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Developmental Impact of a Familial GABA_A Receptor Epilepsy Mutation

Cindy Chiu, PhD¹, Christopher A. Reid, PhD¹, Heneu O. Tan, PhD¹, Philip J. Davies, PhD¹, Frank N. Single, PhD², Irene Koukoulas, PhD¹, Samuel F. Berkovic, MD, FRS³, Seong-Seng Tan, DPhil, MDS¹, Rolf Sprengel, PhD², Mathew V. Jones, PhD⁴, and Steven Petrou, PhD¹

¹Howard Florey Institute, the University of Melbourne, Parkville, Melbourne, Australia

²Molecular Neuroscience, Max Planck Institute for Medical Research, Heidelberg, Germany

³Department of Medicine, Austin Health, the University of Melbourne, Heidelberg West, Melbourne, Australia

⁴Department of Physiology, University of Wisconsin, Madison, WI

Abstract

Objective—A major goal of epilepsy research is to understand the molecular and functional basis of seizure genesis. A human GABA_A $\gamma 2$ gene mutation (R43Q) is associated with generalized epilepsy. Introduction of this mutation into a mouse by gene targeting recapitulates the human phenotype demonstrating a strong genotype to phenotype link. GABA_A receptors play a role in the moment-to-moment control of brain function and also on the long-term wiring of the brain by directing neuronal development. Our objective was to determine whether developmental expression of the mutation alters seizure susceptibility later in life.

Methods—A tetracycline-based conditional model for activation of a hypomorphic Q43 disease allele was created and validated. Seizure susceptibility was assessed using the subcutaneous pentylenetetrazole model.

Results—Seizure susceptibility was significantly reduced in mice where the Q43 allele was suppressed during development.

Interpretation—These results demonstrate that a human epilepsy-causing mutation impacts network stability during a critical developmental period. These data suggest that identification of presymptomatic children may provide a window for therapeutic intervention before overt symptoms are observed, potentially altering the course of epileptogenesis.

Epilepsy, with a lifetime prevalence rate of 3%, is a common and serious neurological disorder. It is characterized by recurrent paroxysms resulting from hypersynchronous discharges in the brain. Mutations in more than a dozen ion channel genes have been associated with familial epilepsy syndromes providing a strong foundation on which to build an understanding of epileptogenesis. Ion channels have an acute role setting the real-time excitability of neurons and networks, and a developmental role in the coupling of neurons in networks. When mutations occur, they may influence either role to varying degrees to cause the clinical phenotype. Distinguishing acute from developmental roles has important

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Address correspondence to Dr Petrou, Howard Florey Institute, The University of Melbourne, Parkville, Victoria 3010, Australia. spetrou@unimelb.edu.au.

C.C. and C.A.R. contributed equally to this work.

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therapeutic implications. Reversing the consequences of developmental dysfunction may not be a simple matter of compensating for the acute receptor deficit because the developmental consequences may be inherent in neural networks of patients with epilepsy.

There is no evidence that familial disease causing ion channel mutations impacts neural development. Mouse models harboring human epilepsy-causing mutations that recapitulate aspects of the human disease are particularly valuable models to test the link between gene mutation and seizures. Manipulation of the temporal expression of a single mutation may provide a means to resolving the impact of a mutation on the developing and adult brain. To this end, we engineered a mouse model in which we can manipulate the temporal expression of a GABA_A $\gamma 2$ mutation, previously reported in a large Australian family with febrile seizures and generalized epilepsy.¹

GABA_A receptors are ligand-gated chloride channels and arbiters of fast inhibitory neurotransmission in the adult brain. Perturbation of fast inhibition has been linked to seizure genesis. During brain development, GABA_A receptors also play an important role in regulating neuronal differentiation, proliferation, and synaptogenesis² that can also influence seizure genesis.

Here, we generate and characterize a conditional mouse model that allows a forebrain-specific switch of a human epilepsy GABA_A $\gamma 2$ (R43Q) mutation at specific times during development. Using this mouse, we demonstrate that expression of GABA_A $\gamma 2$ (R43Q) in the developing brain increases seizure susceptibility in adulthood.

