gene-replacement therapy in *Gabrg2^{+/Q390X}* KI mice by overexpressing the wild-type *GABRG2* gene using a BAC transgenic mouse, the Tg(*hGABRG2^{HA}*) mouse. Similar to other transgenic mice expressing β -actin driven $\gamma 2$ subunits³⁸, Tg(*hGABRG2^{HA}*) mice exhibited normal behavior and life span and had a PTZ seizure threshold that was similar to that of wild-type mice. Although the accumulation of mutant $\gamma 2$ (Q390X) subunits was toxic, the supplementation of wild-type $\gamma 2$ subunits did not seem to be. We found complete recovery of total $\gamma 2$ subunit level and mIPSC amplitude, suggesting a restoration of postsynaptic GABA_A receptors. However, we found that the mutation-induced decrease of partnering α and β subunits as well as mIPSC frequency in the somatosensory cortex was only partially reversed. GABA_A receptors play an important role in establishing inhibitory synapses during development, and loss of postsynaptic GABA_A receptors can affect the presynaptic innervation³⁹. The incomplete recovery of mIPSC frequency and partnering subunits suggested that synaptic deficits might not be fully rescued, which could be caused by the dominant negative effects of mutant $\gamma 2$ (Q390X) subunits, especially during early development. However, the PTZ seizure threshold was still restored to control levels. While we did not compare the severity of spontaneous seizures, which is a more subtle phenotype and highly affected by the genetic background of mice, we found that the intensities of both spontaneous and evoked thalamocortical oscillations, which are hallmarks of generalized epilepsy, was greatly reduced by the transgene. The presence of extra wild-type $\gamma 2$ subunits could not only complement the loss of function of the *hGABRG2* allele, but may also compete with the mutant $\gamma 2$ (Q390X) subunits to reduce the dominant negative effects. As Q390X is the most detrimental epilepsy-associated mutation identified in *GABRG2*, showing not only loss of function but also dominant negative effects, the same strategy is speculated to also rescue other genetic epilepsies caused by *GABRG2* mutations.

In this study, however, we have only provided a proof-of-principle. It would be important to determine whether the severity of genetic epilepsy can still be reduced through gene therapy after the epilepsy onset and interesting to test whether gene therapy could be applied in non-genetic epilepsy models. In the future, gene delivery strategies using AAV or lentivirus and small chemical or genetic molecules regulating the gene expression of $\gamma 2$ subunits will have more promising clinical application. Here we introduced exogenous wild-type $\gamma 2$ subunits from the beginning of gastrulation, but it would be important to study whether there is an important time window for therapeutic intervention as patients cannot be treated until being diagnosed with epilepsy. In addition, in this study we also overexpressed the transgene across the whole brain. Normal thalamocortical oscillation such as sleep spindles have been suggested to be hijacked in generalized epilepsies²⁷ and optogenetic inhibition of VBn neurons in thalamus has been shown to inhibit thalamocortical seizures induced by stroke⁴⁰. Thus it would be interesting to investigate whether overexpressing wild-type $\gamma 2$ subunits in a specific brain region is sufficient to attenuate seizures.

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Key Points

- The Dravet syndrome-associated *GABRG2(Q390X)* mutation affected trafficking of GABA_A receptor subunits and caused epilepsy in both humans and mice.
- Gene-replacement therapy by expressing wild-type *GABRG2* in mice carrying the heterozygous *Gabrg2(Q390X)* mutation increased the wild-type $\gamma 2$ subunit level.
- Restoration of wild-type $\gamma 2$ subunit expression increased partnering subunit levels and inhibitory neurotransmission in somatosensory cortex
- Restoration of wild-type $\gamma 2$ subunit expression was sufficient to decrease the seizure threshold.
- Restoration of wild-type $\gamma 2$ subunit expression was sufficient to decrease thalamocortical oscillations.

Figure 1. Exogenous $\gamma 2^{\text{HA}}$ subunits were introduced in *Gabrg2*^{+/Q390X} KI mice by crossing them with *Tg(hGABRG2*^{HA}*)* mice, and the total amount of full length $\gamma 2$ subunits in KI mice was restored by the transgene

A1. The schematic diagram shows the breeding strategy. Heterozygous *Gabrg2*^{+/Q390X} KI mice were crossed with heterozygous *Tg(hGABRG2*^{HA}*)* BAC transgenic mice, generating offspring with four different genotypes: wt;0 denotes *Gabrg2*^{+/+} mice; het;0 denotes *Gabrg2*^{+/Q390X} mice; wt;Tg denotes *Gabrg2*^{+/+}; *Tg(hGABRG2*^{HA}*)* mice; and het;Tg denotes *Gabrg2*^{+/Q390X}; *Tg(hGABRG2*^{HA}*)* mice. **A2.** PCR and gel electrophoresis were used for genotyping, and the gel presents results from littermates with the four different genotypes. Primers amplifying the endogenous *Gabrg2* allele generated one 323 bp band for the wild-type allele and one 405 bp band for the mutant allele. Primers amplifying the transgenic *hGABRG2*^{HA} allele generated one specific band of 324 bp for the transgene. **B.** Coronal brain sections from adult wt;0 and het;Tg mice were stained by an anti-HA antibody. **C1.** Cortex was collected from adult wt;0, het;0, wt;Tg, and het;Tg mice and blotted by anti-ATPase, anti-HA and anti- $\gamma 2$ subunit antibodies. The anti- $\gamma 2$ subunit antibody only recognized the full length $\gamma 2$ subunits (including endogenous $\gamma 2$ and transgenic $\gamma 2^{\text{HA}}$

