Functional Characterization of Epilepsy Associated *GABRG2* Mutations

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Chapter 1

Introduction: $GABA_A$ Receptors and Epilepsy

Epilepsy

Epilepsy is one of the most common neurological disorders, affecting about 0.5-1% of the general population [1]. Epilepsy is defined as unprovoked, recurrent seizures, which are transient signs and symptoms of abnormal, excessive excitation and synchronous neuronal activity in the brain. Either enhancement of excitatory or reduction of inhibitory neurotransmission (E/I imbalance) could result in these excessive activities. Most seizures arise from the cerebral cortex, although subcortical structures can also generate seizures. Although epilepsy can develop at any age, it is more likely to occur in children or individuals over the age of 65 years. Epilepsy can be controlled often by antiepileptic drugs, and surgery can reduce seizure frequency or abolish seizures in some patients.

Seizures

Based on the recent proposed classification of the International League Against Epilepsy (ILAE) Commission on Classification and Terminology, epileptic seizures can be described as either focal or generalized in terms of their pattern of activity [2]. As shown in electroencephalograms (EEGs), focal seizures are characterized by excessive neuronal activities in focal regions of one hemisphere, while generalized seizures involve the whole cortex. *Clinical seizures* produce overt, noticeable clinical signs or symptoms, while *electrographic (or subclinical) seizures* produce subtle clinical signs or symptoms but are apparent on an EEG.

Focal seizures were called *partial seizures* in the 1989 and 1981 by the ILAE Classification and Terminology [3, 4]. These seizures originate within networks limited to one hemisphere. The behavioral phenotype of each seizure is determined by normal functions served by the cortical site where the seizure originates. Partial seizures were further characterized as *simple partial seizures* and *complex partial seizures*. Simple partial seizures do not affect awareness or memory, while complex seizures affect awareness and/or memory of events before, during, and immediately after the seizure, as well as behavior. Partial seizures can spread to both hemisphere and progress to

secondarily generalized seizures. The EEG features of partial seizures are variable. Partial seizures are proposed to be classified as focal seizures in 2010, and the difference between simple and complex partial seizures are simplified in this proposal [2].

Generalized seizures originate within one network and rapidly engage both cerebral cortex hemispheres [2]. The major subtypes of generalized seizures are absence, myoclonic, tonic, tonic-clonic and atonic.

Absence seizures are brief, usually lasting less than 30 seconds, and feature impaired consciousness and unresponsiveness associated with staring. The EEG signature of absence epilepsy is a generalized 3 Hz spike-wave discharge generated from abnormal thalamocortical oscillations [5].

Myoclonic seizures are rapid, shock-like jerks of a muscle or group of muscles. The EEG signature of myoclonic seizures features a polyspike-and-slow-wave discharge. If several myoclonic seizures occur in succession, they are called *clonic seizures*.

Tonic seizures cause muscle stiffening, generally of those in the back, legs, and arms. They are generalized, involving bilateral musculature in a symmetric or nearly symmetric manner. Tonic seizures are characterized by flexion at the waist and neck, abduction and flexion or extension of the upper extremities, and flexion or extension of

lower extremities. They typically occur during sleep and last 2-20 seconds. The EEG usually shows generalized, low-voltage, fast polyspikes.

Tonic-clonic seizures are the most common generalized seizures. They usually last 1-2 minutes, begin with stiffening of the body (tonic phase) and repeated jerks of the arms and/or legs as well as loss of consciousness (clonic phase).

Atonic seizures cause an abrupt loss of normal muscle tone for seconds, often resulting in falls, or, when milder, head nods or jaw drops. Consciousness is usually impaired. EEG recordings often show an electrodecremental response.

Etiologies of epilepsy

The term **Epilepsy** refers to both recurrent, unprovoked seizures and to more complex syndromes. The ILAE decided not to require the disease-syndrome distinction in referring to epilepsies at this time [2]. Although it is often referred to as epilepsy when seizures are the only neurologic disorder, and epilepsy syndrome when seizures are one of a group of symptoms, these two terms are used depending on context and custom.

In referring to syndromes, each epilepsy syndrome can be characterized according to features including the age at onset, cognitive and developmental antecedents and

consequences, motor and sensory examinations, EEG features, provoking or triggering factors, and patterns of seizure occurrence with respect to sleep [2]. The etiologies of the epilepsies were described as idiopathic, symptomatic, or cryptogenic. With *idiopathic epilepsies,* the disorder is not associated with other neurological or neuropsychiatric abnormalities, and often arises from genetic abnormalities that lead to alteration of basic neuronal regulation. *Symptomatic epilepsies,* in contrast, arise from the effects of a structural lesion, whether that lesion is focal, such as a tumor, or generalized such as a defect in metabolism causing widespread injury to the brain. *Cryptogenic epilepsies* are presumed to be symptomatic, but the presumptive lesion is not identified during evaluation.

Current development in molecular genetics, however, showed that these terms cannot describe accurately the etiologies of epilepsies. For example, Dravet syndrome is associated with genetic mutations in genes encoding sodium and $GABA_A$ receptor channels[6], but it has been classified as a symptomatic generalized epilepsy [2]. The ILAE proposed a rational system for characterizing and classifying etiologies based on mechanisms, which classified epilepsy etiologies as genetic, structural-metabolic, and unknown [2].

Genetic epilepsy directly results from a genetic cause. The associated gene and the mechanism of this association should be identified. Nevertheless, if the genetics studies showed a strong inheritable pattern, the disease can also be referred to as a genetic epilepsy.

Structural-Metabolic epilepsy is the secondary result of structural or metabolic abnormalities. These abnormalities can be associated with genetic defects, such as malformations of cortical development or metabolic disorders.

Unknown epilepsy indicates that the mechanism of epilepsy needs further investigation. It is different from cryptogenic epilepsy because it does not make any assumption about the underlying cause of the epilepsy.

International classification of epilepsies and epileptic syndromes

1. Localization-Related (Local, Focal, Partial) Epilepsies and Syndromes

1.1 Idiopathic (with age-related onset)

Benign epilepsy of childhood with centrotemporal spikes (OMIM no. 117100)

Benign occipital epilepsy of childhood (OMIM no. 132090)

1.2 Symptomatic

Rasmussen's encephalitis

Frontal lobe epilepsies

Occipital lobe epilepsies

Parietal lobe epilepsies

Temporal lobe epilepsies

1.3 Cryptogenic

2. Generalized Epilepsies and Syndromes

2.1 Idiopathic (with age-related onset)

Benign neonatal familial convulsions (OMIM no. 269720)

Benign neonatal convulsions (OMIM no. 121200)

Dravet Syndrome (OMIM no. 607208)

Childhood absence epilepsy (pyknolepsy) (OMIM no. 600131)

Juvenile absence epilepsy (OMIM no. 607631)

Juvenile myoclonic epilepsy (OMIM no. 254770)

2.2 Cryptogenic or Symptomatic

West syndrome (OMIM no. 308350)

Lennox-Gastaut syndrome (OMIM no. 606369)

Tuberous Sclerosis (OMIM no. 191100)

3. Epilepsies and Syndromes Undetermined Whether Focal or Generalized

4. Special Syndromes

Idiopathic/Genetic Epilepsies

Genetic epilepsy is *the direct result of a known or presumed genetic defect(s) in which seizures are the core symptom of the disorder* [2]. The distinction between the old epilepsy classifications as idiopathic and symptomatic epilepsies was often unclear. Idiopathic epilepsy syndromes are primary brain disorders that have no other identifiable neurological defects, and genetic factors are implied. Symptomatic epilepsies are associated with brain lesions resulting from structural diseases such as trauma, tumor, or

cortical malformations. However, some of symptomatic epilepsies are also caused by genetic defects, such as mitochondria diseases, amino-acidopathies, and storage They are also classified as genetic epilepsies in the recent proposal of ILAE and Terminology [2]. In this review, we will focus on mutations in inhibitory $GABA_A$ receptor subunit genes that are associated with genetic epilepsies.

Monogenic Epilepsies

Monogenic epilepsies are caused by mutations in single genes. Monogenic diseases can be passed on to subsequent generations in several ways: autosomal dominant (AD), autosomal recessive, X-linked dominant, X-linked recessive, Y-linked, and mitochondrial. However, most of the monogenetic epilepsies are AD. The genetic studies of epilepsies mediated by monogenic inheritance required many affected members (up to ten for AD diseases) for linkage analysis. Unfortunately, large families are often not found in epilepsy studies, probably because of incomplete penetrance of the epilepsy trait, the small family size, or technical difficulties in accurate diagnosis of disease-affected individuals [7]. Most of the epilepsy associated $GABA_A$ receptor subunit mutations are AD monogenic mutations.

Polygenic Epilepsies

Polygenic epilepsies are also called complex epilepsies or multifactorial epilepsies, which are likely associated with effects of multiple "susceptibility" genes in combination with lifestyle and environmental factors [8]. Identification of susceptibility genes is difficult because complex epilepsies do not have a clear-cut pattern of inheritance, although they often cluster in families. Some of the $GABA_A$ receptor subunit genes are susceptibility genes for polygenic epilepsies.

$GABA_A$ Receptors

GABA is the major inhibitory neurotransmitter in the CNS, and abnormalities in both pre- and post-synaptic GABAergic inhibition could produce epilepsy. The fast neural inhibition is mediated by ionotropic $GABA_A$ receptors while the slow, prolonged neural inhibition is mediated by metabotropic $GABA_B$ receptors. $GABA_A$ receptors are ligand-gated ion channels (LGIC) that belong to the evolutionarily related and similar "Cys-loop" super-family that also includes nicotinic acetylcholine receptors (nAChRs), glycine receptors (GlyRs), and the $5HT_3$ receptor. $GABA_A$ receptors are

heteropentamers assembled from seven different subunit families: α(1-6), β(1-3), γ(1-3), ε, π and θ [9, 10]. The ρ(1-3) subunits are expressed in retina and do not assemble with other subunits [9, 10]. $GABA_A$ receptor sequence diversity is further increased by alternative splicing in some subunit mRNAs [11, 12]. Each $GABA_A$ receptor subunit is a four transmembrane protein. Sequence homology between subunits in the same family is 70-80%, and between subunits in different families is 30-40%. Most $GABA_A$ receptors are composed of two α subunits, two β subunits, and one γ or δ subunit [10]. Interestingly, not all subunit combinations are physiologically relevant [13]. There are α1, α6, β2, β3, γ2, and δ subunits in cerebellar granule cells, but α6 subunit knockout mice had greatly decreased δ subunit membrane expression in cerebellar granule cells, which still expressed α1, β2, β3, and γ2 subunits [14]. Immunochemistry and subtractive immunoprecipitation studies showed that the most common subunit composition in the brain is α1β2γ2, followed by α2β3γ2 and α3β3γ2 [13, 15]. Most of the γ2 subunit-subunit-containing $GABA_A$ receptors are synaptic receptors mediating fast phasic currents, while the δ subunit-containing $GABA_A$ receptors are commonly extrasynaptic receptors mediating slow tonic currents [16].

Antiepileptic drugs that work on $GABA_A$ receptors

$GABA_A$ receptors were identified pharmacologically long before each subunit was cloned. Once assembled, $GABA_A$ receptors form chloride channels containing binding sites for agonists, antagonists and allosteric modulators, such as GABA, picrotoxin, barbiturates, benzodiazepines, and the anesthetic steroids [9]. $GABA_A$ receptors with different subunit compositions have distinct kinetics and pharmacological properties [9]. $GABA_A$ receptors are activated by GABA and the GABA analog muscimol, which bind to the N-terminus at α/β interfaces [17, 18]. Depending on variations of intracellular chloride concentration during development, $GABA_A$ receptors are initially excitatory in immature brain and then inhibitory in adult brain [19]. The potency and efficacy of GABA vary with $GABA_A$ receptor subunit composition. Compared to synaptic $\gamma 2$ subunit-containing $GABA_A$ receptors mediating phasic inhibition, extrasynaptic δ subunit-containing $GABA_A$ receptors mediating tonic have higher GABA potency and higher affinity but lower efficacy [10]. $GABA_A$ receptor currents can be potentiated by benzodiazepines, barbiturates, and neurosteroids, and blocked by Zn^{2+}, bicuculline, penicillin, and picrotoxin. Drugs inhibiting $GABA_A$

receptor currents can induce epilepsy in animals [20]. Benzodiazepines and barbiturates are approved for use as antiepileptic drugs in the United States.

Benzodiazepines are clinically used for antianxiety, antiepileptic, muscle relaxant, and hypnotic activity [21]. Clonazepam, clorazepate, diazepam and lorazepam are approved in the United States for treatment of certain types of seizures. These classical benzodiazepines exert most of their effects by binding to $GABA_A$ receptors at the α/γ interface [22]. The benzodiazepine molecule is oriented such that the C5-phenyl substituent extends approximately parallel to the plane of the membrane [23]. Benzodiazepines increase the agonist-binding affinity of $GABA_A$ receptors and the frequency of gated channel opening [24]. The presence of a γ subunit is essential for benzodiazepine binding, and $\gamma 2$ subunit-containing $GABA_A$ receptors have the highest sensitivity [25]. The $\alpha 1$ subunit residue at histidine 101 is essential for benzodiazepine binding, so benzodiazepines bind non-selectively to $\alpha 1, \alpha 2, \alpha 3$ and $\alpha 5$ subunit-$GABA_A$ receptors, but $\alpha 4$ and $\alpha 6$ subunit-containing $GABA_A$ receptors are not sensitive all benzodiazepines because these two subunits have an arginine at this position [26]. Genetically modified mice showed that different α subunit subtypes mediate different benzodiazepine effects. The H101R mutation abolished diazepam binding to $GABA_A$

receptors containing mutant α1 subunits [27]. The α1(H101R) mouse studies suggested that the motor sedative, anterograde amnesic, anticonvulsant actions and addictive properties of benzodiazepines are mediated by α1 subunit-containing $GABA_A$ receptors, while the sleep continuity-enhancing, anxiolytic-like, myorelaxant, motor-impairing and ethanol-potentiating effects are mediated by $GABA_A$ receptors containing other α [27-30]. A similar strategy to abolish benzodiazepine binding sites in other α subunits showed that anxiolytic and myorelaxant activities are mediated by α2 subunit-containing $GABA_A$ receptors [31, 32]. The α3 subunit-containing $GABA_A$ receptors are involved in mediating anxiolytic activity of diazepam and myorelaxent activity at high diazepam concentrations [32-34]. The α5 subunit-containing $GABA_A$ receptors are involved in mediating myorelaxant activity of diazepam, as well as hippocampus-dependent learning and memory processes, such as trace fear conditioning [35, 36]. Although it has not been confirmed whether $GABA_A$ receptor subtype heterogeneity affects benzodiazepine function in the same way in human brain, $GABA_A$ receptor subtype selective drugs depending on α subunits have been developed to reduce the undesired actions [37].

Phenobarbital was identified as the first effective organic antiseizure agent in 1912, and primidone is an effective treatment for partial and tonic-clonic seizures [21].

Barbiturates bind to $GABA_A$ receptors and enhance GABAergic current by increasing the mean open time but do not affect the channel conductance or opening frequency [38, 39]. Barbiturates bind to $GABA_A$ receptors probably at a different site than GABA or benzodiazepines, because barbiturate binding increases [^{3}H]GABA, [^{3}H]muscimol, and [^{3}H]flunitrazepam binding to $GABA_A$ receptors [40, 41]. Barbiturates at high concentrations can also activate $GABA_A$ receptors in the absence of GABA [42]. Phenobarbital can reach this concentration when used during anesthesia [43].

$GABA_A$ receptor assembly and trafficking

$GABA_A$ receptor assembly in the endoplasmic reticulum (ER)

Because of the complex subunit expression pattern in neurons, most $GABA_A$ receptor assembly and trafficking studies have been performed in heterologous cells overexpressing subunit cDNAs [44]. $GABA_A$ receptor subunits are membrane proteins, and thus each subunit has a leading signal peptide in the premature peptide. During protein translation, the signal peptide targets the polypeptide to the Sec61 translocation machinery cotranslationally or posttranslationally and mediates translocation to the ER lumen [45]. The hydrophobicity pattern of signal peptides is the most important factor

this function [46]. Signal peptides are cleaved from premature subunits by a signal peptidase after translocation and cleaved again in the hydrophobic center between two leucine residues into small pieces [45]. While it has not been demonstrated in $GABA_A$ receptor subunit signal peptides, the cleaved signal peptide may function as a signaling molecule and exert cellular functions [47-49].

$GABA_A$ receptor assembly occurs in the ER lumen [50] and involves classical ER chaperones including BIP and calnexin [50, 51]. Coexpressed $\alpha1\beta2\gamma2L$ and $\alpha1\beta2$ subunits assembled into receptors that were trafficked to the cell membrane, but coexpressed $\alpha1\gamma2L$ and $\beta2\gamma2L$ subunits were retained in the ER [50]. Unassembled and misfolded subunits were retained in the ER and degraded by the proteasome [52, 53]. assembled $GABA_A$ receptors form a clockwise $\gamma2-\alpha1-\beta2-\alpha1-\beta2$ arrangement when viewed from a synaptic gap [54, 55]. The α and β subunits can assemble into heteropentameric receptors and be expressed on the membrane in culture cells or mouse brain [50, 56], and thus, γ subunits are not required for receptor membrane trafficking. Mice lacking $\gamma2$ subunits ($\gamma2^{-/-}$ mice) had slightly reduced GABA binding sites in brain, lost 94% of benzodiazepine binding sites [56]. The sequences important for subunit oligomerization and receptor assembly are at the N-terminus of α, β and γ subunits [57-

[57-59]. Alteration in these N-terminus sequences abolished subunit oligomerization and receptor assembly. *In vivo* studies showed that $GABA_A$ receptor assembly is a preferential process. The α6 subunit knockout mice had decreased δ subunit membrane expression in cerebellar granule cells [14], and the δ subunit knockout mice had decreased α4 subunits level on forebrain membranes [60]. The δ subunit preferentially assembles with α4 and α6 subunits. $GABA_A$ receptor subunits have *N*-linked glycosylation sites at the N-terminus, and the subunit glycosylation pattern affects oligomerization and ER stability [61]. The β3 subunits are also ubiquitinated in the ER an activity-dependent manner [62]. Blocking neuronal activity with the voltage-gated sodium channel blocker tetrodotoxin or glutamate receptor antagonists decreased GABAergic mIPSC amplitude and frequency in cultured neurons. These neurons showed increased β3 subunit ubiquitination and decreased total and surface levels of $GABA_A$ receptors. However, increasing neuronal activity with the $GABA_A$ receptor antagonist picrotoxin increased $GABA_A$ receptor surface levels. This activity-dependent ubiquitination controls the number of $GABA_A$ receptors in the ER and their membrane expression level, suggesting that ER-to-Golgi translocation is an important regulation point for $GABA_A$ receptor membrane trafficking. ER-to-Golgi translocation can be facilitated by the ubiquitin-like protein Plic-1, which binds directly to α and β subunits

inhibits ubiquitin-dependent degradation [63]. Blocking the binding of Plic-1 to α1 subunits reduced GABAergic current in CA1 neurons in hippocampal slices, and overexpressing Plic-1 with β3 subunits increased β3 subunit stability, which led to increased subunit total and surface levels.

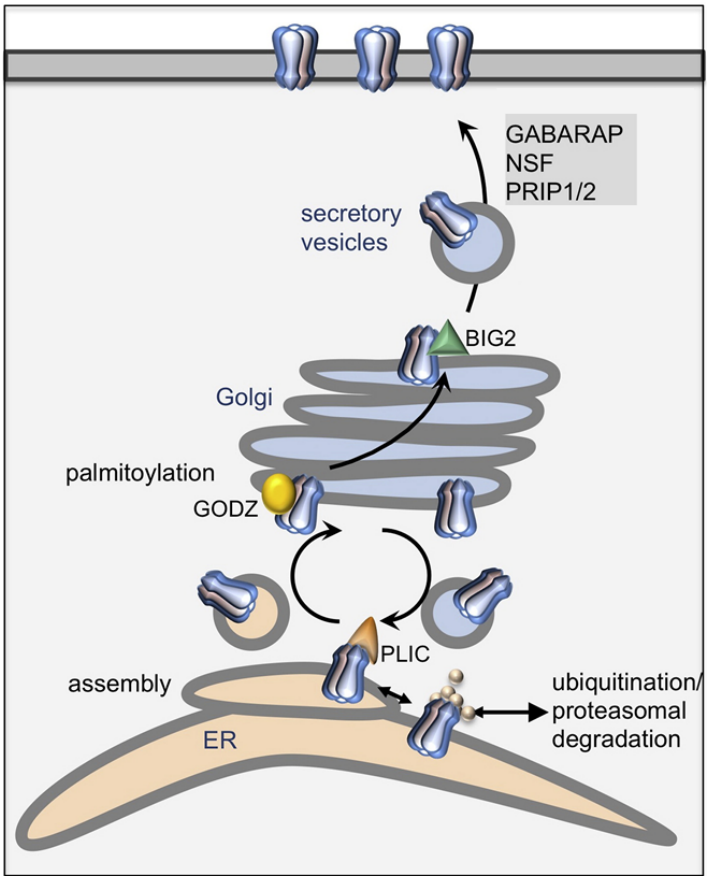

Figure 1.1 $GABA_A$Rs membrane trafficking in the secretory pathway. Adapted from [64]

$GABA_A$ receptor trafficking in the Golgi apparatus

Within the Golgi apparatus, the Golgi-specific palmitoyltransferase DHHC zinc finger domain protein (GODZ) interacts with a cysteine-rich domain in the second cytoplasmic loop of γ1–3 subunits, and palmitoylates γ subunits in assembled receptors [65]. Palmitoylation is a reversible posttranslational modification that enhances protein hydrophobicity and regulates membrane trafficking and clustering [66]. Decreasing GODZ activity in neurons significantly decreased postsynaptic clustering of $GABA_A$ receptors, as well as GABAergic mIPSCs, but did not alter AMPA receptor-mediated glutamatergic transmission [65]. $GABA_A$ receptor subunits glycosylated in the ER carry high mannose *N*-linked glycans that are sensitive to Endoglycosidase H (EndoH) digestion [52, 61]. The oligosaccharide side chains are further modified in the Golgi apparatus so that they are insensitive to EndoH digestion but can still be removed by Peptide: N-Glycosidase F (PNGase F), an endoglycosidase that can remove all *N*-linked glycans [67]. Abolishing glycosylation in α1β2 receptors decreased peak current amplitudes of whole cell currents and reduced channel mean open time of single channel currents [61]. Thus, the glycosylation pattern affected $GABA_A$ receptor biogenesis and channel function.