Materials and Methods

Animals

All experiments were approved by the Animal Ethics Committee at the Howard Florey Institute (04-102). Tg ^{α} -*CaMKII-tTA/LC1*/*Gabrg-2^{R/Q}Neo* mice in the C57BL/6 background were generated by crossing Tg ^{α} -*CaMKII-tTA* mice³ with Tg^{*LC1*} mice⁴ and the gene-targeted mouse line *Gabrg-2^{R/Q}Neo*. *Gabrg-2^{R/Q}* mice were generated by crossing *Gabrg-2^{R/Q}Neo* to a *Cre*deletor strain.⁵ Transgenic Tg^{*ZEG*} mice⁶ were used as a *Cre*-reporter strain. Triple-transgenic mice, Tg ^{α} -*CaMKII-tTA/LC1/ZEG* were generated by crossing Tg ^{α} -*CaMKII-tTA* with Tg^{*LC1*} and Tg^{*ZEG*}. Animals were toe-clipped at postnatal days 8 (P8) to P12 for genotyping. The Table summarizes the genotypes/alleles used in this study and gives a brief description of each.

Conditional Expression

The Tg ^{α} -*CaMKII-tTA/LC1* line was used to regulate *Cre*-recombinase (*Cre*) expression using doxycycline (Dox). This line expresses the tetracycline-controlled transcriptional activator (tTA) under the control of the α -calmodulin-dependent protein kinase II (α -*CaMKII*) promoter. In the absence of Dox, tTA binds to the tetracycline-responsive element in the Tg^{*LC1*} line and activates the bidirectional expression of luciferase and *Cre*. Dox binds tTA, preventing it from interacting with the tetracycline-responsive element, thus inhibiting subsequent transcription of *Cre*.⁴ Doxycycline Hyclate (Sigma-Aldrich, Castle Hill, Australia) was fed to the parental mice from conception via drinking water at a concentration of 50mg/L supplemented with 1% sucrose as described elsewhere.⁷

Genotyping

With the exception of the Tg^{*ZEG*} mouse line, mice were genotyped using toe DNA with the following different primer sets (polymerase chain reaction [PCR] product sizes are given in brackets): Tg ^{α} -*CaMKII-tTA*: forward primer tTA1 (5'-GTG ATT AAC AGC GCA TTA GAG

C-3') and reverse primer tTA3 (5'-CGC CGT CTA AGT GGA GCT CGT CC-3'), (800bp); Tg^{LCL}: forward primer luc1 (5'-CTT TTA CAG ATG CAC ATA TCG AGG-3') and reverse primer luc2 (5'-TAG GTA ACC CAG TAG ATC CAG AGG-3'), (400bp); *Gabrg-2^{R/Neo}*: forward primer CC21 (5'-CAC TGT CAT CTT AAA CAA CCT GCT GGA A-3') and reverse primer PGKprom2 (5'-CAG ACT GCC TTG GGA AAA GCG-3'), (364bp); *Gabrg-2^{R/Q}/Gabrg-2^{R/R}*: forward primer SP031 (5'-GTA GAA GCC AAC TCA GGA GTC ATC-3') and reverse primer SP032 (5'-CCC TGT CTG TCC TAG AAC TCA CTC-3') (475bp and 509bp corresponding to the *Gabrg-2^R* and *Gabrg-2^Q* alleles, respectively). PCR was performed using the following conditions: 96°C for 3 minutes, 35 cycles of 96°C for 20 seconds, 57°C for 30 seconds, and 72°C for 50 seconds; then a final extension at 72°C for 10 minutes using a Biometra TGradient Thermocycler (Biometra, Goettingen, Germany). Tg^{ZEG} mice were genotyped by staining ear clippings from P15 to P21 animals with a LacZ stain using standard protocols as described elsewhere.⁸