subunits). **C2.** Expression levels of wild-type $\gamma 2$ subunits in cortex and thalamus from adult wt;0, het;0, wt;Tg, and het;Tg mice were plotted. The band intensity of $\gamma 2$ subunits was normalized to that of ATPase and was further normalized to that of wt;0 littermates ($n = 7$ for each genotype for cortical samples and $n = 4$ for each genotype for thalamic samples, mean $\pm$ SEM). Differences among littermates were analyzed by one way ANOVA followed by two-tailed paired t test (** $p < 0.001$; * $p < 0.01$; * $p < 0.05$).

Figure 2. The expression levels of partnering $\alpha 1$ and $\beta 2/3$ subunits were restored in the somatosensory cortex by the hGABRG2^{HA} transgene

A. Immunostaining of $\alpha 1$ and $\beta 2/3$ subunits in somatosensory cortex of wt;0, het;0 and het;Tg mice was performed. Brains from 2 month old wt;0, het;0, and het;Tg mouse littermates were fixed and sectioned on a cryostat at 30 μ m. The brain cortices were stained with anti- $\alpha 1$ or anti- $\beta 2/3$ subunit antibody, and cortical layer VI was visualized. The nuclei were stained with TO-PRO-3 (blue). Images were acquired under confocal microscopy. **B.** Expression levels from 12 sections from 3 different mice for each genotype were plotted. The total raw fluorescence values of the $\alpha 1$ or $\beta 2/3$ subunits in somatosensory cortex were quantified by ImageJ. (* $p < 0.05$; ** $p < 0.01$ vs wt, * $p < 0.05$ vs het), unpaired t test).

Figure 3. Cortical mIPSCs were restored by the hGABRG2^{HA} transgene

A. Representative mIPSC traces from layer 6 cortical pyramidal neurons were obtained from wt;0, het;0 and het;Tg mice. The top traces represented 50 continuous overlapped sweeps. The lower traces represented a single 5 s trace that has representative mIPSCs time-expanded to show rising and decaying current phases. Scale bars were indicated as labeled. **B.** The averaged mIPSC amplitudes (left panel) and the averaged mIPSC frequencies (right panel) recorded from slices from wt;0 (black, $n = 11$ cells), het;0 (red, $n = 7$ cells) and het;Tg mice (green, $n = 11$ cells) mice (* $p < 0.05$; t test) were plotted. **C.** Normalized

cumulative curves of mIPSC amplitude (left) and event interval (right) recorded from slices from wt;0 (black), het;0 (red) and het;Tg (green) mice were plotted. The mIPSCs were recorded from the same number of cells ($n = 7$, randomly chosen) and for the same duration of recording (10 min).

Figure 4. Increased PTZ seizure sensitivity was reversed by the hGABRG2^{HA} transgene
A, B. Mice were injected i.p. with PTZ (55 mg/kg) to induce seizures. The susceptibility to (A) PTZ-induced GTCS and (B) death were assessed by survival curves. Differences between littermates were analyzed by the Mantel-Cox method. (A. wt;0 vs. het;0: $p = 0.0009$; het;0 vs. het;Tg: $p = 0.0001$; wt;0 vs. het;Tg: $p = 0.7713$. B. wt;0 vs. het;0: $p = 0.0034$; het;0 vs. het;Tg: $p = 0.0004$; wt;0 vs. het;Tg: $p = 0.5050$).

Figure 5. Spontaneous and evoked thalamocortical oscillations were less intense in het;Tg mice than in het;0 mice

A. Representative extracellular multiple unit recordings (A1, spontaneous and A2, evoked) from slices from wt;0, het;0 and het;Tg mice were presented. One burst was expanded to show the multiple spikes in the burst. Scale bars were indicated as labeled. **B.**

Autocorrelograms generated from spontaneous (B) and evoked (not shown) burst activity calculated using Clampfit. **C.** Summaries of averaged oscillatory index (C1) from both spontaneous and evoked burst activity for wt;0 (n = 8 slices), het;0 (n = 8 slices) and het;Tg

mice ($n = 11$ slices) and averaged duration of oscillatory activity (C2) were presented. (* $p < 0.05$, t-test).

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