Golgi-to-plasma membrane translocation

The translocation of $GABA_A$ receptors from the Golgi apparatus to the plasma membrane is facilitated by Brefeldin-A-inhibited GDP/GTP exchange factor 2 (BIG2), $GABA_A$ receptor associated protein (GABARAP), *N*-ethylmaleimide-sensitive factor (NSF), and Phospholipase-C-related catalytically inactive protein 1 and 2 (PRIP-1, PRIP-2) [68-71].

BIG2 is a Sec7 domain-containing guanine nucleotide exchange factor (GEF) that catalyzes GDP/GTP exchange on the class I small G-protein ADP-ribosylation factor 1 and 3 (ARF1/3) [72]. Activation of these G-proteins regulates coated vesicle formation from the Golgi apparatus and facilitates cargo translocation from Golgi to plasma membrane [73]. BIG2 directly interacts with the human exocyst protein Exo70 in the trans-Golgi network and in microtubules, suggesting that this protein complex has a functional association in both early and late stages of vesicular trafficking [74]. BIG2 pulled down $GABA_A$ receptors from rat brain lysate and colocalized with $GABA_A$ receptors in cultured hippocampal neurons [68]. BIG2 binds to β1−3 subunits in the second intracellular loop at a position that overlaps the Plic-1 binding site. Coexpressing BIG2 and β3 subunits in HEK293 cells improved β3 homopentamer membrane trafficking.

GABARAP interacts with the intracellular loop of γ subunits and microtubules *in vitro* and *in vivo* [69, 75]. GABARAP is enriched in the Golgi apparatus and postsynaptic cisternae, but not at inhibitory synapses [76]. Overexpressing GABARAP in cultured neurons and heterologous cells increased $GABA_A$ receptor membrane levels [77], suggesting enhanced Golgi-to-membrane translocation. This enhancement was abolished by a mutation at the GABARAP C-terminus that disrupted adding phospholipid to GABARAP [78]. Activating NMDA receptors increased $GABA_A$ receptor binding with GABARAP and expression of $GABA_A$ receptors at the dendritic surface of hippocampal neurons [79]. GABARAP binds to the postsynaptic marker gephryin, but this interaction is not important for $GABA_A$ receptor postsynaptic anchoring [80]. GABARAP might be involved only with intracellular trafficking of $GABA_A$ receptors.

NSF is an ATPase associated with various cellular activities protein. It is critical for intracellular membrane fusion and protein membrane trafficking [70]. NSF binds to SNARE (soluble NSF attachment proteins receptor) complexes and utilizes the energy of ATP hydrolysis to disassemble them, thus facilitating SNARE recycling [81]. NSF directly binds to GABARAP and the intracellular loop of β1-3 subunits [70, 76]. NSF GABARAP colocalization was detected in cultured neurons by confocal microscopy and

in hippocampal/spinal slices by electron microscopy [76]. Together with GABARAP, NSF is also essential for enhanced $GABA_A$ receptor membrane expression after NMDA activation [79]. Abolishing GABARAP C-terminus modification disrupted subcellular localization of both NSF and $GABA_A$ receptors and blocked GABARAP-promoted $GABA_A$ receptor surface expression [78]. Mutations in the γ2 subunit-binding domain of GABARAP had similar negative effects [77]. NSF and GABARAP might work together to facilitate $GABA_A$ receptor Golgi-to-membrane translocation. Interestingly, overexpressing NSF alone decreased surface $GABA_A$ receptor level, but overexpressing GABARAP alone increased $GABA_A$ receptor surface level [70, 77]. NSF ATPase activity is required for down regulation of $GABA_A$ receptor membrane trafficking, which can be increased by PKCε phosphorylation at serine 460 and threonine 461 residues of NSF [82].

PRIPs, PRIP-1 and PRIP-2, were isolated from rat brain as inositol 1,4,5-1,4,5-trisphosphate-binding proteins [71]. PRIP-1 is expressed mainly in brain, while PRIP-2 is a ubiquitous protein [83]. The regional expression of PRIP-1 and PRIP-2 mRNAs were identified in hippocampal pyramidal cells, dentate granule cells, pyramidal and granule cell layers of the cerebral cortex, and granule cell and Purkinje cell layers

cerebellar nuclei of the cerebellum [83]. This expression pattern is close to $GABA_A$ receptors [71]. PRIPs directly interacted with β subunits, γ2 subunits, and GABARAP [71, 84, 85]. PRIPs and γ2 subunits bind to the same region of GABARAP, and PRIPs inhibited γ2 subunit binding to GABARAP *in vitro* [71]. However, PRIP-$1^{-/-}$ and PRIP$1/2^{-/-}$ mice both had normal GABAergic currents in hippocampal neurons, but decreased γ2 subunit membrane levels, and behavioral response to pharmacological treatments suggesting decreased γ2 subunit-containing $GABA_A$ receptor expression in the mouse brain [71, 84, 86]. Furthermore, the PRIP$1/2^{-/-}$ mice did not show a decrease in membrane γ2 subunit membrane levels [87]. PRIP$1/2^{-/-}$ mice also had increased α6 subunit membrane levels, and pharmacological and electrophysiological studies showed that more γ2 subunits assembled with α6 subunits into functional receptors in cerebellar granule cells, so the normal γ2 subunit membrane level might be due to increased expression of α6βγ2 receptors. PRIPs are important for γ2 subunit-containing $GABA_A$ receptor surface expression.

$GABA_A$ receptor clustering at synapses

$GABA_A$ receptors integrate into cell membranes at extrasynaptic locations and diffuse laterally in the membrane where they are recruited to postsynaptic sites after synaptic formation [88, 89]. The induction of GABAergic inhibitory synapses is poorly understood. Secreted proteins such as Narp and Ephrin B1 and cell adhesion molecules such as SynCAM and Neuroligin directly induce formation of excitatory synapses [90]. Although Neuroligin-2 and Neurexin 1β are suggested to regulate inhibitory neuron synaptogenesis [91-93], mice lacking neuroligin expression had a normal density of synaptic contacts, suggesting that neuroligins are required for proper synapse maturation and brain function, but not for the initial formation of synaptic contacts [94, 95]. The detailed mechanism of inhibitory synapse formation is still unknown.

The heterogeneity resulting from varying subunit composition affects $GABA_A$ receptor subcellular targeting [96]. α1, α2, β2/3, and γ2 subunit-containing $GABA_A$ receptors often locate at postsynaptic membranes in receptor clusters [97-99]. α4, β1, δ subunit-containing receptors are often distributed diffusely or at perisynaptic [99]. α5 subunit-containing receptors are predominately extrasynaptic, including α5βγ2 receptors [100, 101]. Extrasynaptic α6 or δ subunit-containing $GABA_A$ receptors are

major mediators of tonic inhibition, while γ2 subunit-containing receptors normally mediate phasic inhibition [16, 101, 102].

$GABA_A$ receptor neuronal subcellular targeting is strongly associated with interaction between $GABA_A$ receptor subunits and *gephyrin*, an inhibitory synaptic scaffold protein for both GABAergic and glycinergic synapses [97, 103-105]. Disrupting postsynaptic gephyrin clusters not only decreased postsynaptic $GABA_A$ receptor clusters, but also reduced the number of GABAergic presynaptic boutons contacting the pyramidal cells, suggesting that gephyrin clustering is required for maturation of GABAergic synapses [106]. Gephyrin consists of three major domains, a 20 kDa N-N-terminal G domain and a 43 kDa C-terminus E domain connected by a central linker domain of 18-21 kDa [107]. The E domain contains a common binding site for glycine receptor β subunits and $GABA_A$ receptor α2, α3, and α1 subunits [108-111], and they to gephyrin in an exclusive manner [112]. Although the $GABA_A$ receptor γ2 subunit is required for recruiting gephyrin to the surface membrane [113], the γ2 subunit binding in gephyrin is still unknown. The gephyrin binding sites on glycine or $GABA_A$ receptor subunits are predominantly in the M3-M4 intracellular loop, except for the $GABA_A$ receptor γ2 subunit, which is at TM4 [113]. The number of α2, α3, β2/3, and γ2 subunit

immunoreactive synaptic clusters were significantly decreased in *geph*$^{-/-}$ mice, but the punctate staining of $GABA_AR$ α1 and α5 subunits was unaltered [114]. Recent studies, however, also showed that gephyrin directly bound to α1 subunits and decreased membrane diffusion of α1 subunit-containing receptors [115]. All of this evidence supported a model in which $GABA_A$ receptors on the membrane recruit gephyrin to stabilize formation of inhibitory synapses [106, 114-116]. Loss of gephyrin decreased spontaneous IPSCs but not whole cell GABA currents in cultured hippocampal neurons [106].

The interaction between $GABA_A$ receptors and gephyrin also requires *collybistin* [117]. Collybistin is a member of the Dbl family of guanine nucleotide exchange factors, but the small GTPase, Cdc42, is not involved in gephyrin and $GABA_A$ receptor synaptic targeting [118]. Collybistin-deficient mice had a region-specific loss of postsynaptic gephyrin and $GABA_A$ receptor clusters in the hippocampus and the basolateral amygdala, but glycine receptor clusters were not affected [119]. Interestingly, the SH3 domain at collybistin N-terminus and $GABA_A$ receptor subunits binds to gephyrin at the same binding site [112], and both N-terminus and lipid binding C-terminus of collybistin are required for recruiting $GABA_A$ receptor-gephyrin complexes to postsynaptic membranes

[117, 118, 120]. The presence of gephyrin or collybistin strengthened the tri-hybrid interactions between α2 subunits and collybistin or α2 subunits and gephyrin, suggesting that $GABA_A$ receptors, collybistin and gephyrin form a trimeric complex [109].

$GABA_A$ receptors can form clusters independent of gephyrin. Gephyrin-deficient *geph*$^{-/-}$ mice had unaltered numbers of $GABA_A$ receptor α1 and α5 subunit-containing postsynaptic puncta and inhibitory presynaptic terminals [114], and α2 and γ2 subunit-containing $GABA_A$ receptors in hippocampal cultures from *geph*$^{-/-}$ mice clustered at pyramidal synapses at reduced levels [116]. The ERM family protein radixin directly binds to α5 subunit-containing receptors and cytoskeleton protein F-actin and facilitated α5 receptor clustering [121]. Depletion of radixin in cultured neurons drastically decreased α5 receptor clustering, but the surface level of α5 receptors was not altered. The radixin knockout mouse also had background levels of α5 subunit-containing receptor clustering but normal surface α5 subunit-containing receptor levels in brain. However, electron microscopy studies demonstrated α5 subunit-containing receptor clusters at synaptic membranes that colocalized with gephyrin and γ2 subunit-containing receptors [122].

$GABA_A$ receptor endocytosis and degradation

$GABA_A$ receptors are internalized exclusively at extrasynaptic sites in a clathrin- and dynamin-dependent manner [89, 123]. More than 25% of surface $GABA_A$ receptors were internalized after 30 minutes [124], 70% of the internalized $GABA_A$ receptors were rapidly recycled back to the surface, and 30% of neuronal $GABA_A$ receptors were degraded by lysosome after 6 hours [124]. The interaction between $GABA_A$ receptors and clathrin is mediated by β or γ subunits and the clathrin adaptor protein AP2 [85, 123, 125, 126]. The μ2–AP2 binding sites on β and γ subunit intracellular domains can be phosphorylated by a variety of kinases including PKA, PKC, CaMKII or Akt [127-131], and phosphorylation decreases the binding affinity between $GABA_A$ receptors and μ2–AP2, allowing for activity- and subunit-dependent modulation of surface receptor levels. Applying a non-phosphorylated peptide corresponding to the β3 subunit AP2 binding site to cultured hippocampal neurons increased mIPSC amplitude and frequency [125]. Co-applying non-phosphorylated peptides corresponding to the γ2 and β3 subunit AP2 binding sites increased mIPSC amplitude in additive manner [126]. These resulted demonstrated that $GABA_A$ receptor endocytosis directly regulates inhibitory synaptic transmission. Mice carrying homozygous phosphorylation defective

$\gamma2^{Y365/7F}$ subunits died early in development, and heterozygous $\gamma2^{Y365/7F}$ mice had mIPSC amplitude in the hippocampal CA3 region because of increased $GABA_A$ receptor levels, but also deficits in CA3-dependent spatial memory [132]. However, overexpression of γ2 subunits in transgenic mice did not significantly alter mouse behavioral or biochemical phenotypes except ethanol tolerance [133], suggesting that $GABA_A$ receptor-dependent synaptic inhibition strength regulation is paramount for mouse development and normal brain function.

Huntingtin-associated protein (HAP1) directly binds to the intracellular domain of β1–3 subunits. When HAP1 was overexpressed in cultured neurons, it inhibited endocytosed $GABA_A$ receptor degradation and facilitated receptor recycling [124]. *HAP1*$^{-/-}$ mice lacking HAP1 had substantially decreased surface $GABA_A$ receptor levels, as well as mIPSC amplitudes [134]. HAP1 links $GABA_A$ receptors to the kinesin family motor protein 5 (KIF5) [135]. KIF5 mediates $GABA_A$ receptor membrane insertion, and disrupting the HAP1-KIF5 complex decreased synaptic $GABA_A$ receptor number and reduced the amplitude of inhibitory postsynaptic currents. The decreased inhibitory synaptic currents may contribute to Huntington's disease because mutant huntingtin interrupts HAP1 function and disrupts $GABA_A$ receptor recycling [135].

Endocytosed $GABA_A$ receptors that are not recycled are degraded by lysosomes [124]. This degradation is dependent on γ2 subunit ubiquitination [136]. Blocking γ2 subunit ubiquitination, disrupting the trafficking of ubiquitinated cargo to lysosomes, or blockade of lysosomal activity increased the efficacy of synaptic inhibition. Mutations at γ2 subunit ubiquitination sites also blocked the loss of synaptic $GABA_A$ receptors after anoxic insult. In addition to ubiquitin-mediated proteasomal degradation of $GABA_A$ receptors in the ER, the synaptic γ2 subunit-containing $GABA_A$ receptor level is also regulated by ubiquitin-dependent lysosomal degradation.

$GABA_A$ receptor epilepsy channelopathies

Overview

Mutations and variations in $GABA_A$ receptor subunits have been associated with several genetic epilepsy syndromes [44, 137, 138]. Epilepsy associated $GABA_A$ receptor subunit mutations were identified from genes encoding α1, β3, γ2 and δ subunits (*GABRA1, GABRB3, GABRG2* and *GABRD*) [44]. These are rare mutations, but studying their mechanisms has further elucidated the function of $GABA_A$ receptors and

inhibitory neurotransmission [139, 140]. The functional characterization of epilepsy-epilepsy-associated $GABA_A$ receptor mutations has been performed in heterologous cells, cultured neurons, and genetically modified mice as model systems. A comprehensive comparison about advantages and disadvantages of these systems can be found in Ref [141]. Monogenic epilepsies associated with $GABA_A$ receptor subunit mutations vary in severity from the relatively benign childhood absence epilepsy (CAE; MIM no. 600131) the severe epilepsy syndrome, Dravet syndrome (DS; MIM no. 607208). These either directly or indirectly reduce inhibitory GABAergic neurotransmission, which cause brain hyperexcitability and thereby predispose patients to seizures. Investigators have designated the numbering of mutations in *GABRG2* based on the position in the mature peptide (i.e. *GABRG2(R43Q)*), but mutant amino acids in *GABRA1, GABRB3* and *GABRD* are in the immature peptide that includes the signal sequence (i.e. *GABRA1(A322D)*). For consistency, the numbering of all $GABA_A$ receptor subunit gene mutations will be designated in the immature peptide (i.e. *GABRG2(R82Q)*) in this thesis, including *GABRG2* gene mutations.

GABRG2 gene mutations

GABRG2 *gene expression pattern and γ2 subunit function*

There have been 19 epilepsy mutations identified in $GABA_A$ receptor subunit genes, 8 of which were identified in the *GABRG2* gene, suggesting its strong association with epilepsy [142]. The human $GABA_A$ receptor γ2 subunit is encoded by the *GABRG2* gene, which is located on chromosome 5q34 in a cluster with *GABRB2, GABRA6*, and *GABRA1* genes encoding β2, α6, and α1 subunits, respectively [143]. The mouse *GABRG2, GABRA1 GABRA6* and *GABRB2* genes also form a cluster on mouse chromosome 11. The temporal and spatial expression of $GABA_A$ receptor subunits is strictly regulated [144-149]. In mouse brain, the γ2 subunit is the most abundant γ subunit, followed by the γ1 subunit [150]. The γ3 subunit is rare. Approximately 75-75-80% of $GABA_A$ receptors contain the γ2 subunit [10]. The γ2 subunit mRNA level is prominent at birth, increases to a maximum level in the second or third postnatal week, then decreases slightly to the adult level [144, 150, 151]. *In situ* hybridization showed γ2 subunit mRNA is expressed in the cerebral cortex, pyramidal cell layer of the hippocampus, granule cell layer of the dentate gyrus, inferior and superior colliculi, caudate, and cerebellar cortex [144-146, 151]. The expression in the cerebellar cortex is mainly in the internal granule cell layer; the mRNA level in the external granule cell

is lower. The γ2 subunit mRNA is expressed in the olfactory bulb in mitral cell, glomerular, and internal granular layers. The brainstem also has prominent γ2 subunit mRNA expression. This pattern is consistent with γ2 subunit protein level shown in immunohistochemistry studies [148].

There are three splice variances of γ2 subunits, γ2L, γ2S, and γ2XL subunits [11, 12]. The γ2XL subunits retained a 40 amino acid peptide between Ser 171 and Tyr172 from alternative splicing of intron 5. The subunit, however, did not oligomerize with αβ subunits and was not expressed on cell surface membrane. Thus, the function of this variant is still unknown. In the presence of the neuron-specific RNA binding protein Nova, a 24 bp extra exon was retained in the γ2L subunit mRNA after *GABRG2* gene intron 8 alternative splicing [152]. The retained exon translates to an eight amino acid peptide, LLRMFSFK, in the second intracellular loop that encodes a potential consensus serine phosphorylation site for protein kinase C. Both α1β2γ2L and α1β2γ2S receptors were endocytosed at similar levels after activating PKC with PMA [153]. either γ2L or γ2S variant in transgenic mice using an actin promoter did not affect motor activity, acute effects of benzodiazepines and alcohol, or responses to alcohol withdrawal [133]. Expressing either γ2L or γ2S subunits in $\gamma 2^{-/-}$ mice as a transgene fully

rescued the KO mice behavior, making it indistinguishable from wildtype mice [154]. The extra 24 bp exon was knocked out from intron 8 to generate $\gamma 2L^{-/-}$ knockout mice [155]. Homozygous $\gamma 2L^{-/-}$ knockout mice are viable and indistinguishable from wildtype mice, but they were more sensitive to benzodiazepines because of an increase in affinity brain membrane receptors for benzodiazepine agonists [156]. However, the extra predicted phosphorylation site in the γ2L subunit introduced novel functions compared to γ2S subunits. When expressed in HEK293 cells alone, γ2S subunits were expressed on cell membranes as homopentamers but γ2L subunits were retained in ER [157]. After mutating the extra 8 amino acid peptide in γ2L subunits to 8 alanines, the mutated γ2L subunits were expressed also on the membrane. The γ2L subunits more efficiently accumulate at inhibitory synapses than γ2S subunits [158]. PKC activation facilitated the postsynaptic clustering of γ2L, but not γ2S, subunits, and this effect was blocked by mutating Ser343 to Ala343. Therefore, it is not surprising that γ2L and γ2S subunits are differentially expressed in brain regions [159, 160]. The γ2S subunit mRNA is expressed at a fairly constant level during brain development, while γ2L subunit mRNA levels are low at birth and increase dramatically with maturation [159]. In new born mice, 85% of γ2 subunit mRNAs is the γ2S variant, which decreases to 45% at 6 weeks of age. In adult rat brain, γ2S and γ2L subunit protein levels vary depending on brain region [160]. The

γ2S subunit level is higher than γ2L subunit level in the hippocampus, cerebral cortex, olfactory bulb. In contrast, the γ2L subunit level is higher than the γ2S subunit in the inferior colliculus, medulla, and the cerebellar Purkinje cells. The relative ratio of γ2L to γ2S subunit levels is altered in aging brain, during pregnancy, and with schizophrenia [161-163]. It is not clear whether the relative ratio of γ2L to γ2S subunit levels changes epilepsy.