Histology

Mice were killed via intraperitoneal injection of 100mg/kg sodium pentobarbital (Nembutal, Merial, Australia), then injected with heparin sodium salt in saline (100U, intraperitoneally; Sigma). This was followed by transcardial perfusion of 20ml ice-cold 0.9% NaCl, then 20ml 4% formaldehyde in 0.1M phosphate buffer. Brains were removed and postfixed in 4% formaldehyde for a further hour, washed in phosphate-buffered saline, then cryoprotected in 30% sucrose at 4°C for 24 to 48 hours. Brains were placed in a 1:1 mix of 30% sucrose and Optimal Cutting Temperature Compound (Tissue Tek; Sakura, Torrance, CA) at 4°C 24 hours before cryosectioning. Brains were mounted in OCT, and 16µm coronal sections were collected at two depths (bregma -1.70 and 0.80mm) onto SuperFrost Plus electrostatic slides (BDH Laboratory Supplies, Toronto, Ontario, Canada). Sections were dried overnight in the dark, then mounted with Cytoseal mounting solution (Fischer Scientific, Hampton, USA). Images were captured using a conventional upright confocal microscope with a standard fluorescein isothiocyanate filter, 30% laser power and a 10× objective. All cell counts were performed manually using ImageJ 1.36b (National Institutes of Health, Bethesda, MD).

Quantitative Polymerase Chain Reaction

Total RNA was extracted from whole brains (RNeasy Lipid kit; Qiagen, Chatsworth, CA) and reverse transcribed into complementary DNA (cDNA; TaqMan Reverse Transcription Kit; Applied Biosystems, Foster City, CA). TaqMan probes complementary to the genomic region containing the mutation were designed for wild-type (5' FAM—TAT GAC AAC AAA CTT CGA CCT GAC ATC GG—TAMRA 3') and mutant (5' VIC—TAC GAC AAC AAG CTT CAG CCT GAC ATA GG—TAMRA 3') alleles with differences at the R43Q site and four adjacent silent mutations engineered into the original mouse line. Nonmultiplex reactions were performed with 5ng cDNA, 1× TaqMan Universal Mastermix, 900nM of forward CC11 (5'—GTC ATC TTA AAC AAC CGC TGG AA—3') and reverse CC 12 (5'—CCA ATG CTG TTC ACA TAC ATA TCT GT—3') primers, and either 200nM of wild-type or mutant probes in 25µl reactions. The reactions were performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) at 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 62°C for 1 minute. Specificity of the probes to their corresponding alleles was demonstrated by performing reactions using wild-type cDNA and homozygous cDNA with the mutant and wild-type TaqMan probes, respectively. Quantitative polymerase chain reaction (qPCR) was also performed for the endogenous control 18S ribosomal RNA on the same plate as the reactions described earlier, where 1× Platinum SYBR Green qPCR Supermix (Invitrogen, La Jolla, CA) was used with the ribosomal 18S forward (5'—CGG CTA CCA CAT CCA AGG AA—3') and reverse (5'—GCT GGA ATT ACC GCG GCT—3') primers. Relative gene expression was quantified using standard curves. mRNA levels were normalized to the endogenous 18s ribosomal

RNA. These normalized values were then averaged for each treatment group and relative gene expression calculated. The group with the highest expression was normalized to 100%, and other groups were scaled to this for comparison.

Pentylenetetrazol Seizure Testing

Mice between the ages of P63 and P70 were injected subcutaneously with pentylenetetrazol (PTZ; 85mg.kg⁻¹; Sigma) and video monitored for a maximum of 1 hour. Latencies to minimal (clonic) and maximal (tonic hindlimb extension) seizures were recorded and tested for significance using Kaplan–Meier survival analysis (GraphPad Prism 4.01) using the Gehan–Breslow test. In all the survival plots, animals that did not reach the end point by 60 minutes were censored. Multiple-comparison correction was applied using Keppel's modified Bonferroni correction of the α value.

Electroencephalographic Recordings

P19 to P21 mice were anesthetized with 1 to 3% isoflurane and implanted with chronic epidural electroencephalographic electrodes. Four silver electrodes were implanted on each quadrant of the skull, and a ground electrode was placed just posterior to the olfactory bulb. Mice were allowed to recover for at least 24 hours. Signals were band-pass filtered at 0.1 to 200Hz and sampled at 1KHz using Powerlab 16/30 data acquisition system (ADInstruments, Bella Vista, Australia) with simultaneous synchronized video monitoring (Quicktime capture; ADInstruments, Bella Vista, Australia). Mice were recorded for 4-hour periods over 3 consecutive days during daylight hours. Electroencephalographic traces were screened for 6 to 7Hz spike and wave discharges and corresponding behavioral arrest, characteristic of the absence seizures these mice display. These conditions were sufficient to record spike and wave discharges in the same mice in an earlier study that first described the phenotype of the *Gabrg-2^{R/Q}* knock-in mouse.⁹