Although γ2 subunits are not required for $GABA_A$ receptor membrane expression, they are important for normal brain function. Homozygous $\gamma2^{-/-}$ knockout mice had normal body weight and brain histology, but they died within two weeks after birth [56]. The γ2 subunit is required for maintaining postsynaptic $GABA_A$ receptor clustering [97]. The $\gamma2^{-/-}$ mice lost both $GABA_A$ receptor clustering and scaffold protein gephyrin at the postsynaptic sites in cerebral cortex and cultured neurons, which were restored by ectopically express γ3 subunits [164]. However, endogenous γ3 subunit expression was not changed in the $\gamma2^{-/-}$ mice to compensate for the loss of γ2 subunit [56]. Recordings obtained from $\gamma2^{-/-}$ mouse dorsal root ganglion neurons showed GABAergic currents that were similar to αβ receptor currents recorded from heterologous cells [56, 165]. These $\gamma2^{-/-}$ mice lost 94% of their benzodiazepine binding sites but GABA binding sites were

slightly decreased. The heterozygous $\gamma2^{+/-}$ mice had significantly decreased benzodiazepine binding sites *and* increased extrasynaptic $GABA_A$ receptor radioligand binding sites in cortex, striatum, thalamus, hippocampus, inferior colliculus, and granule cell layer, but muscimol binding sites were not different [166]. Immunohistochemistry study also showed these animals had decreased $GABA_A$ receptor clustering in hippocampus and cerebral cortex [167]. The $\gamma2^{+/-}$ mice showed increased anxiety characterized by harm avoidance behavior and an explicit memory bias for threat cues, probably resulting from disinhibition in CA3 of hippocampus [167]. Diazepam treatment reversed the anxiety phenotypes. These behavioral phenotypes were recapitulated in γ2 subunit knockdown mice [168]. The $GABA_A$ receptor dysfunction might be associated with anxiety traits in patients. No studies of possible seizure phenotypes in $\gamma2^{-/-}$ or $\gamma2^{+/-}$ mice have been reported.

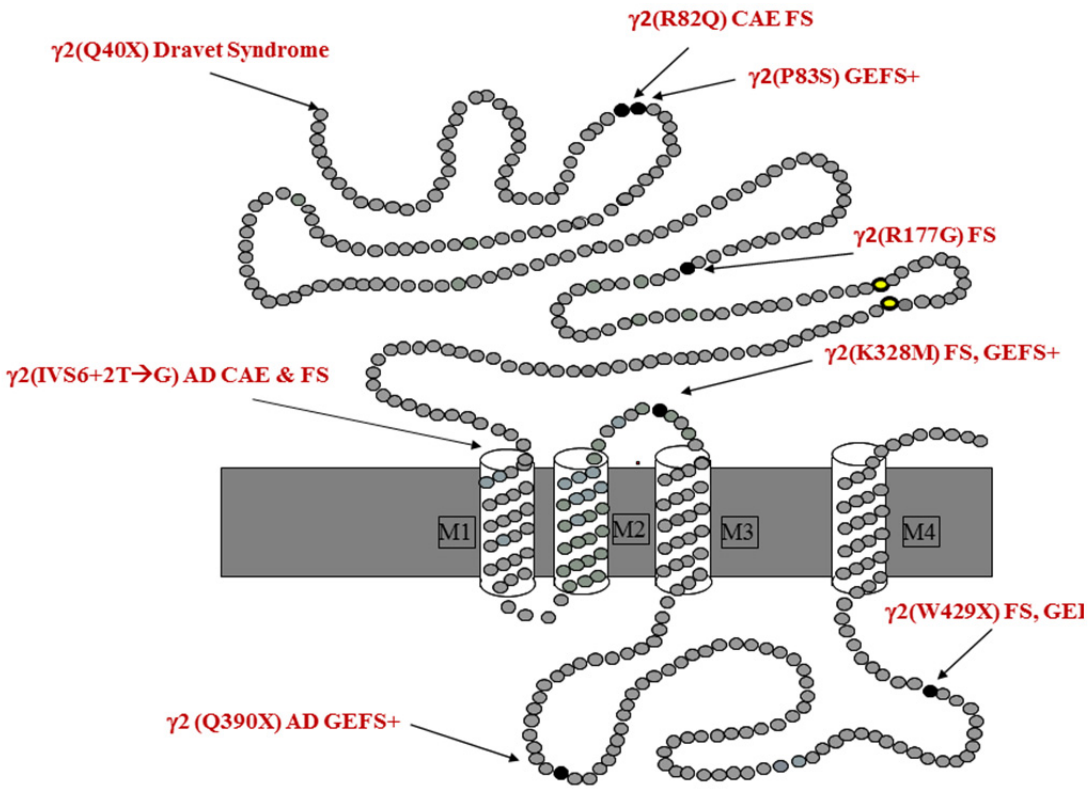

Figure 1.2 $GABA_A$ receptor γ2 subunit mutations associated with genetic epilepsy syndromes.

Predicted membrane topology of $GABA_A$ receptor γ2 subunit consists of a large extracellular domain at the N-terminus, four transmembrane domains (M1–M4) and a large cytoplasmic domain. In this figure, we depicted $GABA_A$ receptor γ2 subunit mutations associated with genetic epilepsy syndromes at their appropriate protein domain within the subunit.

GABRG2 ***gene missense mutations***

The first identified epilepsy mutation in $GABA_A$ receptor subunits was the *GABRG2(K328M)* mutation, which is an AD mutation associated with a family with generalized epilepsy with febrile seizures plus (GEFS+) [169]. A lysine residue in the short extracellular domain between transmembrane domain M2 and M3 is mutated to a methionine residue in the mutant γ2 subunit. When expressed in Xenopus oocytes, mutant α1β2γ2(K328M) receptors had decreased peak current amplitude and diazepam enhancement compared to wildtype α1β2γ2 receptors [169]. However, GABAergic current recorded from HEK293T cells expressing the α1β2γ2(K328M) receptors had unchanged current amplitude but accelerated desensitization [170, 171]. Transient analysis suggested that the mutant α1β2γ2(K328M) receptors decreased channel opening rate constant k_{op} by 5 fold, which decreased GABA-induced channel-opening equilibrium constant between the closed- and open-channel forms of the receptor ($\Phi^{-1} = k_{op}/k_{cl}$) [172]. The anticonvulsant pentobarbital and neurosteroid 5α-THDOC increased the ratio of channel-opening and closing rate constants and partially rescued the malfunction of receptors [173, 174]. Single channel currents in HEK293T cells showed that α1βγ2(K328M) receptors had reduced mean open times [170, 171]. When γ2(K328M)

subunits were overexpressed in cultured hippocampal neurons, they were assembled into $GABA_A$ receptors, expressed on the surface membrane, formed clusters as wildtype γ2 subunits, and accelerated deactivation of mIPSCs [175, 176]. These studies suggested that the mutant γ2(K328M) subunits were assembled to $GABA_A$ receptors and trafficked postsynaptic membrane at normal efficiency, but decreased GABAergic mIPSCs by accelerating channel deactivation. The *GABRG2(K328M)* mutation might associate with epilepsy because it decreases GABAergic inhibitory neurotransmission.

The *GABRG2(R82Q) mutation* is an AD mutation identified from a large family patients with epilepsy syndromes including CAE and febrile seizures [177]. The alone is associated with febrile seizures (fifteen individuals) and possibly GEFS+ (three) this family. The possible interactions with other yet to be identified modifier genes contributed to other epilepsy symptoms including typical CAE (eight individuals), myoclonic astatic epilepsy (two individuals), generalized epilepsy with tonic–clonic seizures alone (one individual), partial epilepsy (one individual) and unclassified epilepsy despite evaluation (two individuals) [178]. Transcranial magnetic stimulation (TMS) studies demonstrated that the subjects affected by the *GABRG2(R43Q)* mutation had increased intracortical excitability because of reduced net short-interval intracortical

inhibition and increased intracortical facilitation; the motor thresholds were not altered either at rest or with weak voluntary activation [179]. The *GABRG2(R82Q)* mutation replaced an arginine residue located in the distal N-terminus of the γ2 subunit between interface to a glutamine residue [59, 176]. The mutant subunit might have altered benzodiazepine binding affinity because the Arg82 residue in γ2 subunit is in the of the first high-affinity benzodiazepine binding site at the α/γ2 interface [177, 180]. When expressed in HEK293T cells, mutant α1βγ2(R82Q) receptors had decreased peak current amplitude, but macroscopic current activation and deactivation were not altered, nor was [^{3}H]flunitrazepam binding affinity [170, 180]. However, the total benzodiazepine binding site was decreased in both HEK293T cells expressing α1β2γ2(R82Q) receptors and in the brain of patients carrying the *GABRG2(R82Q)* mutation, reflecting the decreased surface γ2 subunit-containing $GABA_A$ receptor expression [180, 181]. Statistical parametric mapping showed that the greatest change in benzodiazepine binding in human brain occurred in insular and anterior cingulate Further studies showed that the R82Q mutation impaired mutant γ2 subunit oligomerization with patterning subunits and $GABA_A$ receptor assembly [59, 175, 176, 180, 182]. When the γ2(R82Q) subunits were coexpressed with α1β2 subunits in HEK293T cells, most of the mutant γ2(R82Q) subunits were retained in the ER so the

majority of functional $GABA_A$ receptors on the cell membrane are composed of αβ subunits. The γ2(R82Q) subunits in postsynaptic $GABA_A$ receptor clusters were decreased too. The γ2 subunit increases $GABA_A$ receptor channel conductance and opening duration, so the α1β2γ2 receptors are more efficient in conducting Cl^- currents than α1β2 receptors [183]. However, the γ2(R82Q) subunits suppressed endogenous α5 subunit surface expression in cultured hippocampal neurons and decreased tonic current amplitude, suggesting possible dominant negative function [176].

Although no studies of possible seizure phenotypes in $\gamma2^{-/-}$ or $\gamma2^{+/-}$ mice have been reported, decreasing γ2 subunit expression in mouse whole brain ($\gamma2^{+/-}$ mice) or cortex (Emx1Cre × fγ2/+ mice) decreased postsynaptic γ2 subunit expression and induced abnormal mouse behaviors [167, 184]. Knocking out the γ2 subunit at embryonic day 10 in cerebral glutamatergic neurons in heterozygous mice (Emx1Cre × fγ2/+ mice) recapitulated the anxiety behaviors of constitutive γ2 subunit knockout $\gamma2^{+/-}$ mice and reduced adult hippocampal neurogenesis, but heterozygous mice (CaMKIICre2834 × fγ2/+ mice) with knockout of the γ2 subunit from forebrain glutamatergic neurons since postnatal day 17 were not different from wildtype mice [184]. The γ2 subunit expression during embryonic and early postnatal developmental stages accounted for the abnormal

mouse behaviors. Homozygous $\gamma2^{R82Q/R82Q}$ mice died before postnatal day 19, which similar to $\gamma2^{-/-}$ mice [185] and had decreased surface and total γ2 subunit levels in brain tissue and cultured neurons. Heterozygous $\gamma2^{R82Q/+}$ mice carrying only one *GABRG2(R82Q)* allele had decreased surface γ2 subunit expression in cultured neurons, but surface α1 subunit level in cultured neurons was not changed. The $\gamma2^{R82Q/+}$ mice also showed decreased $GABA_A$-mediated synaptic currents in layer II/III cortical pyramidal neurons, but not in thalamic neurons, and behavioral arrest associated with 6-to 7-Hz spike-and-wave discharges (SWDs), which is the clinical hallmark of human CAE (3 7- in humans). The genetic background modified the penetrance of the *GABRG2(R82Q)* mutation as well as the EEG pattern during seizures, which supported the possible gene effects in the human pedigree. The *GABRG2(R82Q)* allele containing a neomycin cassette decreased mRNA expression by 76-91%, which was fully restored after the neomycin cassette was removed [186]. When the neomycin cassette was removed in the forebrain from the temporally and spatially regulated conditional heterozygous $\gamma2^{R82Q/+}$ knockin mice since conception, the mice had similar pentylenetetrazol-induced seizure susceptibility in adulthood as the constitutive heterozygous $\gamma2^{R82Q/+}$ knockin mice. However, when the neomycin cassette was removed from these animals at postnatal day 21, the mice had significantly higher pentylenetetrazol-induced seizure susceptibility in

adulthood than those mice expressing the γ2(R82Q) subunits from conception. Decreasing the γ2(R82Q) subunit expression from conception to P21 made the mice less likely to have seizures, suggesting that the γ2(R82Q) subunit has the potential to impair brain E/I balance with an unknown mechanism. Therefore, the *GABRG2(R82Q)* associates with epilepsy through multiple mechanisms: impairing subunit oligomerization and mutant γ2 subunit-containing $GABA_A$ receptor membrane trafficking, enhancing modifier gene effects, and other dominant negative effects, possibly inhibiting tonic $GABA_A$ receptor currents [176].

The *GABRG2(R177G)* mutation was identified from a family of patients with febrile seizures [187]. The mutation is located in exon 4 of the *GABRG2* gene and changed the highly conserved Arg177 residue in the second benzodiazepine binding site in the γ2 subunit N-terminus to glycine. Mutant α1β2γ2(R177G) receptors have increased desensitization and reduced sensitivity to diazepam, but the same peak current amplitude and Zn^{2+} inhibition as wildtype α1β2γ2 receptors. The molecular mechanisms of the *GABRG2(R177G)* mutation are still unclear. Future studies focusing on the mutant protein maturation and mutant receptor biogenesis are needed to elucidate the molecular defect of this mutation in epilepsy.

The *GABRG2(P83S)* mutation was identified in a large French Canadian family exhibiting febrile seizures and idiopathic generalized epilepsy over three generations [188]. The nine individuals carrying this mutation in heterozygous form had febrile seizures and/or other epilepsy syndromes. The mutation changed the highly conservative γ2 subunit Pro83 residue next to the Arg82 residue described above into a serine residue. The α1β2γ2(P83S) receptors had normal channel kinetics and responded normally to $GABA_A$ receptor modulators Zn^{2+} and diazepam. Future studies are needed to explore how the *GABRG2(P83S)* mutation induces epilepsy.

GABRG2 ***gene nonsense mutations***

The *GABRG2(Q390X)* mutation was identified in a family with generalized epilepsy and febrile seizures [189, 190]. Genetic study showed that the mutation interacted with an unidentified modifier gene and caused Dravet syndrome in individual carrying the mutation in a heterozygous form [189]. The mutation changed the Gln390 residue to a premature-translation termination codon (PTC) in the last exon, is translated to a truncation γ2 subunit that lacks its C-terminal 78 amino acids [191]. mutant subunit mRNA was not degraded by nonsense mediated decay (NMD), consistent with the previous study that the PTC at the last exon did not activate NMD [192]. The

mutant γ2(Q390X) subunit was retained in the ER as an immature peptide, but it oligomerized with partnerning wildtype subunits, retained them in the ER, and the degradation of wildtype subunits though ER-associated degradation dependent on ubiquitinylation and proteasome degradation [191]. Currents recorded from $\alpha1\beta2\gamma2^{Q390X/+}$ receptors were reduced compared to those from hemizygous $\alpha1\beta2\gamma2^{+/-}$ receptors. Mutant γ2(Q390X) subunits formed SDS-resistant, high-molecular-mass complexes shortly after they were synthesized and were degraded significantly slower wildtype γ2 subunits [193]. The function of the γ2(Q390X) subunit-containing SDS-SDS-resistant, high-molecular-mass complexes is not clear, but protein aggregation has been linked to neurodegenerative diseases, such as Alzheimer's disease (beta-amyloid aggregation), “mad cow” disease (prion aggregation), and Huntington's disease aggregation) [194]. The *GABRG2(Q390X)* mutation generated a mutant γ2 subunit that was not trafficked to the cell membrane, decreased surface wildtype receptor expression a dominant negative manner, and formed protein aggregations that could interfere with normal cell functions.

The *GABRG2(Q40X)* mutation is a heterozygous nonsense mutation identified from twin sisters with Dravet syndrome [195]. The mutation changed the Gln40 residue

at the end of the γ2 subunit signal peptide to a PTC, which is located at the second exon γ2 subunit mRNA. The mutant mRNA is likely to be degraded by NMD, so the *GABRG2(Q40X)* mutation may associate with epilepsy though haplo-insufficiency, but this has not been demonstrated yet.

The *GABRG2(W429X)* mutation was identified in a family with GEFS+ [196]. The mutation changed the Trp390 residue to a PTC and truncated the γ2 subunit in the intracellular loop between the third and fourth transmembrane segments. Because the PTC is located at the last exon, the mutant mRNA should not be degraded by NMD. Therefore, the mutant allele is expected to be expressed as a truncated γ2 subunit with loss of the C-terminal 39 amino acids. Further studies are needed to elucidate the function of the γ2(W390X) subunit and demonstrate how the *GABRG2(W390X)* mutation associates with epilepsy.

The *GABRG2(R136X)* mutation was identified from a family with febrile seizures and GEFS+ [197]. The mutation changed Arg136 residue to a PTC, which is located at the exon 4 of γ2 subunit mRNA. The mRNA should be degraded by NMD, but this has not been reported. The undegraded mRNAs would be translated as truncated γ2 subunits containing the wildtype signal peptide and the N-terminal 97 amino acids. When

expressed in a cultured neuronal cell line, mutant γ2(R136X) subunits were detected in ER and decreased $GABA_A$ receptor clusters on the cell surface relative to wildtype γ2 subunits. Further studies are needed to elucidate whether and how the γ2(R136X) subunits affects $GABA_A$ receptor biogenesis, and whether the GABRG2(R136X) is associated with epilepsy through mechanisms other than haplo-insufficiency.

GABRG2 ***gene intron splice donor site mutation***

The *GABRG2(IVS6+2T→G)* mutation is an AD mutation identified from a two generation family with CAE and febrile seizures [198]. The mutation changed the *GABRG2* gene intron 6 splice donor site sequence from GT to GG and prevented the splicing from happening at this site [199]. It was predicted that the mutant intron 6 might splice out between the wildtype acceptor site and donor site of intron 5 or an alternative splice donor site 375 bp or 758 bp downstream of the original wildtype donor site, a mutant mature mRNA containing PTC in the middle, and get degraded by NMD. However, not all the NMD-susceptible mRNAs are removed by NMD [200, 201]. NMD efficiency is different among cell types, and undegraded mRNAs are translated to It is possible that the mutant γ2(IVS6+2T→G) subunit mRNAs are expressed to proteins, and their amount is higher in cells that have lower NMD efficiency. Further studies are

needed to decide the intron splice pattern of the mutant mRNA, evaluate the function of possible translation products, and demonstrate the association with epilepsy.

GABRA1 gene mutations

GABRA1 *gene expression pattern and α1 subunit function*

The human $GABA_A$ receptor α1 subunit is encoded by the *GABRA1* gene, which also located on human chromosome 5q34 in a cluster with *GABRB2, GABRA6*, and *GABRG2* [143]. In adult mouse brain, the α1 subunit is the most abundant α subunit and is colocalized with β2 and γ2 subunits in virtually all brain regions [13, 147]. The α1 subunit mRNA level is weak at E18 and during the first prenatal week, dramatically increases during the second postnatal week and then progressively increases to the adult level [144, 150, 202]. Using *in situ* hybridization, α1 subunit mRNA was shown to be expressed at high levels in the cerebral cortex, pyramidal cell layer of the hippocampus, granule cell layer of the dentate gyrus, thalamus, septum, inferior colliculi, and globus pallidus in adult rat brain [146, 147, 202]. The expression in the cerebellar cortex is mainly in the Basket cells, Purkinje cells, and granule cells [145, 146, 202]. The expression in the Bergmann glia is low. The α1 subunit mRNA is expressed in the

olfactory bulb in mitral cells, and in the glomerular layer. The brainstem also has prominent α1 subunit expression. This pattern is consistent with α1 subunit levels shown in immunohistochemistry studies [148].

Despite the fact that the α1 subunit is the most abundant α subunit and ubiquitously expressed in brain, $\alpha1^{-/-}$ mice are viable, fertile, and show no spontaneous seizures [203, 204]. The loss of α1 subunit induced adaptive responses [205, 206]. The α2 and α3 subunits were upregulated by 37% and 39% in $\alpha1^{-/-}$ mice cerebral cortex; the α6 subunit in the cerebellum is decreased by 38%, while β2/3 subunits were decreased by 65% and γ2 subunits were decreased by 47%. The total $GABA_A$ receptor number was decreased more than 50% [204]. These compensatory responses probably happened at the protein level [206-208], which is also suggested by microarray studies demonstrating that genes involved in protein trafficking and metabolism were over-represented in $\alpha1^{-/-}$ mice [206].