Results

Suppression of the Q43 Allele

Intronic retention of loxP flanked Neomycin resistance (Neo) cassettes may suppress allele expression, and thereby provides the basis for a conditional gene regulated model.¹⁰ To verify the expression of the *Gabrg-2^{Qneo}* allele that contains floxed Neo in intron 2 of the *Gabrg-2^Q* allele, we performed qPCR analysis in mice before and after Neo excision (Fig 1). Comparative expression analysis of *Gabrg-2^{R/Qneo}* and *Gabrg-2^{R/Q}* mice demonstrated a significant hypomorphic effect of Neo within the *Gabrg-2^Q* gene locus (see Fig 1A). *Gabrg-2^Q* (Q43) allele expression was reduced by 91% in the *Gabrg-2^{R/Qneo}* mice relative to Q43 allele expression in *Gabrg-2^{R/Q}* at P15 and by 76% at P66 (see Fig 1B). This establishes the basis of an “off” state for the Q43 mutation that can be switched “on” by Neo removal using temporally and spatially restricted expression of *Cre*.⁷

Reporter Mice Demonstrate Spatial and Cell-Type Extent of Cre Excision

For in vivo activation of the hypomorphic *Gabrg-2^{Qneo}* allele, we chose tetracycline-controlled, *Cre*-mediated Neo removal.⁷ We first profiled the temporal and spatial characteristics of *Cre* expression using the Tg^{Z/EG} reporter mouse.⁶ Forebrain specific activation can be achieved by transgenes from the Tg ^{α} -*CaMKII-tTA/LC1* mouse (Fig 2A) as demonstrated in Tg^{Z/EG} *Cre*-reporter mice (see Fig 2B).⁶ Coronal brain slices of the triple-transgenic Tg ^{α} -*CaMKII-tTA/LC1/Z/EG* mice showed enhanced green fluorescent protein (EGFP) expression in several regions of the forebrain including hippocampus, cortex, striatum, piriform cortex, cingulate cortices, thalamus (ventrobasal thalamus only), and amygdale (see Fig 2C). Examination of brain regions known to almost exclusively harbor

inhibitory interneurons including the thalamic reticular nucleus and the molecular layer of CA1 were devoid of EGFP-positive cells, presumably as a consequence of lack of α -*CaMKII* promoter activation (see Fig 2D).

In *Tg α -CaMKII-*tTA/LC1*/Gabrg-2^{R/Qneo}* mice, the qPCR analysis of forebrain showed that the Q43 transcript levels are similar to that of *Gabrg-2^{R/Q}* mice, indicating efficient Neo removal of the *Gabrg-2^{Qneo}* allele (Q43 on always; see Fig 2E) at postnatal day 66. Although there was no significant difference between these groups, there was a trend for the α -*CaMKII*-driven Q43 allele group to be lower, as expected given the lack of inhibitory neuron expression (see Fig 2D). This result confirms that in vivo conversion of the *Gabrg-2^{Qneo}* back to *Gabrg-2^{R/Q}* by *Cre*-mediated Neo excision is accompanied by a full restoration of *Gabrg-2^{R/Q}* levels.

Conditional Activation of Q43neo Allele Maintains Subcutaneous Pentylentetrazol Susceptibility

Having established the extent of activation to be expected in our conditional mouse model, it was necessary to compare the phenotypic consequences of this restricted pattern of Q43 activation with heterozygous mutant mice. *Gabrg-2^{R/Q}* mice display a heightened sensitivity to subcutaneous pentylentetrazol (scPTZ), providing a useful measure of seizure susceptibility in animals with R43Q mutation⁹ and, potentially, the effects of the conditional activation of the Q43neo allele. Mice were injected with scPTZ (85mg.kg⁻¹) and monitored for two clearly defined end points: (1) time to first clonic seizure, and (2) time to first tonic hindlimb extension. *Gabrg-2^{R/Q}* mice showed increased seizure susceptibility compared with *Gabrg-2^{R/R}* (Fig 3) as previously reported.⁹ Importantly, there was no significant difference in scPTZ susceptibility between *Tg α -CaMKII-*tTA/LC1*/Gabrg-2^{R/Qneo}* and *Gabrg-2^{R/Q}* mice (see Fig 3). This demonstrates that expression of Q43 allele in the forebrain is sufficient to produce a scPTZ susceptibility phenotype.