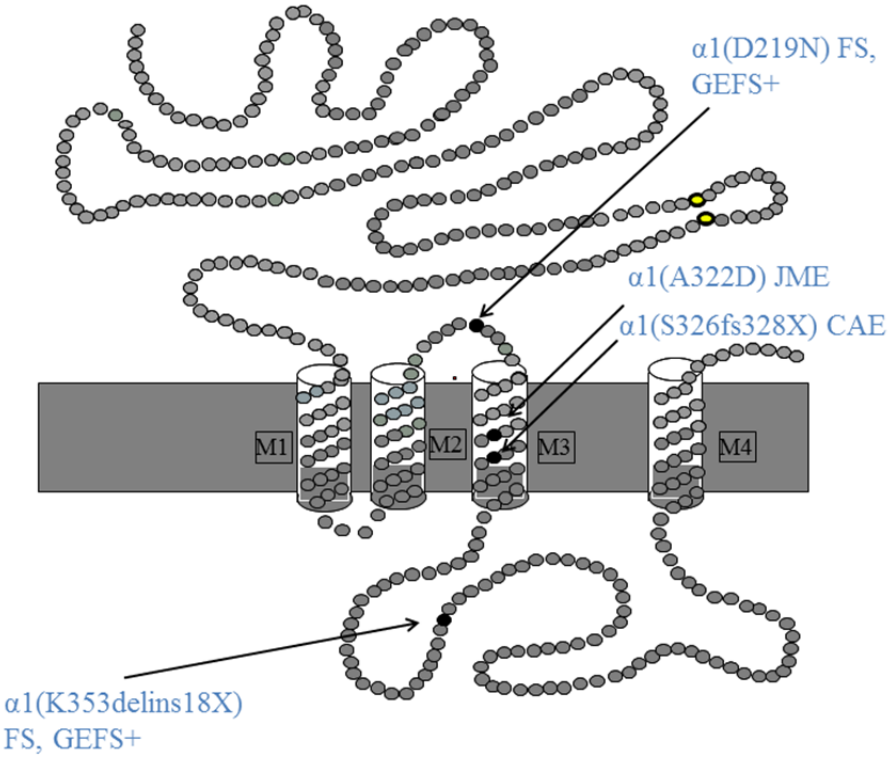

Figure 1.3 $GABA_A$ receptor α1 subunit mutations associated with genetic epilepsy syndromes.

Predicted membrane topology of $GABA_A$ receptor α1 subunit consists of a large extracellular domain at the N-terminus, four transmembrane domains (M1–M4) and a large cytoplasmic domain. In this figure, we depicted $GABA_A$ receptor α1 subunit mutations associated with genetic epilepsy syndromes at their appropriate protein domain within the subunit.

GABRA1 ***gene missense mutations***

The *GABRA1(A322D)* mutation is a missense mutation found in all family members who were affected with an AD form of JME (ADJME) [209]. All patients had one wildtype and one mutant α1(A322D) subunit allele. Homozygous α1(A322D)βγ receptors had ~10% of the peak current and an ~200-fold higher GABA EC_{50} value compared with wildtype α1βγ receptors [209]. Heterozygous expression of mutant and wildtype α1 subunits at a 1:1 ratio produced smaller currents than wildtype and much larger currents than homozygous mutant transfections [210]. The mutation introduces a negatively charged aspartate into the middle of the α1 subunit third transmembrane domain, thus destabilizing insertion of the M3 domain into the lipid bilayer and α1 subunit misfolding [53]. The misfolded α1(A322D) subunit was primarily degraded by ER-associated degradation before receptor assembly [211], or rapidly endocytosed membrane insertion and degraded by lysosomal degradation [212]. Recent studies demonstrated the α1(A322D) subunit oligomerized with other $GABA_A$ receptor subunits and retained them in the ER, thus decreasing membrane levels of $GABA_A$ receptors in a dominant negative manner [213]. Interestingly, it preferentially decreased α3β2γ2 receptor membrane level by a greater amount than α1β2γ2 receptors. The

GABRA1(A322D) mutation would induce epilepsy though both *GABRA1* gene haplo-haplo-insufficiency and α1(A322D) subunit dominant negative effects.

The AD *GABRA1(D219N)* mutation was identified from the same studies in the French Canadian family with febrile seizures and idiopathic generalized epilepsy that identified the *GABRG2(P83S)* mutation [188]. The negatively charged residue Asp219 at the extracellular N-terminal domain was mutated to a non-charged polar residue N219 in the mutant α1(D219N) subunit. The mutation was proposed to reduce the interaction between D129 and K247, thus destabilize the $GABA_A$ receptor opening state similar to the *GABRG2(K328M)* mutation. Consistent with this hypothesis, the α1(D219N) subunit was assembled with β2 and γ2 subunits, trafficked to cell membrane, but the GABA-evoked currents in α1(D219N)β2γ2 coexpression had faster desensitization [188]. However, the γ2(K328M) mutation only affected receptor kinetics, and α1β2γ2(K328M) receptors had peak current amplitudes similar to α1β2γ2 wildtype receptors [170], while α1(D219N)β2γ2 receptors had peak current amplitudes in between of α1β2γ2 and α1β2 receptors, suggesting that the α1(D219N) subunit had decreased oligomerization and receptor assembly. Further studies are needed to explore how the *GABRA1(D219N)* mutation induces epilepsy.

GABRA1 ***gene deletion mutations***

The *GABRA1(S326fs328X)* mutation is a *de novo* AD mutation identified from a patient with CAE [214]. A single nucleotide Cytosine975 was deleted from codon of residue S326 in exon 8, causing a frameshift, and creating a PTC at residue L328, 74 bps upstream of the last exon–exon junction. The mutant α1(S326fs328X) subunit was truncated from the middle of the third transmembrane domain. It totally abolished GABA-evoked current recorded from cells cotransfected with α1(S326fs328X)β2γ2 subunits [214]. The mutant mRNA was degraded by NMD, and the undegraded mRNA was translated to protein that was further degraded by ER-associated degradation through the ubiquitin-proteasome system [201]. The process of both NMD and ER-associated degradation decreased the total level of α1(S326fs328X) subunits to less than 3% of wildtype α1 subunit total levels. The *GABRA1(S326fs328X)* mutation would induce epilepsy in patients through *GABRA1* gene haplo-insufficiency. Because $\alpha 1^{-/-}$ mice lacking α1 subunits did not have spontaneous seizures [203, 204], the pathogenesis of *GABRA1(S326fs328X)* mutation might also involve other unidentified modifier genes.

The AD *GABRA1(K353delins18X)* mutation was the third mutation identified the genetic screening in the family that identified *GABRG2(P83S)* and *GABRA1(D219N)*

mutations [188]. The four patients carrying this mutation over two generations all had epilepsy. The mutant allele had a 25 bp insertion close to the intron 10 splice acceptor site. RT-PCR of the patient's mRNA demonstrated a 1242 bp fragment of intron 10 in mature mutant mRNA, resulting in deletion of 103 amino acids from the α1 subunit C-C-terminus containing the last transmembrane domain TM4, and translation of an 18 amino acid intronic sequence until the PTC [188]. The mutant α1(K353delins18X) subunit was truncated and retained inside of the cell. No GABA-evoked current was recorded from cells cotransfected with α1(K353delins18X)β2γ2 subunits. The *GABRA1(K353delins18X)* mutation might induce epilepsy by *GABRA1* gene haplo-haplo-insufficiency, but further studies are needed to elucidate the mechanism of epileptogenesis associated with this mutation.

GABRB3 **gene mutations**

GABRB3 ***gene expression pattern and β3 subunit function***

The human $GABA_A$ receptor β3 subunit is encoded by the *GABRB3* gene, which also located at human chromosome 15q11.2-q12 in a cluster with *GABRA5*, and *GABRG3* genes [143]. The mouse *GABRB3, GABRA5* and *GABRG3* gene cluster is located at

mouse chromosome 7. The *GABRB3* gene is located in the Angelman/Prader-Willi of human chromosome 15 (15q11q13) [215]. Angelman syndrome results from deletion of a chromosome 15 fragment carrying the *GABRB3* gene [216]. The β3 subunit mRNA level is high during development, peaks at P12, and then decreases to adult levels during the first and second postnatal weeks [144, 151]. Studies using *in situ* hybridization showed that β3 subunit mRNA was detected in E14 embryonic brain in the olfactory thalamus, and spinal cord, then drastically increased in the hippocampus, cerebral cortex, and internal granule cell layer of the cerebellum until the first postnatal week [144, 151]. The β3 subunit mRNA was also detected in the external granule cell layer of the cerebellum, caudate, thalamic nuclei, and superior and inferior colliculi at this age. In adult rat brain, β3 subunit mRNA is expressed at high levels in olfactory bulb, cortex, caudate-putamen, accumbens nucleus, hypothalamus, amygdala, hippocampal formation, and at moderate levels in thalamus, superior and inferior colliculus, and many areas of the brainstem [146, 147, 217]. Expression in the cerebellar cortex is mainly in Purkinje cells and granule cells. This pattern is consistent with β3 subunit protein level as shown in immunohistochemistry studies [148].

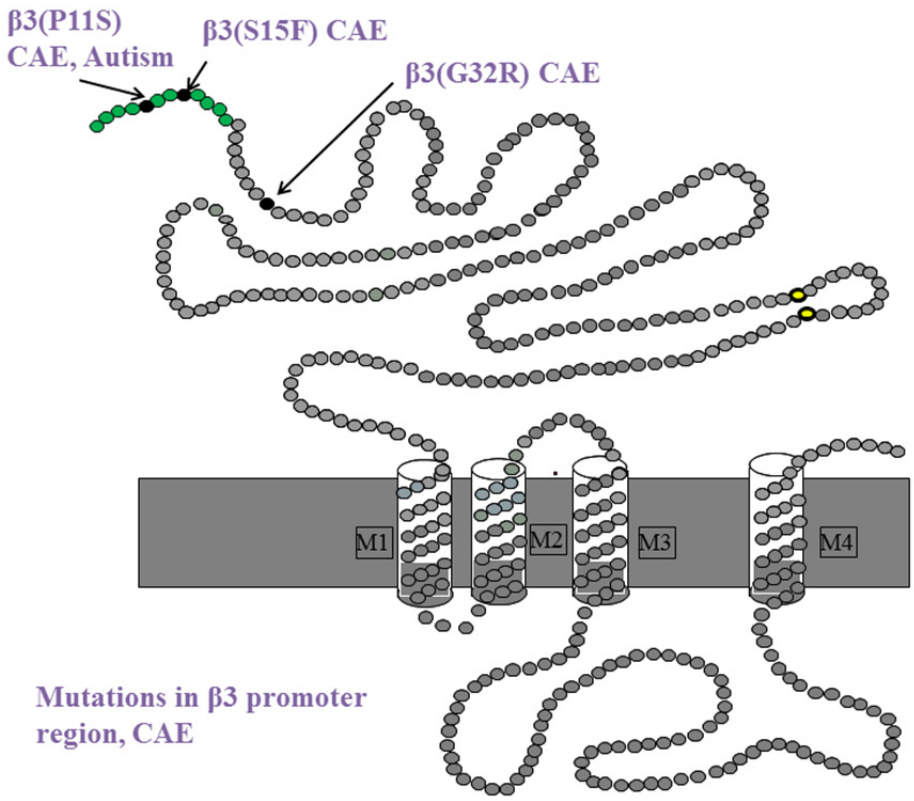

Figure 1.4 $GABA_A$ receptor β3 subunit mutations associated with genetic epilepsy syndromes.

$GABA_A$ receptor β3 subunits are translated as a precursor protein whose signal sequence (green) is removed leaving a mature protein consisting of a large extracellular domain at the N-terminus, four transmembrane domains (M1–M4) and a large cytoplasmic domain. In this figure, we depicted $GABA_A$ receptor β3 subunit mutations associated with genetic epilepsy syndromes at their appropriate protein domain within the subunit.

GABRB3 ***gene mutations***

The *GABRB3* mutations P11S, S15F and G32R were identified from families with CAE [218]. The *GABRB3(P11S)* mutation was also identified from multiple families with autism spectrum disorders [219]. The *GABRB3(P11S)* and *GABRB3(S15F)* mutations are in exon 1a of the *GABRB3* gene and encodes the β3 subunit signal peptide. The *GABRB3(G32R)* mutation is in the β3 subunit mature peptide next to the first N-glycosylation site, N33 residue, in the N-terminus. All three mutations were predicted to change β3 subunit N-terminus secondary structure, but not the signal peptide cleavage [218]. All three mutant subunits had increased glycosylation and decreased peak amplitudes of GABA-evoked whole cell currents when they were coexpressed with α1 β2 subunits [218-220]. Further studies demonstrated that these three mutations might affect $GABA_A$ receptor biogenesis through different mechanisms. With $\alpha1\beta3(P11S)^{HA}\gamma2S$ subunit coexpression, there were reduced surface levels of $\beta3(P11S)^{HA}$ subunits [219], while with $\alpha1\beta3(G32R)^{HA}\gamma2L$ subunit coexpression there was increased surface expression of β3 subunits but decreased surface expression of γ2L subunits, suggesting that the mutation altered surface $GABA_A$ receptor stoichiometry [220]. The

α1β3(G32R)γ2L receptors had significantly reduced macroscopic current density and impaired gating with shorter mean open time in single channel currents, which could be because the mutation altered salt bridges at γ2−β3 and β3−β3 subunit interfaces that are important for subunit oligomerization [220]. These three mutations might induce epilepsy through *GABRB3* gene haplo-insufficiency. This hypothesis is consistent with EEG studies that homozygous *GABRB3* knock-out mice demonstrated epileptiform complexes and seizures responsive to antiepileptic drugs [221].

Also consistent with the important role of *GABRB3* in epilepsy, SNPs identified in the region from the exon 1a promoter to the beginning of intron 3 in *GABRB3* haplotype 2 were found to have a significant association with CAE [222]. The disease-associated haplotype 2 promoter reduces the nuclear protein binding affinity, resulted in significantly lower transcription activity than the haplotype 1 promoter that is over-represented in the controls. *In silico* analysis suggested the decreased binding of the neuron-specific transcriptional activator N-Oct-3 in haplotype 2 promoter. The *GABRB3* haplotype 2 promoter could significantly decrease β3 subunit mRNA and protein level, and induce epilepsy through *GABRB3* halpo-insufficiency.

GABRD variants.

GABRD *gene expression pattern and δ subunits function.*

Human $GABA_A$ receptor δ subunit is encoded by the *GABRD* gene, which is located on human chromosome 1 (1p36.3) but not clustered with any other $GABA_A$ receptor subunit genes [223]. Deletion of the 1p36 region is associated with neurological defects such as severe psychomotor retardation, seizures, growth delay, and dysmorphic features [224-227]. Angelman syndrome results from deletion of a chromosome 15 fragment carrying the *GABRB3* gene [216]. The neurobehavioral symptoms of Angelman syndrome patients are very similar to 1p36 deletion syndrome patients, so the *GABRD* gene has been suggested to underlie these defects [223]. Further studies confirmed that the *GABRD* gene is a susceptibility locus for epilepsy [228].

Similar to the α1 subunit, the δ subunit mRNA level is only detected at low levels after birth and progressively increases to the adult level [144]. Studies using *in situ* hybridization showed that the δ subunit mRNA level is lower than γ2 subunit levels in adult rat brain [145-147]. The δ subunit is mostly expressed at high levels in the cerebellar cortical gray matter, at low level in dentate gyrus and subiculum in hippocampus, cerebral cortex, and granule layer of olfactory bulb [146, 147]. An

immunohistochemistry study confirmed this expression pattern and showed that the δ subunit distribution is similar to that of high affinity $GABA_A$ receptors identified by autoradiography [148, 229, 230].

GABRD *gene variants*

GABRD(E177A) and *GABRD(R220H)* are both *GABRD* variants identified from two small families with an AD generalized epilepsy similar to GEFS+ [228]. Both variants are located in the δ subunit N-terminus, either adjacent to one of the two that form a disulfide bond (E177A), or between the cys-loop and the beginning of the transmembrane domain (M1) (R220H). Compared with wildtype receptors, α1β2Sδ(E177A) and α1β2Sδ(R220H) receptors had significantly decreased $GABA_A$ receptor current amplitudes [228]. This could be because both variants decreased surface levels of mutant receptors, as well as the single channel mean channel open time [231]. Although δ subunit-containing $GABA_A$ receptors are extrasynaptic [99, 232, 233], these receptors have slower and less extensive desensitization and high sensitivity to GABA, thus transporting more Cl^- into the cell [16]. The *GABRD(E177A)* and *GABRD(R220H)* variants could cause disinhibition by impairing δ subunit-containing $GABA_A$ receptor function, thus inducing epilepsy. However, a genetic study showed that the frequency of

the *GABRD* His220 allele in epilepsy patients was not different from controls [234], suggesting that the *GABRD(R220H)* variant could be a modifier or susceptibility gene for epilepsy.

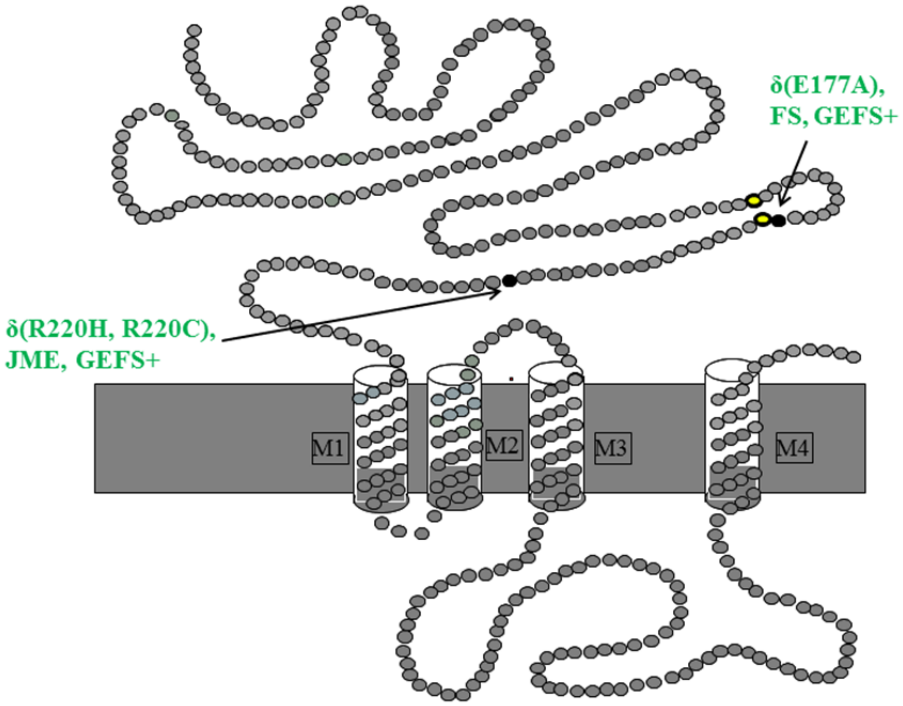

Figure 1.5 $GABA_A$ receptor δ subunit variations associated with genetic epilepsy syndromes.

Predicted membrane topology of $GABA_A$ receptor δ subunit consists of a large extracellular domain at the N-terminus, four transmembrane domains (M1–M4) and a large cytoplasmic domain. In this figure, we depicted $GABA_A$ receptor δ subunit variants associated with genetic epilepsy syndromes at their appropriate protein domain within the subunit.

Chapter 2

The Intronic *GABRG2* Mutation, IVS6+2T→G, Associated with CAE Altered Subunit mRNA Intron Splicing, Activated Nonsense-Mediated Decay and Produced a Stable Truncated γ2 Subunit

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Abstract

The intronic *GABRG2* mutation, IVS6+2T→G, was identified in an Australian family with childhood absence epilepsy (CAE) and febrile seizures [198]. The *GABRG2* intron 6 splice donor site was found to be mutated from GT to GG. We generated wildtype and mutant γ2S subunit bacterial artificial chromosomes (BACs) driven by a CMV promoter and expressed them in HEK293T cells and expressed wildtype and γ2S subunit BACs containing the endogenous *hGABRG2* promoter in transgenic mice. Wildtype and mutant *GABRG2* mRNA splicing patterns were determined in both BAC transfected HEK293T cells and transgenic mouse brain, and in both, the mutation abolished intron 6 splicing at the donor site, activated a cryptic splice site, generated partial intron 6 retention and produced a frame shift in exon 7 that created a premature translation-termination codon (PTC). The resultant mutant mRNA was either degraded partially by nonsense mediated mRNA decay (NMD) or translated to a stable, truncated subunit (the γ2-PTC subunit) containing the first 6 *GABRG2* exons and a novel frame-frame-shifted 29 aa C terminal tail. The γ2-PTC subunit was homologous to the mollusk acetylcholine binding protein (AChBP) but was not secreted from cells. It was retained

the ER and not expressed on the surface membrane, but it did oligomerize with α1 and subunits. These results suggested that the *GABRG2* mutation, *IVS6+2T→G,* reduced surface αβγ2 receptor levels, thus reducing GABAergic inhibition, by reducing *GABRG2* transcript level and producing a stable, nonfunctional truncated subunit that had a dominant negative effect on αβγ2 receptor assembly.

Introduction

Epilepsy is one of the most common neurological disorders, affecting up to 3% of the general population. One-third to one-half of all epilepsy syndromes have a genetic basis [235], and patients with genetic epilepsy syndromes (GESs) have absence, myoclonic, and/or generalized tonic-clonic seizures [236]. Most mutations associated with genetic epilepsies have been identified in genes encoding voltage- or ligand-gated ion-channels [237].

$GABA_A$ receptors mediate the majority of inhibitory neurotransmission in the Epilepsy mutations have been identified in $GABA_A$ receptor subunit genes (*GABR*) *GABRA1, GABRB3* and *GABRG2* [142], but most of the mutations are in *GABRG2*. The

α1, β2 and γ2 subunits form the most abundant $GABA_A$ receptor subtype in the CNS [13, 15, 16], and the γ2 subunit plays a critical role in brain function. In mouse brain, approximately 75-80% of $GABA_A$ receptors contain the γ2 subunit [10]. Mice lacking γ2 subunits ($\gamma2^{-/-}$ mice) died shortly after birth [56]. These $\gamma2^{-/-}$ mice lost 94% of their benzodiazepine binding sites but GABA binding sites were only decreased slightly. The γ2 subunit is required for maintaining postsynaptic $GABA_A$ receptor clustering [97]. Heterozygous $\gamma2^{+/-}$ mice had significantly decreased benzodiazepine binding sites and increased extrasynaptic $GABA_A$ receptor radioligand binding sites in the CNS, but unchanged muscimol binding sites [166], and these animals had decreased $GABA_A$ receptor clustering in hippocampus and cerebral cortex [167]. The $\gamma2^{+/-}$ mice had increased anxiety [167], a behavior recapitulated in γ2 subunit knockdown mice [168]. Epilepsy, however, has not been reported in $\gamma2^{-/-}$ or $\gamma2^{+/-}$ mice.

GABR mutations have been associated with seizures ranging from relatively absence and/or febrile seizures to severe myoclonic seizures [138]. The most well characterized γ2 subunit missense mutation is *GABRG2(R82Q)* associated with childhood absence epilepsy and febrile seizures [177]. This mutation impaired α1β2γ2 receptor assembly, retained mutant γ2 subunits in the endoplasmic reticulum and reduced receptor

surface trafficking [59, 170, 176]. Knock-in mice harboring the *GABRG2(R82Q)* mutation had reduced cell surface γ2 subunit expression and reduced cortical inhibition, even in heterozygous animals [185]. Mice heterozygous for the mutation also had absence seizures.

GABRG2(IVS6+2T→G) is a mutation of the intron 6 splice donor site from GT to GG identified in an Australian family with CAE and febrile seizures [198]. The basis for the epilepsy in this family results from the specific alteration in splicing of *GABRG2(IVS6+2T→G)* mRNA and on subsequent translation of protein. To determine this splicing pattern, we generated wildtype and mutant *GABRG2*(IVS6+2T→G) BACs and determined how the IVS6+2T→G mutation altered intron 6 splicing and γ2 subunit expression in HEK293T cells and transgenic mouse brain. We then characterized the biogenesis and function of the translated mutant γ2 subunit.

Materials and Methods

Expression vectors with $GABA_A$ receptor subunits

The coding sequences of human α1, β2, γ2S and γ2L $GABA_A$ receptor subunits from the translation initiation codon ATG to the stop codon were cloned into pcDNA3.1 expression vectors (Invitrogen) or pLVX-IRES-ZsGreen1 vectors (Clontech) as previously described [211]. The cDNA encoding the HA peptide, YPYDVPDYA, was introduced between the 4th and 5th amino acids of mature γ2S and γ2L subunits, which has been reported to be a functionally silent position [50]. In recent studies, the position of the mutant and variant amino acids in α1, β3 and δ subunits have been specified in the immature peptide that includes the signal peptide, but mutations in γ2 subunit have been reported in the mature peptide, excluding the signal peptide. For consistency, in this study the positions of γ2 subunit mutations were designated also in the immature peptide.

The BAC clone number RP11-1035I20 (BACPAC Resources; http://bacpac.chori.org) contains a human chromosome 5 fragment that included the wildtype human *GABRG2* gene genomic sequence (and thus a complete intron 6) and 20 kb upstream and 40 kb downstream human chromosome 5 sequences (Figure 1A). The

BAC sequence was confirmed by restriction enzyme digestion and direct DNA The BAC clone was recombined with the pEHHG vector [238], which contained the reporter gene driven by the HSV early gene promoter. In target cells expressing these BAC vectors, eGFP fluorescence was detected. In this BAC clone *hGABRG2* was predicted to be driven by the promoter sequence in the 20 kb upstream human sequence, while the eGFP was driven by a separate HSV promoter, and thus, expression eGFP was independent of expression of *hGABRG2*. To introduce the point mutation in *hGABRG2* at the IVS6+2 position, we used *galK* facilitated recombineering [239, 240]. The *galK* gene encodes galactose kinase and provides both positive and negative factors in this technique. Using *galK* facilitated BAC recombineering, the human chromosome sequence upstream of the *GABRG2* translation initiation sequence ATG was replaced with a CMV promoter (Figure 1A). Unless otherwise specified, wt and mutant *hGABRG2* BACs were driven by the CMV promoter and contained the eGFP reporter gene.

Cell culture, transfection and RNAi

Human embryonic kidney cells (HEK293T) (ATCC, CRL-11268) and HeLa cells (ATCC, CCL-2) were incubated at 37°C in humidified 5% CO_2, 95% air and grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were transfected with cDNAs using the FuGENE 6 transfection reagent (Roche Applied Science) or Lipofectamine 2000 (Invitrogen) at a DNA:Transfection Reagent ratio of 1:3 according to the manufacturer's instructions. The transfected cells were harvested after 36 hrs in culture for the following experimental protocols.

Sprague Dawley rat cortex was dissected from E18 embryos and dissociated using 0.25% trypsin and mild trituration [241]. Neurons were plated on poly-l-ornithine-coated coverslips in DMEM (Invitrogen) supplemented with 10% horse serum, 2 mM glutamine and 1 mM Na-Pyruvate. After 4 hours, medium was replaced by 1 ml of serum-free culture medium containing Neurobasal with B27 supplement, glutamine (2 mM) and penicillin/streptomycin. Cultures were maintained at 36°C in a humidified CO_2 incubator for up to 4 weeks and fed once a week. Cultured neurons were transfected with Lipofectamine 2000 (Invitrogen) at 7 DIV according to the manufacturer's instructions.

mixture of 1 μg of DNA and 3 μl of lipofectamine in 60 μl of Opti-MEM (Invitrogen) added to the well. One hour after incubation, the culture media containing the Lipofectamine/DNA complex was completely replaced with fresh serum-free Neurobasal/B27 culture media. Neurons were immuno-stained 7 days after transfection.

NMD efficiency was decreased by knocking down the essential factor UPF1. Silencer select pre-designed and validated siRNA (Ambion, siRNA ID s11926) was transfected to cells using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's manual. Twenty-four hours later the same cells were transfected again with the wildtype or mutant BAC constructs and harvested two days later for RT-PCR. The efficiency of UPF1 knockdown was confirmed by Western blot.

RNA extraction, RT-PCR and Taqman real-time qPCR

Total RNAs were extracted from transfected HEK293T cells by using the PerfectPure RNA Cultured Cell kit (5 Prime) following the manufacturer's protocol. RNA in mouse brain tissue was expressed by TRIzol reagent (Invitrogen) and PureLink RNA mini kit (Invitrogen) according to manufacturer's manual, and human total brain RNA was obtained from Ambion. 200 ng total RNA of each sample was reverse

transcribed to cDNA in a 10 μl volume using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems). The transcribed cDNA was used as a template perform regular PCR using Expand High Fidelity PCR Kit (Roche Applied Sciences) following the manufacturer's manual. One μl of the 50 times diluted transcribed cDNA was mixed with Taqman® Universal PCR Master Mix (Applied Biosystems) and Taqman® probes in a total volume of 5 μl for the Taqman® qPCR experiments. Taqman® probe detecting human *GABRG2* gene mRNA, human GAPDH gene, 18S rRNA, or eGFP (part number 4331348 [Custom Taqman Gene Expression Assay were used. Each sample was run in triplicates, and the average threshold cycle (Ct) value of each sample was calculated by the Sequence Detection System v2.3 Standard Edition (Applied Biosystems). The average Ct values of *GABRG2* gene mRNA were normalized to the endogenous human GAPDH, 18S rDNA or eGFP amount, and the normalized Ct values of samples were compared to get the relative RNA abundance.

Generation and maintenance of *hGABRG2* BAC transgenic mice

The cesium chloride density centrifugation purified BAC DNAs were microinjected into the male pronucleus of C57BL/6J F1 fertilized mouse embryos and

implanted into pseudopregnant ICR surrogate mice by the Vanderbilt Transgenic/ES Cell Shared Resource Facility. Founder mice were bred to C57BL/6J mice to establish transgenic lines. All animals used in these studies were handled in strict compliance with the guidelines of the American Association for Laboratory Animal Science and the Vanderbilt University Institutional Animal Care and Use Committee Protection of Research Subjects.

Transgenic mouse genotyping PCR

Mouse tail samples collected at P14-21 were extracted using red Extract-N-AMP tissue PCR kit (Sigma) according to manufacturer manual. Forward primers binding to either HA tag (primer sequence: TACCCCTACGACGTGCCCGACTACGCC) or intron 1/exon 2 border (GTAATCTATGTGTTTTTTGACCAATATGTTTTTTCTTAGCTTCACTAGCCAGA AATCTG) and reverse primer binding to intron 2 (CACCTCTCCCACTCATAGGCCTGAATG) were used for genotyping. PCR cycling conditions were: 95 °C 5 min initial denature step; 95 °C 1 min/68 °C 1 min/72 °C 1 min (30 cycles); 72 °C 5 min final step.

Immunohistochemistry

Brains were removed from CO_2 euthanized mice, fresh frozen in powdered dry ice, and stored at -80°C until sectioned. Parasagittal sections, 20-μm thick, were prepared with a cryostat (CM1950, Leica Microsystems), and stored at -80°C until immunostaining [242]. Brain slices were fixed and permeablized with 2% paraformaldehyde (Sigma) in PBS for 2 minutes, and washed with PBS. Slices were incubated overnight in rabbit monoclonal anti-HA epitope-tagged antibodies (1:500; clone C29F4, Cell Signaling) in PBS with 0.2% Triton-X (Sigma) to detect HA epitope-tagged γ2 subunits, following by two hour incubation in IRDye800 conjugated donkey anti-rabbit IgG secondary antibodies at 1:1000 dilution in PBS with 0.2% Triton-X. Immunolabeled slices were scanned with Odyssey imaging system (Li-cor) after air dry. Scan parameters were: resolution 21μm, quality highest, focus offset 0.0 mm, intensity 5.0 in both 700 and 800 channels. The 700 channel fluorescence signal was scanned to show autofluorescence of the brain sections. Scanned images were analyzed with Odyssey V3.0 (Li-cor).

Immunocytochemistry and confocal microscopy

HEK293T cells were plated on poly-L-ornithine-coated, glass-bottom imaging dishes at a density of 3 x 10^5 cells/dish and cotransfected with 0.5 μg each of human subunit plasmid. Cells were fixed with 1% paraformaldehyde for 15 minutes to stain surface proteins, or permeabilized with CytoPerm (BD Biosciences) for 15 minutes to stain total proteins. The fixed/permeabilized cells were stained with rabbit polyclonal BIP antibodies (Abcam) for an hour, then a mixture of Alexa 568 conjugated donkey anti-rabbit secondary antibodies, Alexa 488 conjugated mouse monoclonal HA antibodies, and Alexa 647 conjugated mouse monoclonal α1 subunit antibodies (Millipore) for an hour. BIP protein (GRP78) is an ER specific marker. BIP antibodies visualized ER in total staining, and showed membrane integrity in surface staining.

Neurons were fixed with 4% paraformaldehyde/4% glucose in PBS for 15 to stain surface proteins, or permeablized with CytoPerm (BD Biosciences) for 15 to stain total proteins. Coverslips were then blocked for 1 hour with 10% BSA in PBS, and incubated in mouse monoclonal antibody against the HA-epitope tag (Covance) and rabbit polyclonal antibodies against ER marker BIP (Abcam) for 2 hours, followed by Alexa568 conjugated donkey anti-mouse IgG antibodies (Invitrogen) and Alexa647

conjugated donkey anti-rabbit IgG antibodies (Invitrogen) for 1 hour. Antibodies were diluted in 4% BSA in PBS for surface staining, or in 4% BSA in PBS containing 0.2% Triton X-100 for total staining. Coverslips were mounted with 5% n-propyl gallate (Sigma) in PBS/Glycerol. The ZsGreen translated from the pLVX-IRES-ZsGreen1 vector (Clontech) was a marker for transfected neurons.

Confocal experiments were performed in part using the VUMC Cell Imaging Shared Resource (supported by NIH grants CA68485, DK20593, DK58404, HD15052, DK59637 and EY08126). Images were obtained using a Zeiss LSM 510 META inverted confocal microscope. Stained HEK293T cells or cultured neurons were excited with the 488 nm laser for the Alexa 488 fluorophore or ZsGreen signal, 543 nm laser for the Alexa 568 fluorophore signal and 633 nm laser for the Alexa 647 fluorophore signal. We adjusted the pinhole of all channels to obtain 1 μm sections from HEK293T cells, or 2 μm sections from cultured neurons. In each experiment, we adjusted the laser intensity and detector sensitivity to utilize the full linear range of detection. Images were obtained with 8-bit, 1024 × 1024 pixel resolution, and an average of 4 scans was taken to decrease the background noise.

Flow cytometry

To collect cells for flow cytometry analysis, monolayer cultures of HEK293T cells were dissociated by 37 °C trypsin (Invitrogen) for 2 min, then isolated to single cells in 4 °C PBS containing 2% fetal bovine serum and 0.05% sodium azide (FACS buffer) by pipette up and down ten times. Surface levels of each subunit were also quantified in 2 mM EDTA dissociated cells compared to trypsinized cells. The relative surface levels were not affected by trypsinization (data not shown). To evaluate total subunit levels, cells were permeablized with CytoPerm (BD Biosciences) for 15 minutes, and washed with CytoWash (BD Biosciences).

Following washes with FACS buffer for surface staining or CytoWash for total staining, cells were incubated with anti-HA epitope-tagged antibodies (clone 16B12, Covance) conjugated to the Alexa-647 fluorophore (Invitrogen) for 1 hour. Cells were then washed three times and fixed with 2% paraformaldehyde. Flow Cytometry experiments were performed in the VMC Flow Cytometry Shared Resource, which is supported by the Vanderbilt Ingram Cancer Center (P30 CA68485) and the Vanderbilt Digestive Disease Research Center (DK058404). Data were acquired using FACSDiva 6.0 (BD Biosciences) and analyzed off line using FlowJo 7.5 (Treestar, Inc.). The mean

fluorescence intensity of each sample was evaluated, and normalized to the 100% control ($\alpha1\beta2\gamma2L^{HA}$ or $\alpha1\beta2\gamma2S^{HA}$ as noted in each figure legend). The normalized mean fluorescence intensity was represented as a percentage of the 100% control.

Immunoblotting

Transgenic mouse brain tissue samples or cultured HEK293T cells were sonicated in radioimmune precipitation assay (RIPA) buffers (Pierce) and a protease inhibitor cocktail (Sigma Aldrich). Total tissue or cell lysates were cleaned by centrifugation at 20,000 X *g* for 30 min in 4 °C. The supernatants were mixed with Nupage LDS sample buffer (Invitrogen) then subjected to SDS-PAGE. Proteins in gels were transferred to Millipore Immobilon® FL PVDF Membrane (Millipore). Non-specific binding on the membrane was blocked with the Odyssey blocking buffer (Li-cor). Rabbit polyclonal anti-$GABA_A$ receptor γ2 subunit antibodies (final concentration, 2 μg/ml; Alomone) and monoclonal anti-HA epitope-tagged antibodies (0.2 μg/ml; clone 16B12, Covance) were used to detect endogenous mouse γ2 subunits and HA epitope-tagged γ2 subunits, respectively. Monoclonal anti-$GABA_A$ receptor α1 subunit antibodies (final concentration, 5 μg/ml; clone BD24, Chemicon) and monoclonal anti-$GABA_A$ receptor

β2/3 antibodies (4 μg/ml; clone 62-3G1, Upstate) were used to detect wildtype human α1 and β2 subunits, respectively. The polyclonal anti-human Upf-1 (hUpf-1) antibodies (Abgent, AP1905c) were used at a final concentration of 125 ng/ml. Anti-Na+/K+-Anti-Na+/K+-ATPase antibodies (0.2 μg/ml; clone ab7671, Abcam) were used to check loading variability. Following incubation with primary antibodies, IRDye® secondary antibodies were used at a 1:10,000× dilution (Li-cor) for visualization of specific bands with the Odyssey imaging system (Li-cor). The band intensities of scanned images were quantified with the Odyssey analysis software (Li-cor).

Glycosidase Digestion

Whole cell lysates obtained from 10 mm-Tris RIPA buffer (10 mm Tris-HCl, 150 mm NaCl, 1.0 mm EDTA, 1% Nonidet P-40, and 0.25% sodium deoxycholate) extraction were subjected to endo H and peptide *N*-glycosidase-F digestion (New England Biolabs) following the manufacturer's recommended protocol. The digestion reactions were carried out at 37 °C for 3 hours and terminated by addition of sample buffer.

Immunoprecipitation

Protein complexes containing HA-tagged $GABA_A$ receptor subunits were immunoprecipitated using EZview Red anti-HA M2 beads (Sigma) 30 minutes at room temperature following manufacture's manual. After three washes with extracting RIPA buffer, protein complexes were eluted with 100 μg/ml HA peptide (Sigma).

Electrophysiology

Lifted whole cell recordings were obtained from transfected HEK293T cells as previously described [170]. Briefly, cells were bathed in an external solution consisting of (in mM): NaCl 142; KCl 8; $MgCl_2$ 6; $CaCl_2$ 1; HEPES 10; glucose 10, pH 7.4, 325 mOsm. Electrodes were fire-polished to resistances of 0.8-1.5 MΩ and filled with an internal solution consisting of (in mM): KCl 153; $MgCl_2$ 1; MgATP 2; HEPES 10; EGTA 5, pH 7.3, 300 mOsm. The combination of internal and external solutions produced a chloride equilibrium potential of ~0 mV. For all recordings, cells were voltage clamped -20 mV. GABA (1 mM) was applied to cells for 4 seconds and cells were then washed with external solution for 40 seconds. Zn^{2+} (10 μM) was then preapplied for 10 seconds followed by coapplication of GABA (1 mM) and Zn^{2+} (10 μM) for 4 seconds. Finally,

cells were washed with external solution for 10 seconds followed by application of (1 mM) for 4 seconds. Whole cell currents were low-pass filtered at 2-5 kHz and at 10 kHz, and peak current amplitudes were quantified using the pClamp9 software suite (Axon Instruments).

Statistical analysis

Data are presented as means ± SEM. We used Student's *t*-test for two group comparisons, and one-way or two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test for multiple comparisons. Data were plotted and analyzed with GraphPad Prism 5 (GraphPad Software).

Results

The *GABRG2(IVS6+2T→G)* mutation generated a mutant *hGABRG2(IVS6+2T→G)* BAC transcript that retained a 53 bp intron 6 fragment

The *GABRG2(IVS6+2T→G)* mutation altered the *GABRG2* intron 6 splice donor site sequence. As a result, it was proposed that intron 6 is spliced out either with the site from another intron, resulting in exon skipping, or with an alternative donor site downstream of the wildtype site, resulting in cryptic splice donor site activation and intron 6 retention in the mutant mature mRNA [198]. However, the actual splice pattern of the mutant mRNA is unknown, and patient tissues or RNA samples are not available. Our approach to determine the splicing pattern of the mutant gene was to study splicing *vitro* and *in vivo* of a BAC construct that contained *hGABRG2* genomic sequence, and a full length intron 6 (Figure 1A, see methods for construct details). Intron splicing is type specific, and the optimal approach to study splicing of *GABRG2* is to do so in cells with endogenous *GABRG2* expression. We used Lipofectamine 2000 to transfect either wildtype or mutant *hGABRG2* BACs containing their native promoter and the eGFP reporter gene into PC12 cells, which have been reported to have endogenous *GABRG2*

expression [243]. Although GFP expression was observed in BAC-transfected PC12 using RT-PCR we were unable to demonstrate hγ2 subunit mRNA. As an alternative strategy, we replaced the *hGABRG2* promoter with a CMV promoter and expressed the CMV promoter-driven *hGABRG2* BAC in HEK293T cells (Figure 1A, see methods for construct details), and using RT-PCR, we were able to demonstrated hγ2 subunit mRNA expression. Thus unless otherwise specified, all *hGABRG2* BAC constructs in the remainder of the *in vitro* studies utilized the CMV promoter.

To determine wildtype and mutant *hGABRG2* splicing patterns, we expressed both wildtype *hGABRG2* BAC and control γ2S cDNA constructs in HEK293T cells and collected total RNA. DNA sequencing of *hGABRG2* BAC RT-PCR products using primers binding to exons 5 and 9 of the *GABRG2* coding sequence showed that the intervening introns 6, 7 and 8 were completely spliced out, and only γ2S subunit mRNA was transcribed from the *hGABRG2* BAC (not shown). This was consistent with the finding that the γ2S subunit splice variant is the default splicing product and that generation of the γ2L subunit splice variant requires positive regulation such as the function of neuron-specific RNA binding protein Nova-1 [152, 244, 245].

The mutant *hGABRG2(IVS6+2T→G)* BAC transcript was expressed in HEK293T cells and cloned and sequenced (Figure 1B). The mutant *hGABRG2(IVS6+2T→G)* BAC intron 6 utilized a cryptic splice donor site 53 bp downstream of the wildtype splice donor site, and thus, the mutant transcript retained a 53 bp intron 6 fragment (Figure 1C, D). None of the intron splice donor site prediction models that we employed detected this site, suggesting that its sequence did not comply with general splice donor site rules. The mutant splice donor site was predicted to be much weaker than the wildtype site [246], having less hydrogen bonding with the splice machinery, and hence, forming a less stable mRNA-protein complex. These are all common properties of mutant splice donor sites [199].