Temporal Regulation of Q43 Expression

Q43 allele activation is triggered by absence or removal of Dox from the drinking water of the conditional mice. There is, however, a lag anticipated with *Cre* activation associated with epigenetic silencing of the Tet operon.¹¹ Profiling of the *Cre* activation in *Tg α -CaMKII-*tTA/LC1*/Z/EG* reporter mice was performed by removal of Dox at P21 followed by comparison of EGFP-positive neurons from these mice to controls that never received Dox and hence expressed *Cre* from conception (Fig 4). The α -*CaMKII* promoter fragment of the *Tg α -CaMKII-*tTA/LC1*/Z/EG* reporter mouse used in this study has a different temporal profile of activation than the full-length promoter that switches on postnatally around day 17.¹² In contrast, the promoter fragment we used switches on embryonically at around E10.^{13–15} Confocal imaging of the hippocampi of these mice showed the temporal profile of *Cre* expression (see Fig 4A). EGFP-positive cells were counted to provide a quantitative assessment of this profile and showed that the first detectable signs of *Cre*-recombinase activation occurred at around 9 days after Dox removal. In CA1, EGFP-positive cell counts from Dox-treated mice approached (84%) those seen in the Dox naive mice (see Fig 4B). The efficiency of reactivation appears to be correlated with the expression level of the Dox-dependent transcription factor tTA, from the time point of activation.^{7,11}

The qPCR analysis of relative expression of Q43 alleles from the *Gabrg-2^{R/Qneo}* mice and the *Tg α -CaMKII-*tTA/LC1*/Gabrg-2^{R/Qneo}* mice kept under Dox from conception until P66 (conditionally suppressed) showed an identical poor activation of Q43 allele expression (see Fig 4C) highlighting the effectiveness of the conditional silencing of the Ptet-Bi-controlled *Cre*-recombinase. qPCR analysis was performed on entire forebrains of *Tg α -CaMKII-*tTA/LC1*/Gabrg-2^{R/Qneo}* mice treated with Dox from conception to P21 (Q43 on at P21) or not treated

(Q43 on always). Comparison of relative Q43 transcript levels from these groups at age P66 demonstrated an almost complete recovery of Q43 allele expression in the Dox-treated group (see Fig 4D). Collectively, these data provide the basis for examining the impact of Q43 allele expression during conception to death versus P21 to death. With this validation in mind, comparison of seizure susceptibility in these two groups will probe the developmental role of the Q43 allele.

Activation of the Hypomorphic Q43 Allele during Development Increases Seizure Susceptibility

Having established the validity of the conditional model, we next investigated the developmental impact of the Q43 mutant allele. Comparison of scPTZ susceptibility in $Tg^{\alpha-CaMKII-tTA/LC1}/Gabrg-2^{R/Qneo}$ mice treated with doxycycline from conception to P21 (Q43 on at P21) or not Dox treated (Q43 on always) demonstrated that reduced Q43 expression during development (conception to P21) significantly increased the time to first clonic seizure (Fig 5A) and to first hindlimb extension (see Fig 5B). Thus, mice that expressed the Q43 mutation throughout life had an increased susceptibility to scPTZ seizures compared with mice that expressed the mutation only after postnatal day 21.