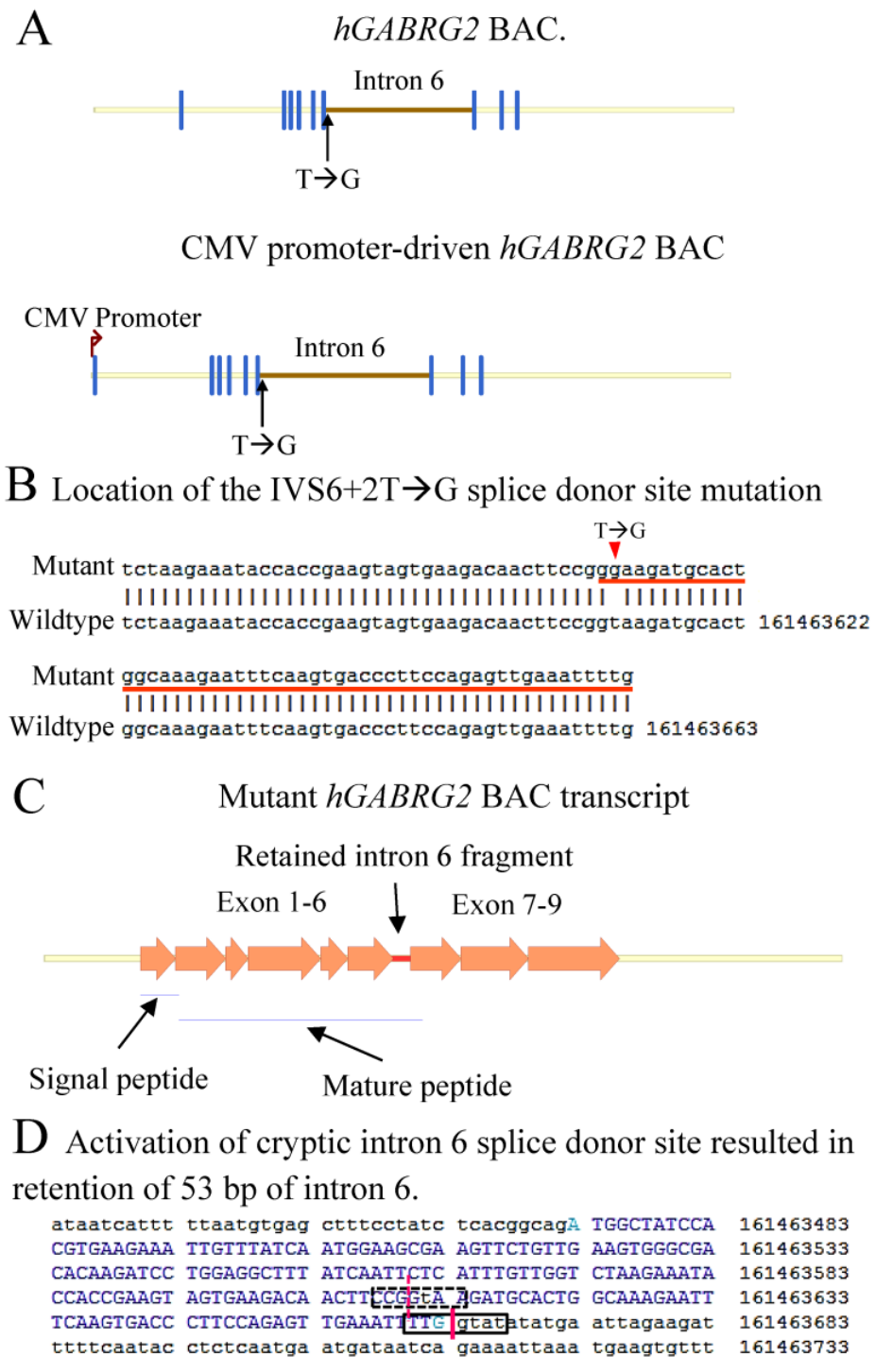


Figure 2.1 The *GABRG2(IVS6+2T→G)* BAC transcript retained 53 bp of intron 6 sequence

A. Structures of the human GABRG2 genomic sequence used in this study. The genomic sequence of GABRG2 in the BAC used in this study (RP11-1035I20) and the CMV promoter-driven GABRG2 BAC are shown. The yellow horizontal lines represent introns and 3'- and 5'-UTRs. The brown horizontal line represents intron 6. The vertical dark blue lines represent exons. The black arrow points to the mutation. The brown arrow at the left end represents the CMV promoter. The length of each line is proportional to the molecular size of the represented region (nucleic acid number). B. The mutant mRNA was cloned into the TOPO cloning vector (upper) and human genomic mRNA (lower) sequences were aligned. The last 91 bp of the mutant exon 6 alignment result including the retained intron 6 sequence are presented. The number on the lower line shows the position of each nucleotide in human chromosome 5. The red arrow points to the IVS6+2T□G mutation, and the red line underlines the retained 53 bp intron 6 sequence. C. The structure of the GABRG2(IVS6+2T→G) BAC transcript is shown. The arrows represent exons 1-9. The red line represents the retained intron 6 fragment. The yellow lines represent 3'- and 5'-UTRs. The blue lines underline the signal and mature peptides. The length of each arrow and line is proportional to the molecular size of the represented region (nucleic acid number). D. The sequence of the mutant intron 6 splice donor site is shown. In the full mutant exon 6 sequence, a "t" in lower case is shown at the T→G mutation site. The dotted black box shows the wildtype intron 6 splice site conservative sequences, and the dotted red line marks the wildtype splice site. The solid black box encloses the 7 nucleic acids at the mutant donor site sequence, and the solid red line marks the splice site. The nucleotides in upper case are exon 6 sequences, and the nucleotides in lower case are intron sequences.

The mutant *GABRG2(IVS6+2T→G)* mRNA should be translated to a truncated subunit containing the signal peptide and N-terminal 217 amino acids of the wildtype γ2 subunit

In silico translation, using Vector NTi (Invitrogen), showed that the mutant transcript should be translated to a polypeptide containing the signal peptide and N-

N-terminal 217 amino acids of the wildtype γ2 subunit. The retained 53 bp intron 6 fragment caused a frame shift in exon 7, which generated a stop codon 33 bp from the of the fragment. The retained intron 6 fragment and the exon 7 frame shift sequence are predicted to be translated to a novel 29 amino acid peptide tail at the C-terminus of the mutant protein (Figure 2A), so the mutant protein contained the N-terminus of the subunit and the novel C-terminal tail (γ2-PTC subunit) (Figure 2B). The hydrophobicity of the 29 amino acid tail was evaluated by ProtScale at Expasy.org [247] and was found be hydrophilic at the N-terminus and hydrophobic at the C-terminus (Figure 2C). The maximum hydrophobic region was the C-terminus, where the calculated maximum hydrophobicity was 1.43 [248]. This was very close to the maximum hydrophobicity of the wildtype γ2S subunit, which was 1.75 when evaluated by the same model. The structure of the mutant γ2-PTC subunit was unknown, but bioinformatics models did not predict that any secondary structure formed in this fragment.

This truncated subunit was reminiscent of the soluble acetylcholine-binding proteins (AChBPs) found in mollusk glial cells [249-251]. AChBP sequences are homologous to the N-terminal extracellular domains of cys-loop family ligand gated ion channel (LGIC) subunits, and the crystal structure and protein function are similar to the

ligand-binding domain of the nicotinic acetylcholine receptor α-subunit. $GABA_A$ receptors also belong to the cys-loop LGIC family. AChBPs oligomerize to form homopentamers containing binding sites for agonists and antagonists including acetylcholine. Upon acetylcholine release, AChBPs are released from glia cells into synaptic gaps and inhibit cholinergic neurotransmission by binding free acetylcholine molecules [249]. Sequence alignment showed that the γ2-PTC subunit has the highest homology with the *Aplysia californica* AChBP (Ac-AChBP) (Figure 2D). ClustalW alignment showed that the γ2-PTC subunit had a 21-29% sequence identity with AChBPs (not shown), which was even higher than the 13-25% sequence identity between AChBPs and LIGC subunit N-terminal extracellular domains [251]. The *GABRG2(IVS6+2T→G)* mutation might generate a mutant protein, the γ2-PTC subunit, that structurally resembles AChBPs and interferes with GABAergic neurotransmission in a similar way.

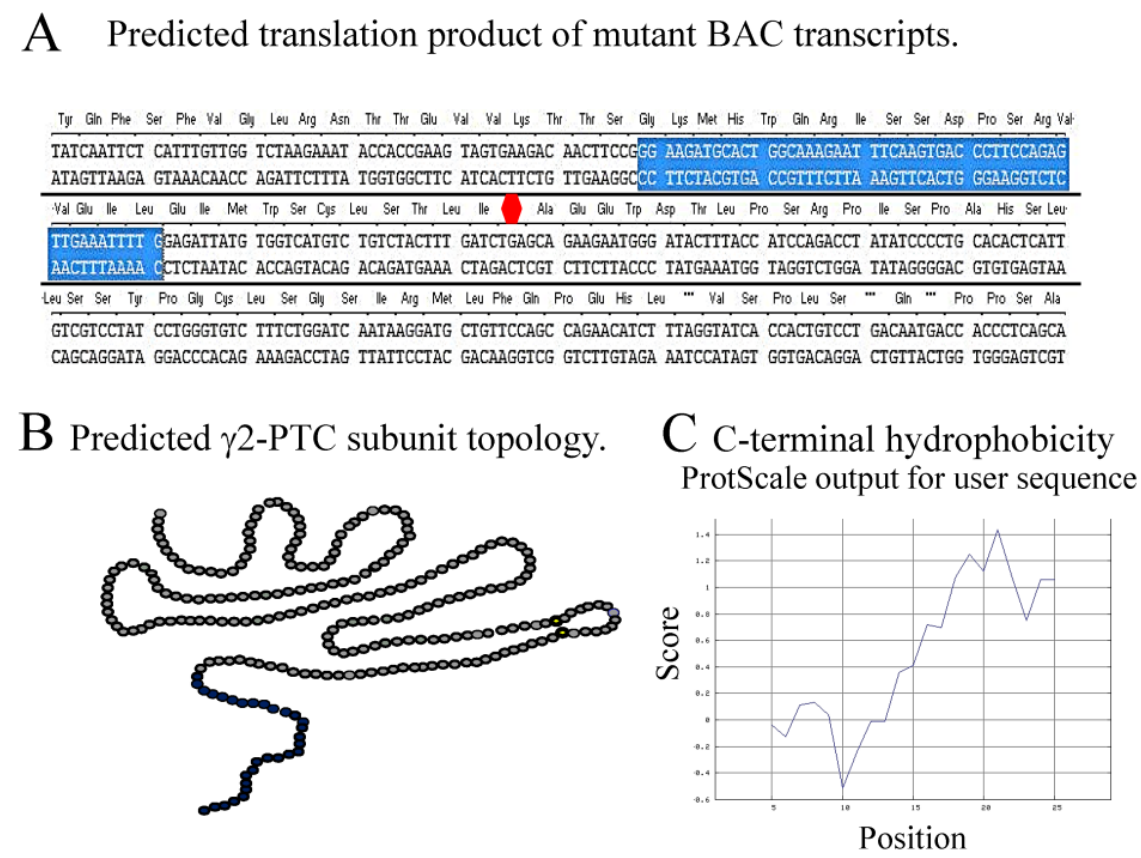


Figure 2.2 The mutant BAC transcript was predicted to encode a truncated protein containing most of the γ2 subunit N-terminus and a novel hydrophobic C-terminal tail translated from the retained intron 6 fragment and the exon 7 frame shift product

A. The predicted sequence of the C-terminal tail of the *GABRG2(IVS6+2T→G)*

BAC transcript (γ2-PTC subunit) is shown. The blue background shows the retained 53 bp intron 6 fragment, and the red octagon shows the position of the PTC in exon 7. Sequences of both DNA strands are shown. The predicted amino acids are shown in blue blue above the DNA sequences. B. The predicted membrane topology of the γ2-PTC subunit is shown. The grey circles represent the wildtype γ2 subunit N-terminus 217 aa peptide. The dark blue circles represent the 29 aa novel C-terminus translated from retained the intron 6 and exon 7 frame shift product. C. The hydrophobicity of the novel novel C-terminal tail translated from retained intron 6 and exon 7 frameshift product was determined based on an amino acid scale [248]. The average hydrophobicities of 5 adjacent amino acids (Y axis) are plotted against the amino acid positions in the peptide (X axis). D. Peptide sequence alignment by ClustalW2 showing the γ2-PTC subunit is homologous to AChBP identified from different species. Ac-AChBP, *Aplysia californica*; Bt-AChBP, *Bulinus truncatusi*; Ls-AChBP, *Lymnaea stagnalis*.

The expression pattern of *hGABRG2*^{HA} BACs in transgenic mouse brain was similar to that of the endogenous *GABRG2*

The transcription product of the CMV driven *hGABRG2(IVS6+2T→G)* BAC in HEK293T cells, the γ2-PTC subunit mRNA, retained a 53 bp intron 6 fragment in the mutant exon 6. However, it has been reported that promoter usage can affect intron splicing pattern [252], and thus, the mutant *hGABRG2(IVS6+2T→G)* BAC might be spliced to another mRNA when driven by its endogenous promoter. The intron splicing pattern is also cell type dependent. To minimize possible artifacts, we studied intron splicing of the *hGABRG2(IVS6+2T→G)* BAC with its endogenous promoter region in transgenic mouse brain (Figure 3). We first expressed an HA-tagged *hGABRG2* BAC in

C57BL/6J mice, which is the C57BL/6-Tg(*hGABRG2*^{HA})RLM mouse line (according to Jackson Laboratory mouse nomenclature, Tg(*hGABRG2*^{HA} mice)). The HA-tag was introduced to a functionally silent position in the wildtype subunit coding sequence. The HA-tagged γ2 subunits were not recognized by polyclonal γ2 subunit antibodies (not shown). These antibodies recognized a strong nonspecific band below 50 kDa and weak γ2 subunit specific bands below the nonspecific band (Figure 3A). The Western blot on adult mouse total brain tissue lysate showed that both transgenic mice (Figure 3Aa; lanes 4-5) and wildtype littermates (Figure 3Aa; lanes 1-3) had endogenous mouse γ2 subunits, but only transgenic Tg(*hGABRG2*^{HA}) mice had both endogenous mouse and HA-tagged human γ2 subunits (Figure 3Ab; lanes 4-5). The merged image showed that the HA-band molecular mass was similar to the endogenous mouse γ2 subunit band (Figure 3Ac). We repeated this Western blot four times with 15 adult Tg(*hGABRG2*^{HA}) mouse brain and detected the same HA-tag band in addition to the endogenous mouse γ2 subunit band. Although the HA-tagged *hGABRG2* BAC construct had human *GABRG2* gene genomic sequence and transcription regulatory elements, it was translated to protein in the transgenic mouse brain.

We then collected brain samples from Tg($hGABRG2^{HA}$) mice and wildtype littermates, cryosectioned them to 20 μm sections, stained the sections with HA antibodies and IRDye800 conjugated donkey anti-mouse IgG secondary antibodies, and scanned the immuno-labeled sections with the Odyssey imaging system (Figure 3Ba, b). Wildtype mouse brain sections only showed weak fluorescence signal in the 800 channel (Figure 3Bb). Its pattern was similar to the pattern of its auto-fluorescence scanned in the 700 channel, which did not receive any antibody labeling (not shown). The Tg($hGABRG2^{HA}$) mouse brain section had enhanced 800 channel fluorescence signal primarily in olfactory bulb, cortex, hippocampus, thalamus, midbrain, pons and cerebellum. The expression pattern of HA-tagged human γ2 subunits in the Tg($hGABRG2^{HA}$) mouse brain was similar to endogenous mouse γ2 subunits as reported previously [149, 253]. The human *GABRG2* gene promoter in $hGABRG2^{HA}$ BACs and the endogenous mouse *GABRG2* gene promoter functioned similarly.

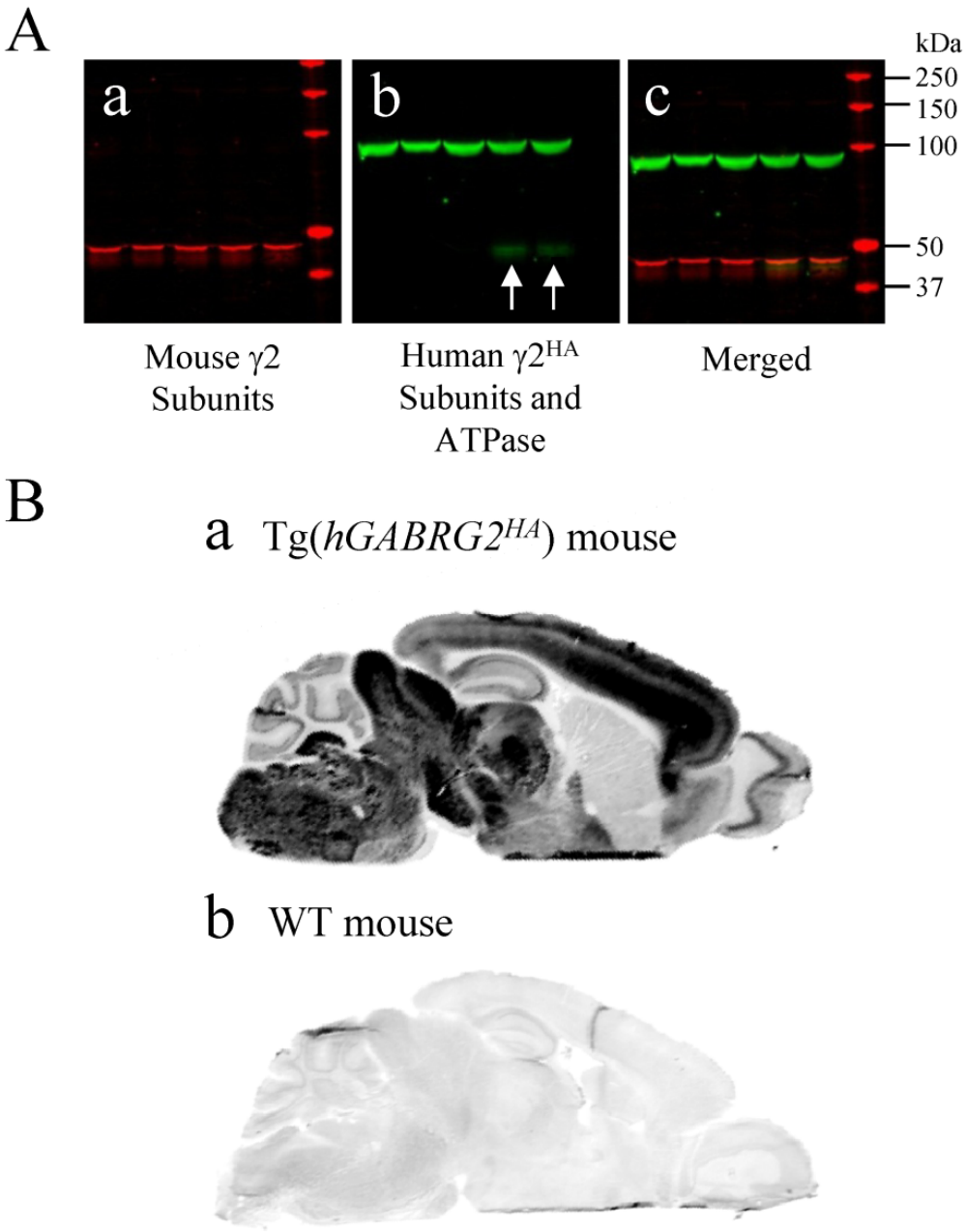


Figure 2.3 The wildtype human *hGABRG2^{HA}* BAC in transgenic mouse brain had the same expression pattern as the endogenous mouse *mGABRG2*.

A. Western blot on brain total lysate of Tg(*hGABRG2*^{HA}) transgenic mice showing transgenic mice expressed both endogenous mouse γ2 subunits and HA-tagged human γ2 subunits (n = 4). Endogenous mouse γ2 subunits were labeled in the red channel. ATPase and HA-tagged proteins were labeled in the green channel. The merged image showed the molecular size of HA-tagged human γ2 subunits and endogenous mouse γ2 subunits were similar. White arrows in the middle panel point to the HA-bands. B. The expression pattern of $\gamma2^{HA}$ subunits in the Tg(*hGABRG2*^{HA}) mouse brain was similar to that of γ2 subunits in wt mouse brain. HA-antibodies stained parasagittal sections of adult Tg(*hGABRG2*^{HA}) BAC transgenic mouse brain (a) or adult wildtype littermate brain (b). Sections were scanned in Odyssey scanner as one image after immuno-labeling, which was presented in gray scale. The signal in the Tg(*hGABRG2*^{HA}) section was over-saturated in some regions because the setting was chosen to visualize the non-specific binding in the wildtype littermate section (n = 3).

The γ2-PTC subunit was expressed as a stable protein in HEK293T cells and Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain

Having confirmed the expression pattern of *hGABRG2* BACs, we next the effect of the mutation on mRNA splicing in the transgenic mouse brain. We introduced the IVS6+2T→G mutation into the BAC without the HA-tag, expressed the mutant BAC in C57BL/6-Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$)RLM mice (Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mice), collected transgenic mouse and wildtype littermate brain total RNAs and them to total cDNAs. To determine the effect of the mutation on mRNA splicing pattern in transgenic mice, we performed RT-PCR using primers binding to exon 5-7 of the γ2 subunit cDNA. The amplified fragment from wildtype human or mouse γ2 subunit

was 320 bp, and the fragment amplified from the CMV-driven BAC transcript in HEK293T cell, the γ2-PTC subunit, was 373 bp. The primers amplified only one band from wildtype littermate total cDNAs but two bands that were almost overlapping from mutant Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse total cDNAs (data not shown). The IVS6+2T→G mutation generated an NciI restriction enzyme site in the middle of the γ2-PTC subunit cDNA. NciI should cut the amplified mutant exon 5-7 fragment into two fragments of 204 and 169 bp, thus allowing more separation on the gel between the amplified mouse fragment (320 bp) and the digested mutant transgene products (204 and 169 bp). We repeated the exon 5-7 RT-PCR in mutant transgenic mouse brain total RNAs and in wildtype human γ2S subunit or γ2-PTC subunit cDNA transfected cell total RNAs. We then digested the RT-PCR products with NciI and separated digested products in bromide stained agarose gel (Figure 4A). The human γ2S subunit PCR product was undigested and remained about 320 bp as expected (Figure 4A, lane a), but the γ2-PTC subunit PCR product was digested to a broad ~200 bp band, consistent with two 204 and 169 bp products (Figure 4A, lane b). The Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse total RNA RT-RT-PCR product showed two bands of about 320 and 200 bp (again consistent with two 204 and 169 bp products) after digestion (Figure 4A, lane 5), while their wildtype littermate RT-PCR fragments had only one 320 bp fragment after digestion (Figure 4A,

lanes 6-8). Direct DNA sequencing of the cloned RT-PCR products showed that the Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain had human γ2-PTC subunit cDNA identical to the transcription product of *CMV-hGABRG2(IVS6+2T→G)* BAC in HEK293T cells, as well as endogenous mouse γ2 subunit cDNA. This RT-PCR was repeated in seven Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain total RNA samples at different ages (three at P0, at P35), and the same mutant human BAC transcript was detected in these animals. Thus, the splicing pattern of the mutant *hGABRG2(IVS6+2T→G)* BAC intron 6 in mouse brain was the same as the splicing pattern of the mutant CMV driven BAC intron 6 in HEK293T cells.

Although mutant *GABRG2(IVS6+2T→G)* BAC γ2 subunit mRNAs were susceptible to degradation by NMD, we still detected γ2-PTC subunit mRNAs in transfected HEK293T cells and Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain. *In silico* predicted that the γ2-PTC subunit retained most of the wildtype γ2 subunit N-terminus. We transfected wildtype γ2S subunit cDNA and γ2-PTC subunit cDNA in HEK293T separated total cell lysates using a 4-12% gradient NuPage Novex Bis-Tris gel and ran Western blots using polyclonal γ2 subunit antibodies (Figure 4Ba). All samples had a faint nonspecific band slightly smaller than 50 kDa, and a specific γ2 subunit band

wildtype γ2 subunit that was around 40 kDa (Figure 4Ba, lanes 1-2, upper arrow). The γ2-PTC subunit transfected cells had the same non-specific band and showed a γ2 subunit specific doublet band smaller than 37 kDa (Figure 4Ba, lane 3-4, lower arrow). The faint lower band in the doublet was visible only at the higher γ2-PTC subunit amount and was obvious in 3 μg cDNA transfected cells (Figure 4Ba, lane 3) but not in the 1 μg cDNA transfected (Figure 4Ba, lane 4). It was probably generated by a different pattern of subunit glycosylation [61]. The predicted γ2-PTC subunit encodes an ~33 kDa protein containing a signal peptide of 4 kDa and mature peptide of 28 kDa. The molecular mass of this protein band in SDS-PAGE gel was larger than predicted for the mature peptide, probably because of posttranslational modifications. Thus, the γ2-PTC subunit was translated as a stable protein in HEK293T cells.

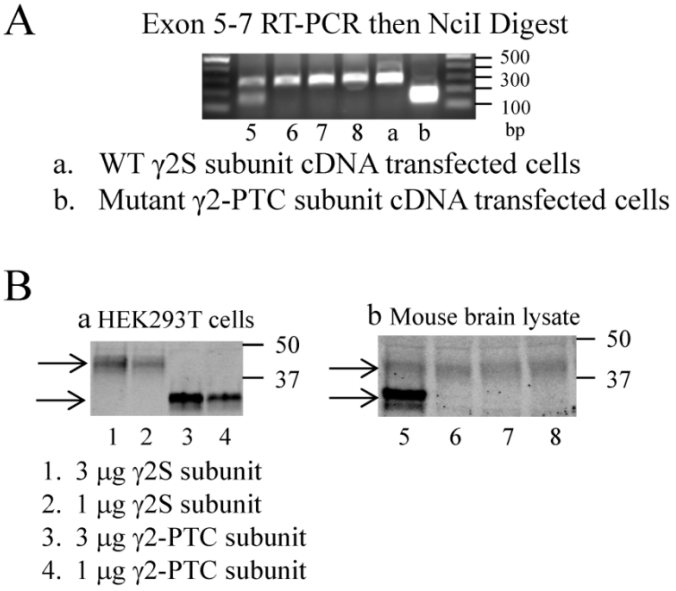


Figure 2.4 The γ2-PTC subunit was expressed as a stable protein in HEK293T cells and Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain.

A. Mutant γ2-PTC subunit mRNA was detected in Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain total RNA. RT-PCR experiment amplifying exon 5-7 of γ2 subunit, followed by NciI digestion, showed that the IVS6+2T→G mutant γ2 subunit mRNAs were expressed in Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mutant BAC transgenic mice brain total RNA. **B. (a)** The γ2S or γ2-PTC subunits cDNAs were transfected in HEK293T cells at different amounts, total protein level were evaluated by western blot using antibodies against the epitope at γ2 subunit mature peptide N-terminus. **(b)** Western blot on the Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse and wildtype littermates total brain lysate showing mutant γ2-PTC subunits were expressed in Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain. Numbers below each lane represent the mouse numbers. Experiment was repeated three times and seven Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mice were studied.

We then collected Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain total lysate and blotted with the same antibodies (Figure 4Bb). All mouse samples had the γ2 subunit specific band at the same size as the wildtype γ2S subunit in HEK293T cells, which were endogenous mouse γ2 subunits (Figure 4Ba, lane 5-8, upper arrow). The Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain sample (from mouse #5), however, had an extra doublet band at the same size as the γ2-PTC subunit in HEK293T cells (Figure 4Ba, lane lower arrow). We evaluated 12 Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain samples at ages varying from P0 to P80 and detected the same staining pattern. We also introduced the

hGABRG2(IVS6+2T→G) BAC to the B6D2F1/J mouse and made a B6D2-B6D2-Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse. Western blot on four B6D2-B6D2-Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain samples at P30 showed the same mouse γ2 and human γ2-PTC subunit band migration pattern as the C57BL/6- mouse brain samples. The mutant human *GABRG2(IVS6+2T→G)* BAC transgene was detected as stable γ2-PTC subunits in the Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain. These mice expressed both endogenous mouse γ2 and human γ2-PTC subunits in brain. Therefore, both Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mice and transfected HEK293T cells can be used to study the function of the γ2-PTC subunit.

NMD decreased mutant γ2-PTC subunit mRNA levels

The *GABRG2* mutation, IVS6+2T→G, generated a PTC in exon 7. The mature mutant γ2-PTC subunit mRNA, therefore, should be degraded by the NMD machinery, since it contains a PTC that is more than 55 bp upstream of an exon-exon junction In contrast, the *GABRG2(Q390X)* mutation is an autosomal dominant mutation associated with Dravet Syndrome [189]. The mutation generates a PTC in the last exon, so the mature mutant *GABRG2(Q390X)* mRNA should not be degraded by NMD and should be

translated to truncated γ2(Q390X) subunits [191]. To determine if NMD was activated these PTCs, wildtype *hGABRG2*, NMD-susceptible mutant *hGABRG2(IVS6+2T→G)* and NMD-resistant mutant *hGABRG2(Q350X)* BACs were expressed in HEK293T cells expressing siRNAs against the NMD essential factor UPF1, and γ2 subunit mRNA levels were quantified using the Taqman real-time PCR assay. BACs were also transfected into HEK293T cells expressing siRNAs without cellular function according to the manufacturer's manual. The Taqman probe was designed to bind to the borders of exons 4 and 5. The levels of BAC-derived γ2 subunit mRNAs were compared to GFP mRNA levels for each condition, and then the γ2 subunit mRNA levels of UPF1 siRNA cells were compared to negative control siRNA transfected cells for each BAC construct. Western blot showed that the UPF1 protein level was unchanged in negative control siRNA transfected HEK293T cells compared to untransfected control cells but was decreased to ~20% in UPF1 siRNA transfected cells (data not shown). Real-time PCR results showed that the wildtype γ2 subunit mRNA levels was not changed (1.16 ± 0.14 fold, n = 6) after UPF1 knock down (Figure 5A). The γ2-PTC subunit mRNA level, however, was increased 2.14 ± 0.52 fold after UPF1 knock down ($p < 0.05$, n = 6), while expected the γ2(Q390X) subunit mRNA level was not changed (1.19 ± 0.20 fold; not significant, n = 6). We also evaluated γ2 subunit mRNA level after blocking another

NMD essential factor, SMG6 and obtained similar results (not shown). Thus, γ2-PTC, not γ2 or γ2(Q390X), subunit mRNA was subject to degradation by NMD.

NMD decreased mutant γ2-PTC subunit levels

We collected the BAC transfected cells lysate and blotted proteins using endogenous γ2 subunit antibodies (not shown). We quantified the γ2 subunit band intensity of each lane, normalized to the ATPase band intensity of the same lane, and compared the normalized γ2 subunit band intensity between cells expressing UPF1 siRNA and cells expressing negative control siRNA (Figure 5B). The wildtype band intensity was unchanged (87.2 ± 18.9%, n = 4), the *GABRG2(IVS6+2T→G)* BAC protein band intensity was increased to 232.6 ± 31.5% ($p < 0.01$, n = 4), while the *GABRG2(Q390X)* BAC band intensity was unchanged (116.0 ± 20.5%; $p = 0.095$, n = 4) relative to the wildtype band intensity. The increased amount of *GABRG2(IVS6+2T→G)* BAC protein was consistent with the increased mRNA level. The wildtype and mutant γ2 subunit protein level increases after blocking SMG6 had the same trend (not shown).

These data demonstrated that the amount of the *GABRG2(IVS6+2T→G)* BAC translation product, the γ2-PTC subunit, was increased by decreasing NMD efficiency in

cells. The γ2-PTC subunit protein levels in the brain of the Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse, and presumably human patients, would be determined by both mutant *GABRG2(IVS6+2T→G)* gene transcription level and NMD efficiency.

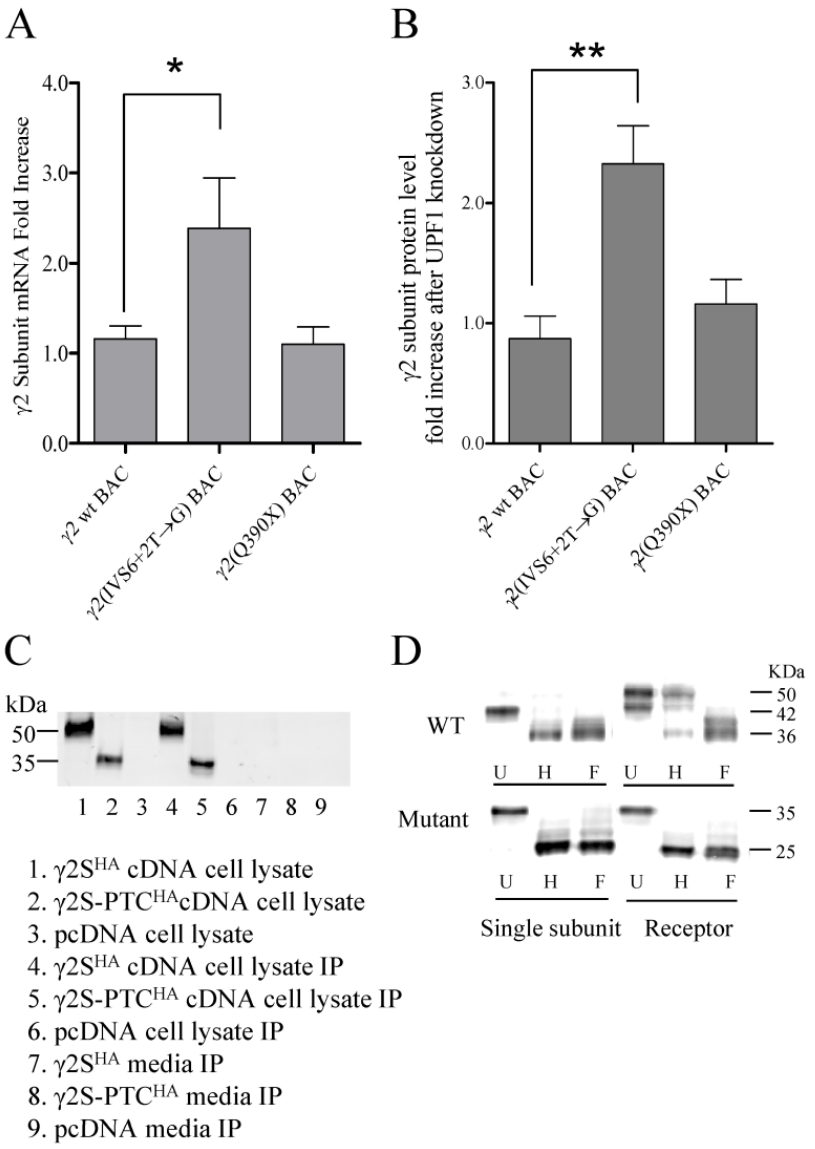


Figure 2.5 The mutant γ2-PTC subunit mRNA level was decreased by NMD, while the undegraded mRNA was translated to the immature γ2-PTC subunit with an ER glycosylation pattern.

A. The mRNA level of the γ2-PTC subunit was increased after UPF1 knockdown (n = 6). RNA levels were evaluated by Taqman quantitative real-time PCR as described in methods. * = $p < 0.05$, one-way ANOVA with Bonferroni's multiple comparison test. B. The protein level of the γ2-PTC subunit was increased also after UPF1 knockdown (n = 4). ** = $p < 0.01$, one-way ANOVA Bonferroni's multiple comparison test. C. In HEK 293T cells the γ2-PC subunit is a stable protein that was not secreted into the culture media. HA-tagged wildtype γ2S subunit cDNA, γ2-PTC subunit cDNA and pcDNA empty vector were expressed in HEK293T cells. Culture media of each cell and total cell lysate were both collected and incubated with HA-beads to pull down HA-tagged proteins. Pull-down proteins were eluted with HA-peptide, separated by SDS-PAGE, and blotted with HA-antibodies. The experiment was repeated three times and a representative gel was shown. D. The γ2-PTC subunit had an ER glycosylation pattern. HA-tagged wildtype γ2L and γ2-PTC subunits were expressed in HEK293T cells as either single subunits or were coexpressed with α1β2 subunits (Receptor). Total cell lysates from each condition were collected and digested with endoglycosidase Endo H or PNGase F. Digested and undigested proteins were blotted with HA-antibodies. U undigested; H Endo H digested; F PNGase F digested. The experiment was repeated four times and a representative gel was shown.The γ2-PTC subunit was not secreted into the culture medium.

The γ2-PTC subunit was not secreted into the culture medium

As noted above, the γ2-PTC subunit is homologous to the AChBPs [254], which are secreted into the extracellular space by glial cells where they bind acetylcholine to terminate synaptic transmission. When Ac-AChBPs were expressed in HEK293T cells, homopentameric Ac-AChBPs were secreted into the culture media [250]. Thus by analogy, it is possible that γ2-PTC subunits are folded correctly, form pentamers and are secreted from cells. However, due to the increased hydrophobicity at the C-terminal tail,

it is also possible that the subunit has a transmembrane segment that folds and assembles a membrane bound protein and is not secreted. Epitope-tagged γ2-PTC^{HA} subunits were used to determine the cellular fate of the truncated γ2-PTC subunits. The HA-tag was added to the same site that was functionally silent in wildtype subunits. When wildtype γ2S^{HA} or γ2-PTC^{HA} subunits were expressed in HEK293T cells, both were stable proteins (Figure 5C). As the γ2 subunit antibodies showed in Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse samples, HA-tagged mutant γ2-PTC^{HA} subunits (lane 2) were smaller than wildtype subunits (Figure 5C, lane 1). The HA-beads successfully pulled down HA-tagged wildtype γ2S^{HA} or mutant γ2-PTC^{HA} subunits from total cell lysate (Figure 5C, lanes 4-5) but pulled down nothing from the cell culture medium (Figure 5C, lane 7-8). We collected about 15 ml of culture media from each sample. If the γ2-PTC^{HA} subunit was secreted from cells at the same efficiency as Ac-AChBP (1 - 3 mg/L) [250], there would about 15 - 45 μg of γ2-PTC subunit protein in 15 ml of culture media. We used the Odyssey quantitative Western blot system to detect the γ2-PTC^{HA} subunit. According to the manufacturer's (Li-Cor) document, even if the amount of γ2-PTC^{HA} subunit was hundred times less than 15 - 45 μg, it should still be sufficient for detection by our blot. Although the γ2-PTC subunit is highly homologous to the secreted Ac-AChBP, γ2-γ2-PTC^{HA} subunits were present, but not secreted, into the culture medium. In

Tg($hGABRG2^{IVS6+2T\rightarrow G}$) mouse brain or human patients, the mutant allele would be translated to γ2-PTC subunits, which are likely to be also expressed inside of the neurons and not secreted to extrasynaptic spaces.

The γ2-PTC subunit attained altered ER associated glycosylation

While not secreted, γ2-PTC subunits could still form homooligomers or heterooligomers that are trafficked to the surface membrane. During the process of subunit maturation, immature *N*-linked mannose-rich oligosaccharides attached in the ER are replaced by mature glycans that are attached in the *trans*-Golgi region. Wildtype γ2L subunits show only low levels of membrane trafficking when expressed alone, which increased substantially with co-expression of α1 and β2 subunits [157]. To determine if the γ2-PTC subunits had mature glycosylation consistent with surface membrane trafficking, we compared the glycosylation patterns of γ2L and γ2-PTC subunits without or with cotransfection of α1 and β2 subunits.

Endo H cleaves immature *N*-linked mannose-rich oligosaccharides attached in the ER but not the mature glycans attached in the *trans*-Golgi region. In contrast, PNGase F removes all oligosaccharides attached both in the ER and *trans*-Golgi regions [255].

When expressed alone, $\gamma 2L^{HA}$ subunits on Western blot ran as a single band that was sensitive to digestion by endoglycosidases Endo H and PNGase F, consistent with primarily immature glycosylation and suggesting that $\gamma 2L^{HA}$ subunits were retained in the ER (Figure 5D, WT subunit). When co-expressed with α1 and β2 subunits, $\gamma 2L^{HA}$ subunits showed an extra band on Western blots that was insensitive to Endo H digestion, but was sensitive to PNGase F digestion (Figure 5D, WT Subunit). With coexpression of α1 and β2 subunits, $\gamma 2L^{HA}$ subunits had mature glycosylation, suggesting processing in Golgi apparatus and trafficking to the cell membrane.

With expression alone or with coexpression of α1 and β2 subunits, $\gamma 2\text{-PTC}^{HA}$ subunits showed only one band on Western blot that was sensitive to both Endo H and PNGase F (Figure 5D, Mutant subunit), suggesting that $\gamma 2\text{-PTC}^{HA}$ subunits were retained in the ER and not transported to the Golgi apparatus. The size of the digested $\gamma 2\text{ -PTC}^{HA}$ subunit protein band was consistent with the predicted size of the mature $\gamma 2\text{ -PTC}^{HA}$ subunit based on amino acid sequence. This phenomenon is consistent with the finding that $\gamma 2\text{ -PTC}^{HA}$ subunits were not secreted into the culture medium, which requires Golgi translocation. These data further suggested that the γ2-PTC subunit might not be

trafficked to the cell membrane and might instead be retained in the ER under physiological conditions such as in patients or in Tg($hGABRG2^{IVS6+2T\rightarrow G}$) mouse neurons.

The γ2-PTC subunits oligomerized with α1 and β2 subunits

$GABA_A$ receptor subunit oligomerization is determined by sequences at the extracellular N-terminal domain [256], and the γ2-PTC subunit included more than 90% the wildtype γ2 subunit N-terminal extracellular domain. A benzodiazepine-binding site is present at the αγ subunit interface. A radioligand binding study showed that the of radio-labeled benzodiazepine binding in cells expressing α1 and full-length γ2 subunits was comparable to the cells expressing α1 subunits and the N-terminus of γ2 subunits, that cells expressing α1, β2 and γ2 subunits had much higher benzodiazepine binding [257]. There is a 15 amino acid sequence in the γ2 subunit N-terminal extracellular domain around residue R82 (residue numbered in the immature peptide) that was to pull down β2 subunits, but the presence of an R82Q mutation in this peptide abolished the interaction, suggesting that this site was involved in the oligomerization of β2 and γ2 subunits [59]. The γ2-PTC subunit includes this 15 amino acid sequence, and so to explore if γ2-PTC subunit can oligomerize with partnering subunits, wildtype $\gamma2S^{HA}$,

$\gamma 2L^{HA}$ and mutant $\gamma 2\text{-}PTC^{HA}$ subunits were co-expressed with α1 and β2 subunits in HEK293T cells. HA-tagged proteins were pulled down using HA-beads and blotted for α1 and β2 subunits or for the HA tag (Figure 6A). The amount of pulled down α1 or β2 subunits reflected binding between the γ2 subunits and α1 or β2 subunits, respectively. The eluted HA-tagged proteins showed a band pattern that was similar to that of the total HA-tagged proteins. The wildtype $\gamma 2S^{HA}$ or $\gamma 2L^{HA}$ subunits both pulled down substantial amounts of α1 and β2 subunits (Figure 6A, lanes 2, 3). The $\gamma 2\text{-}PTC^{HA}$ subunit pulled down α1 and β2 subunits (Figure 6A, lane 4), but the amounts pulled down were less than those pulled down by wildtype γ2L or γ2S subunits (Figure 6A, lane 4). This was an expected finding because the mutant $\gamma 2\text{-}PTC^{HA}$ subunits contain the entire extracellular N terminal domain of γ2 subunits including the 15 amino acid peptide sequence that has shown to be sufficient to pull down β2 subunits. These data suggested that γ2-PTC subunits in physiological conditions would be nonfunctional and decrease GABAergic inhibition by decreasing surface γ2 subunit levels and having a dominant negative action reduce heteropentameric $GABA_A$ receptor assembly and trafficking because of its direct interaction with α1 and β2 subunits.