The doxycycline-treated mice are conceived with a hypomorphic allele. The absence of full expression of both wild-type R alleles in the hypomorphic conditional mouse may heighten seizure susceptibility similar to that seen in mice expressing the mutant Q disease allele. To eliminate this possibility as a potential confound for interpretation of the earlier data (see Figs 5A, B), we compared seizure susceptibility of the $Gabrg-2^{R/Qneo}$ mice (hypomorphic) with conditional $Tg^{\alpha-CaMKII-tTA/LC1}/Gabrg-2^{R/Qneo}$ mice where the Q allele had been expressed in early embryonic development (Q43 on always). This comparison showed that the hypomorphic mice had significantly lower seizure susceptibility than mice expressing the Q allele (see Figs 5C, D), suggesting that mutant Q allele expression is a stronger determinant of susceptibility than hypomorphism alone. Furthermore, the increased susceptibility seen in mice with lifelong expression of the Q43 mutation is unlikely to be due to a seizure kindling effect because there were no pathological spike-wave discharges in the electroencephalographic records from either group (see Fig 5E). We also tested whether doxycycline has a protective effect on seizure susceptibility by comparing scPTZ sensitivity in wild-type mice treated with doxycycline or vehicle alone. This comparison showed no significant difference in the time to first clonic seizure ($p = 0.21$) or first tonic hindlimb extension ($p = 0.66$), suggesting that the differences in the conditional mice (see Figs 5A, B) were not due to any protective effect of doxycycline. Collectively, these results support the idea that developmental expression of the human epilepsy R43Q GABA_A receptor mutation is critical to defining adult seizure susceptibility.

Discussion

Conditional gene expression in which the levels of a gene can be temporally or spatially regulated is frequently used to determine gene function.¹⁶ To date, no study has attempted to determine the developmental impact of channel dysfunction caused by familial epilepsy mutation. Recently, we described a novel knock-in mouse model carrying the R43Q mutation in the $\gamma 2$ subunit of the GABA_A receptor. This mouse displays spontaneous 6 to 8 Hz spike-wave discharges associated with behavioral arrest,⁹ recapitulating a major phenotype of patients harboring the mutation.¹ A general hyperexcitable phenotype was also described with enhanced susceptibility to proconvulsant challenges.⁹ The known molecular cause of this phenotype, based on a human mutation, provides a useful framework with which to dissect out potential developmental processes involved in the generation of excitable phenotypes.

Developing methods to manipulate gene expression is critical for investigating the roles of ion channel mutations in disease genesis. Viral-mediated gene transfer and transgenic-mediated conditional expression are two broad strategies that can be used to dissect acute from developmental effects of epilepsy-causing mutations. Viral-mediated gene transfer has several drawbacks, including extent of transfer, cell-type specificity of infection, level of expression, and competition with endogenous alleles. Conditional expression of a mutant allele with the simultaneous deletion of a wild-type allele under strict temporal control may potentially be the ideal solution. A similar but simpler approach, which we use in this study, relies on the ability of the Neo selection cassette to suppress gene expression by virtue of its effect on RNA splicing.^{17,18}

In this study, we created a mouse model in which Q43 disease allele expression is regulated using a tetracycline-controlled conditional system.¹⁹ Reduced expression of the Q43 disease allele during early development (inception to P21) significantly reduced seizure susceptibility. This suggests that mutation-mediated dysfunction in channel activity during development can be a critical determinant of seizure susceptibility in later life. Although we have not unequivocally demonstrated neuron-specific activation, our data suggest that inhibitory neurons may be spared, raising the intriguing possibility that excitatory neuron expression of the Q43 disease allele is sufficient to alter seizure susceptibility.

The association between brain development and epilepsy has long been documented.^{20,21} Until recently there has been little experimental evidence that mutations seen in idiopathic epilepsy are associated with demonstrable changes in brain development. A structural magnetic resonance imaging study of members of the Australian family with the GABA_A $\gamma 2$ (R43Q) mutation provided the first strong evidence for such an association. Family members with the GABA_A $\gamma 2$ (R43Q) mutation presented changes in corpus callosum volume that were not observed in family members without the mutation (S. F. Berkovic, personal communication).

GABA_A receptors play a key role during brain development where they impact neuronal differentiation, proliferation, and synaptogenesis.² Dysfunction in any of these could potentially lead to the development of an excitable phenotype. For example, the migration patterns of interneurons in the developing cortex depend on GABA_A receptor function.² Even subtle differences in interneuron placement could explain hyperexcitability as seen in the uPAR^{-/-} knock-out mice with reduced interneurons migrating their correct region in the cortex.²² Modulating synaptic pathways is also a critical role played by GABA_A receptors in early development.² Toward the final phases of neuronal development when networks are formed, spontaneous neuronal oscillations are thought to mediate the functional and structural maturation of neuronal networks.²³ Giant depolarizing potentials (GDP) are such oscillations seen in the hippocampus and are mediated by the excitatory GABA_A receptor current.^{24,25} GABA_A receptor-mediated depolarization during the giant depolarizing potential results in activation of *N*-methyl-D-aspartate receptors and voltage-gated calcium channels causing calcium influx^{26–28} that is believed to activate signaling pathways to direct the establishment and refinement of network circuitry.²⁹ The abundant expression in most brain regions of the GABA_A $\gamma 2$ subunit at an early time point in development positions it as a key player in the developmental role of GABA_A receptors.³⁰ Changes in giant depolarizing potential caused by the R43Q mutation in the GABA_A $\gamma 2$ subunit could impact network formation and thereby contribute to the excitable phenotype seen in patients with this mutation.

In this study, we have demonstrated that a human epilepsy mutation may influence both neuronal development and the acute function of GABA_A receptors. These findings may have important therapeutic implications; reversing the consequences of developmental

dysfunction may not be a simple matter of compensating for the acute receptor deficit. Currently, no known antiepileptic drugs have shown the capability to prevent or alter the course of epilepsy progression.^{31–33} This may stem from our inability to diagnose “epilepsy” before seizure presentation. Early detection facilitated by genetic testing may provide a window for therapeutic intervention before overt symptoms are observed, potentially altering the course of epileptogenesis.

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Fig 1. Effect of Neomycin resistance (Neo) cassette retention on Q43 allele transcript levels. (A) Schematic depiction of genomic structure of the Gabrg-2 alleles used to generate the conditional mouse line. Qneo = neo-retaining mutant allele; Q = neo-excised mutant allele; R = the wild-type allele. White triangles designate loxP sites; black vertical line designates the position of the R→Q mutation. (B) Normalized messenger RNA expression of the Q43 allele for each of the experimental groups. **p* = 0.004; ***p* = 0.001. *n* values are shown in parentheses above each bar graph.

Fig 2.

Spatial pattern of Cre expression. Schematic depiction of the alleles used to regulate and report Cre expression. (A) Tetracycline-regulated, Cre-expressing mouse line $T_g^{\alpha\text{-CaMKII-tTA/LC1}}$ in which the $\alpha\text{-CaMKII}$ promoter drives the tetracycline-controlled transcription activator (tTA) and subsequently Cre. (B) Schematic depiction of the alleles in the Cre-reporter mice line $T_g^{Z/EG}$. In this line, the floxed βgeo (LacZ/Neo) gene is expressed before Cre excision, whereas enhanced green fluorescent protein (EGFP) is expressed after Cre excision. White triangles designate loxP sites. (C) $T_g^{Z/EG}$ reporter mice crossed to $T_g^{\alpha\text{-CaMKII-tTA/LC1}}$ mice reported sites of Cre expression by EGFP fluorescence. Several brain regions express Cre (examined in $n = 5$ mice). (D) Magnification of the thalamic reticular nucleus (TRN) and the stratum radiatum of the CA1 region shown boxed in (C) highlighting the lack of interneuron expression of Cre. (E) Quantitative polymerase chain reaction (qPCR) analysis of mouse forebrains comparing allele expression. Normalized messenger RNA expression levels of the Q43 allele for the in vivo excised $T_g^{\alpha\text{-CaMKII-tTA/LC1}}/\text{Gabrg-2}^{\text{R/Qneo}}$ mouse (Q43 on always) and the mutant mouse $\text{Gabrg-2}^{\text{R/Q}}$ mouse (RQ) were not significantly different ($p = 0.09$). n values are shown in parentheses above each bar graph. Tg = transgenic; CamkII = calmodulin-dependent protein kinase II; SIBF = primary somatosensory cortex barrel field; pir = piriform cortex; cg = cingulum; RSA = retrosplenial agranular cortex; RSG = retrosplenial granular cortex; cg1-2 = cingulate cortex areas 1 and 2; CPu = caudate putamen; ec = external capsule; VB = ventral basal thalamic nucleus; nRT = reticular thalamic nucleus; ic = internal capsule; opt = optic tract; MePD = medial amygdala posterodorsal nucleus; MePV = medial amygdala

posteroventral nucleus; BMA = basomedial amygdala anterior nucleus; DG = dentate gyrus; CA1 = cornu ammonis 1 region of the hippocampus. Scale bars = 100 μ M.

Fig 3.

Kaplan–Meier survival plots for subcutaneous pentylenetetrazol (scPTZ) elicited seizures in the $Tg^{\alpha-CaMKII-tTA/LC1}/Gabrg-2^{R/Qneo}$, $Gabrg-2^{R/Q}$, and $Gabrg-2^{R/R}$ mice. In vivo activation of the hypomorphic $Gabrg-2^{Qneo}$ allele restores PTZ susceptibility. Survival plots illustrating (A) time to first clonic seizure and (B) time to hindlimb extension. Comparison of survival plots from $Tg^{\alpha-CaMKII-tTA/LC1}/Gabrg-2^{R/Qneo}$ (Q43 on always; n = 9; triangles) and $Gabrg-2^{R/Q}$ (RQ; n = 10; squares) mice showed no significant difference for either time to clonic seizure ($p = 0.41$) or time to first hind-limb extension ($p = 0.12$). Circles represent wild-type mice (n = 12).

Fig 4.

Temporal profile of Cre expression. (A) Hippocampal Cre expression as reported by Tg ^{α} -CaMKII-tTA/LC1/Z/EG mice at different time points after Dox removal. (B) Average enhanced green fluorescent protein (EGFP)-positive cell counts in CA1 region of hippocampus. (C) Quantitative polymerase chain reaction (qPCR) analysis showing percentage messenger RNA (mRNA) expression relative to the Q43 allele for the Gabrg-2^{R/Qneo} (neo retained) and Tg ^{α} -CaMKII-tTA/LC1/Gabrg-2^{R/Qneo} mice (conditionally suppressed). No significant difference was seen between these two groups ($p = 0.897$). (D) qPCR analysis showing normalized mRNA expression of the Q43 allele for the Tg ^{α} -CaMKII-tTA/LC1/Gabrg-2^{R/Qneo} mouse subject to developmental Cre suppression by doxycycline treatment from conception to postnatal day 21 (P21; Q43 on at P21) or in vivo excision by no doxycycline treatment (Q43 on always). No significant difference was observed between these two groups ($p = 0.151$). n values for each experiment are shown in parentheses. CA1 = cornu ammonis 1 field of hippocampus; DG = dentate gyrus. Scale bar = 100 μm .

Fig 5.

Kaplan–Meier survival plots for subcutaneous pentylenetetrazol (scPTZ) elicited seizures in the *Tg α -CaMKII-tTA/LC1/Gabrg-2^R/Q^{neo}* mouse subject to developmental Q43 suppression from conception to postnatal day 21 (P21) or with lifelong Q43 allele expression. Survival plots illustrating (A) time to first clonic seizure and (B) time to hindlimb extension. A significant difference was seen for both the time to first clonic seizure ($p = 0.03$) and time to hindlimb extension ($p = 0.004$). Survival plots comparing hypomorphic mice (RQneo) with conditional mice expressing the Q allele throughout life, (C) time to first clonic seizure, and (D) time to hindlimb extension. A significant difference was seen for both the time to first clonic seizure ($p = 0.02$) and time to hindlimb extension ($p = 0.001$). (E) Comparison of electroencephalographic recordings from P20 mice. Triangles represent Q43 on always ($n = 9$); diamonds represent Q43 on at P21 ($n = 9$); squares represent RQneo ($n = 10$).

Table
Genotypes and Alleles Used

Genotype	Description
Gabrg-2 ^{R/R}	Wild type
Gabrg-2 ^{R/Q}	Knock-in mouse (human genotype equivalent model)
Gabrg-2 ^{R/Qneo}	Hypomorphic for R allele caused by Q allele suppression
Tg ^{Z/EG}	Enhanced green fluorescent protein, LacZ conditional reporter mouse
Tg ^{α-CaMKII-TA/LCI}	Doxycycline-regulated Cre-expressing transgenic alleles