The γ2-PTC subunit was a stable intracellular protein

The *hGABRG2*^{HA} BAC had CNS expression pattern that was similar to that of the endogenous mouse γ2 subunit gene (Figure 3B), and mutant γ2-PTC subunits were identified in Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain. Since mutant γ2-PTC subunits had immature glycosylation and impaired oligomerization, it is likely that they had impaired assembly into receptors and impaired membrane trafficking. Furthermore, it may be recognized as a misfolded or misassembled protein that was subject to ER associated degradation by the proteasome. To evaluate this, we quantified mutant subunit and membrane trafficking using high throughput flow cytometry. We expressed wildtype or mutant γ2^{HA} subunits in HEK293T cells either as single subunits or coexpressed with and β2 subunits and evaluated total and surface levels of each subunit in >50,000 cells. All subunits were transcribed from the same pcDNA3.1 vector. The total HA level obtained with cotransfection of α1, β2 and γ2S^{HA} subunits was used as a control (100%) other γ2 subunit levels, and total levels obtained from pcDNA mock transfected cells used as a baseline control (0%). Total levels of the γ2-PTC^{HA} subunit did not differ from those of wildtype γ2L^{HA} or γ2S^{HA} subunits with single subunit expression (p = 0.02, 0.06, respectively, n = 4), or when coexpressed with α1 and β2 subunits (Figure 6B). Thus,

mutant γ2-PTC^{HA} subunit was not degraded and was as stable in these cells as wildtype subunits.

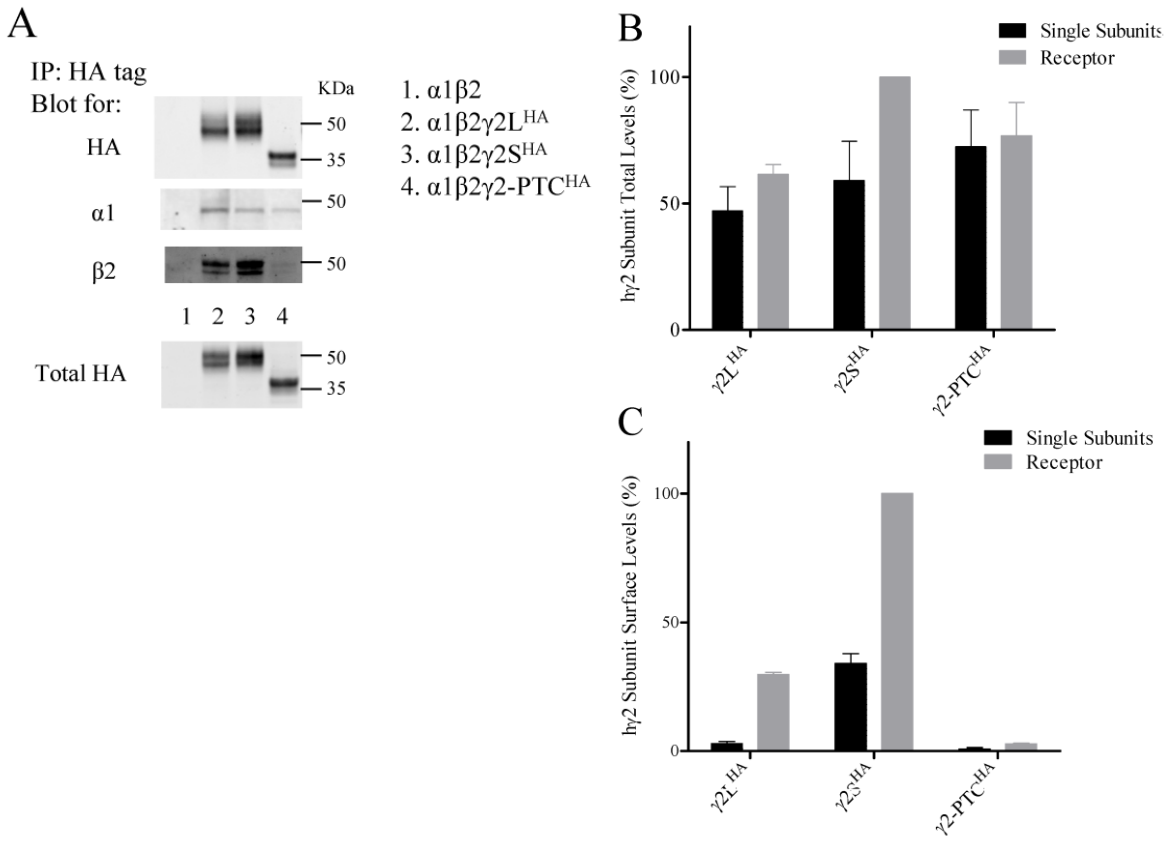


Figure 2.6 The γ2-PTC subunits oligomerized weakly with α1 and β2 subunits and had impaired membrane trafficking.

A. HA-tagged wildtype and mutant γ2 subunits were coexpressed with α1 and β2 subunits in HEK293T cells. Total cell lysate from each condition were collected and incubated with HA-beads. Pull down products were eluted with HA-peptide and blotted with antibodies against α1 subunits, β2 subunits, and the HA-epitope-tag. Western blot on total cell lysate with HA antibodies are also shown. Total cell lysate were also blotted blotted for α1 and β2 subunits in western blot but data not shown. The experiment was

repeated six times and a representative gel was shown. B. The normalized mean fluorescence intensity showing the total HA level from wildtype and mutant $\gamma2^{HA}$ subunits expressed as either single subunit (black) or with $\alpha1\beta2$ subunit coexpression (gray). The total HA level with $\alpha1$, $\beta2$ and $\gamma2S^{HA}$ subunit coexpression was used as 100%. The total HA level of pcDNA mock transfected cells was taken as a 0% baseline. baseline. Data were analyzed with a two-way ANOVA with Bonferroni's multiple comparison test. C. The surface HA level from wildtype and mutant $\gamma2^{HA}$ subunits expressed as either single subunit (black) or with $\alpha1$ and $\beta2$ subunits coexpression (grey). The surface HA level with coexpression of $\alpha1$, $\beta2$ and $\gamma2S^{HA}$ subunits was used as 100%. The surface HA level of pcDNA mock transfected cells is 0% baseline. Data Data were analyzed with a two-way ANOVA with Bonferroni's multiple comparison test.

The γ2-PTC subunit had impaired membrane trafficking

To assess surface trafficking of the mutant $\gamma2\text{-PTC}^{HA}$ subunit, we used technique of flow cytometry without cell permeabilization. We cotransfected cells using the same subunit combinations used to assess total cell levels of $\alpha1\beta2\gamma2S^{HA}$ subunits by measuring surface HA levels for each subunit (Figure 6C). The surface HA level with $\alpha1\beta2\gamma2S^{HA}$ subunit coexpression was used as a 100% normalization control for $\gamma2^{HA}$ subunit surface level, and surface HA level obtained with pcDNA mock transfected cells was used as baseline (0%). The single wildtype $\gamma2L^{HA}$ subunit had a low surface level ($2.93 \pm 0.76\%$, $n = 4$), which was increased substantially ($29.71 \pm 0.88\%$, $p < 0.01$, $n = 4$) by co-expression with $\alpha1$ and $\beta2$ subunits. The wildtype $\gamma2S^{HA}$ single subunit surface

level was much higher than $\gamma 2L^{HA}$ subunit surface level, probably because the $\gamma 2S^{HA}$ subunits have higher trafficking efficiency and lower PKC-dependent endocytosis [153, 157]. Its single subunit surface level (34.08 ± 3.80%, n = 4) was substantially higher than the $\gamma 2L^{HA}$ subunits single subunit surface level. Its surface level with α1 and β2 subunit coexpression was 100%, also substantially higher than with $\alpha 1\beta 2\gamma 2L^{HA}$ coexpression (n 4). Compared to $\gamma 2L^{HA}$ and $\gamma 2S^{HA}$ subunits, the surface levels of the $\gamma 2\text{-}PTC^{HA}$ subunit were substantially smaller with both expression conditions. The $\gamma 2\text{-}PTC^{HA}$ single subunit surface level was low (0.76 ± 0.56%, n = 4) and did not increase significantly with α1 β2 subunit coexpression (2.76 ± 0.30%, n = 4, not significant). The surface levels of γ2-$\gamma 2\text{-}PTC^{HA}$ subunits with or without α1 and β2 subunit coexpression were not greater than the mock control level (*p* value: single subunit > 0.05; with α1 and β2 coexpression: >0.05, n = 4). These results suggest that even though the mutant $\gamma 2\text{-}PTC^{HA}$ subunit oligomerized with α1 and β2 subunits, it was not trafficked to the cell surface.

The γ2-PTC subunits were retained in the ER

We have demonstrated that γ2-PTC subunits are stable in cells and minimally trafficked to the cells surface when coexpressed with α1 and β2 subunits. Given this

impaired trafficking, it is likely that the mutant γ2-PTC subunits with or without oligomerization with α1 and β2 subunits are retained in the ER with little localization in the *trans*-Golgi or surface membrane. Because the mutant *hGABRG2(IVS6+2T→G)* BAC in the Tg($hGABRG2^{IVS6+2T\rightarrow G}$) mouse does not have an HA tag, and the antibodies against endogenous γ2 subunits had a high nonspecific signal, we could not stain the Tg($hGABRG2^{IVS6+2T\rightarrow G}$) mouse brain to detect where the γ2-PTC subunit was expressed. Therefore, we coexpressed wildtype and mutant γ2-PTC^{HA} subunits with α1 and β2 subunits in HEK293T cells, stained the permeabilized cells with fluorescence-conjugated antibodies against the α1 subunit or the HA tag, and obtained confocal microscope to visualize the cellular localization of the subunits (Figure 7A). The ER was visualized using antibodies against the ER marker BIP. In addition, membrane expression was confirmed further by confocal microscope images taken from unpermeablized HEK293T cells cotransfected with α1 and β2 subunits and wildtype or mutant $\gamma2^{HA}$ subunits (not shown). BIP staining was not detected in any of these samples, showing that paraformaldehyde fixation did not permeablize the membrane (not shown). With coexpression of α1 and β2 subunits without γ2 subunits, the α1 subunit signal overlapped the ER signal, but also showed a ring structure that surrounded the ER signal (Figure 7A, 1st row) and outlined the cell membrane (not shown), consistent with low levels of

α1β2γ2-PTC and higher levels of α1β2 receptor expression on the cell membrane. With coexpression of α1, β2 and $\gamma2^{HA}$ subunits, wildtype $\gamma2L^{HA}$ or $\gamma2S^{HA}$ subunits were both visualized in regions that overlapped α1 subunits (Figures 7A, 2nd and 3rd row; surface staining not shown), consistent with co-assembly with α1 and β2 subunits into receptors that were trafficked to the cell surface. The $\gamma2\text{-PTC}^{HA}$ subunit signal overlapped that of the ER signal (Figure 7A, 4th row), and was absent from the surface membrane (not shown). The wildtype α1, γ2L and γ2S subunits often showed HA signals in the region that was recognized by Golgi specific antibodies (not shown), but the $\gamma2\text{-PTC}^{HA}$ subunit was not. Thus, the $\gamma2\text{-PTC}^{HA}$ subunit was retained primarily in the ER, consistent with background levels on the surface membrane and its absence in the culture medium.

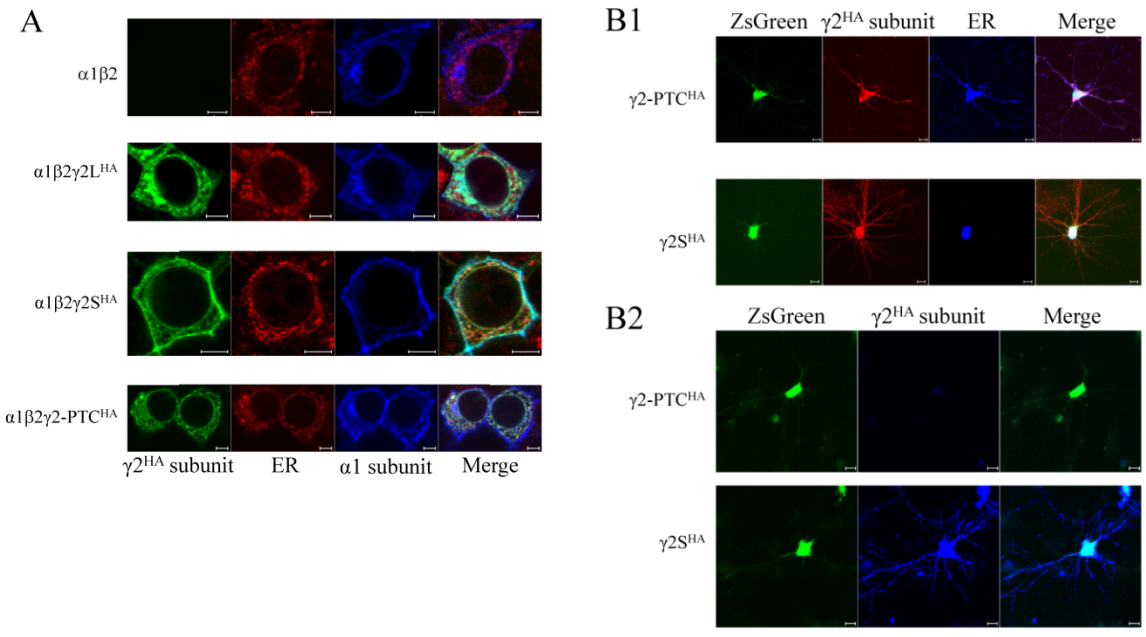


Figure 2.7 When expressed with α1 and β2 subunits, mutant γ2-PTC subunits were trapped in the ER.

A. Confocal images were obtained of the distribution of $GABA_A$ receptor subunits in permeablized HEK293T cells. In HEK293T cells α1 and β2 subunits were coexpressed with a blank pcDNA vector, and wildtype and mutant $\gamma2^{HA}$ subunits were coexpressed with α1 and β2 subunits at 1 μg of each cDNA. The transfected cells were permeablized and stained with Alexa fluorophores conjugated antibodies against HA-tag (Alexa488 conjugated, green) or α1 subunits (Alexa647 conjugated, blue). The ER was visualized with primary rabbit antibodies against the ER marker BIP, and Alexa568 conjugated secondary antibodies against rabbit IgG (red). Scale bars were 5 μm. **B.** Confocal images were obtained of transfected cultured cortical neurons expressing $\gamma2^{HA}$ subunits in pLVX-IRES-ZsGreen1 vectors. **B1**. Cultured neurons were permeablized and immune-stained with mouse monoclonal antibody against the HA-epitope tag and rabbit polyclonal antibodies against ER marker BIP, followed by Alexa568 conjugated donkey anti-mouse IgG antibodies (red) and Alexa647 conjugated donkey anti-rabbit IgG antibodies (blue). The ZsGreen, shown in green, labeled transfected neurons. Scale bars were 10 μm. **B2**. Cultured neurons were immune-stained without permeabilization, showing surface expression of HA-epitope tagged γ2 subunits. Neurons were stained with mouse monoclonal antibody against the HA-epitope tag and Alexa647 conjugated donkey anti-mouse IgG (blue). The ZsGreen, shown in green, labeled transfected neurons. Scale bars were 10 μm.

We then cloned the $\gamma2S^{HA}$ and $\gamma2\text{-}PTC^{HA}$ subunit cDNAs into pLVX-$\gamma2^{HA}$-IRES-ZsGreen1 vectors expressing $\gamma2^{HA}$ subunits and green fluorescent protein ZsGreen from the same mRNA but separated by internal ribosome entry site sequence (IRES) and expressed them in cultured cortical neurons (Figure 7B). Cytoplasmic ZsGreen protein showed green fluorescence in both soma and processes in

transfected neurons. The $\gamma2^{HA}$ subunit fluorescence signal was detected in all ZsGreen positive neurons, but also in a few ZsGreen negative neurons, suggesting that the cDNA downstream of IRES has a lower probability for translation to proteins (not shown). The $\gamma2\text{-PTC}^{HA}$ subunit fluorescence signal in permeablized neurons was diffusely distributed over the neuron soma and less intensely over the process, and it colocalized well with ER marker, BIP (Figure 7B1). On the contrary, the $\gamma2S^{HA}$ subunit signal was present in widespread, large clusters on neuronal somata and dendritic arbors, and spread outside of the ER marker signal (Figure 7B1). The clustered expression pattern of $\gamma2S^{HA}$ subunit immunoreactivity resembled that of endogenous γ2 subunits in cultured neurons [99]. The surface staining on cortical neurons further confirmed that the $\gamma2\text{-PTC}^{HA}$ subunit was absent from cell membranes (Figure 7B2). While the $\gamma2S^{HA}$ subunit showed strong $\gamma2^{HA}$ clusters on unpermeablized transfected neurons, both on cell somata and processes, the $\gamma2\text{-PTC}^{HA}$ subunit had only background level of the HA-epitope tag signal (Figure 7B2), similar to the HA-epitope tag signal from pLVX-IRES-ZsGreen1 empty vector mock transfected neurons (not shown). These data confirmed that the $\gamma2\text{-PTC}^{HA}$ subunit was retained in the ER when expressed in neurons and not able to be expressed on synaptic membranes.

The GABA-evoked current from α1β2γ2-PTC subunit coexpression was similar to α1β2 receptor current

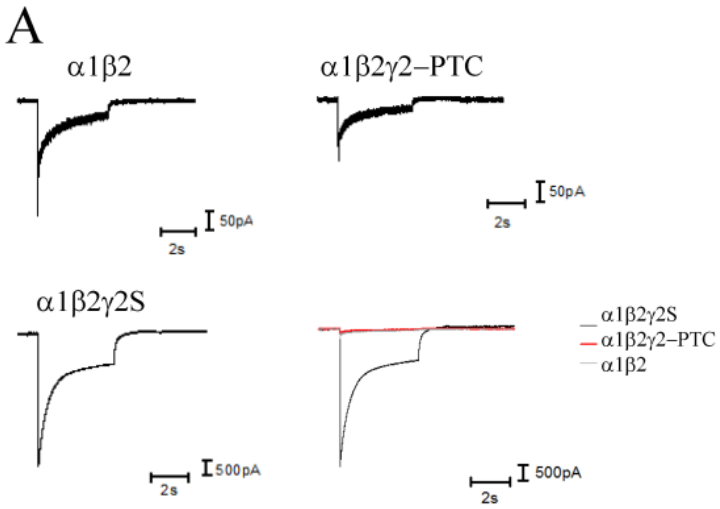
The data above suggest that the majority of receptors on the surface of cells with coexpression of α1, β2 and γ2-PTC subunits would likely be α1β2 receptors. To explore this, we coexpressed α1 and β2 subunits and α1 and β2 subunits with γ2-PTC or γ2S subunits in HEK293T cells and recorded GABA-evoked current evoked by a saturating concentration (1 mM) of GABA (Figure 8A). With coexpression of α1 and β2 subunits, GABA-evoked currents had a small peak amplitude of ~400 pA and very fast desensitization (Figure 8A). With coexpression of α1, β2 and γ2S subunits, GABA-evoked currents were much larger and desensitization was slower. With coexpression of α1, β2 and γ2-PTC subunits, GABA-evoked currents had fast desensitization, and a small peak amplitude that was more similar to α1β2 receptor currents than α1β2γ2S receptor currents.

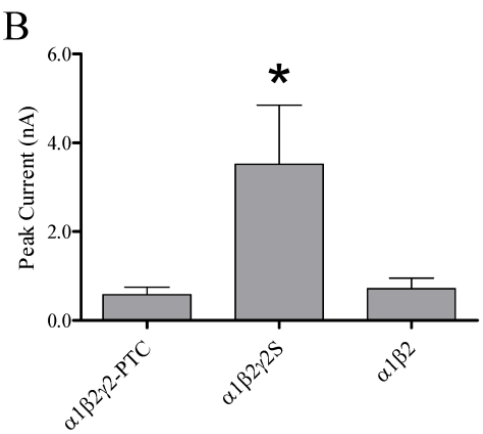
We then recorded from cells coexpressing α1, β2 and γ2S or γ2-PTC subunits (n 8 cells) and measured peak current amplitudes (Figure 8B). With coexpression of α1, β2 and γ2-PTC subunits, the average peak current amplitude was 575.5 pA. With

coexpression of α1, β2 and γ2S subunits, the average peak current amplitude was 3516.0 pA, and with coexpression of α1 and β2 subunits, the peak current amplitude was 710.4 pA. The peak current amplitudes of these two wildtype receptors fall in the normal range of reported values. The α1β2γ2-PTC receptor peak current amplitude was significantly decreased from α1β2γ2S receptors but was similar to that of α1β2 receptors.

The divalent cation Zn^{2+} is an endogenous neuromodulator [258]. It's ability to inhibit $GABA_A$ receptor currents depends on receptor subunit composition [259]. The α1β2 receptors are highly sensitive to Zn^{2+}, with IC_{50} values about 0.1-1 μM, while the α1β2γ2 receptors are very insensitive to Zn^{2+}, with IC_{50} values of about 200-600 μM. bathed cells with an external solution containing 10 μM Zn^{2+}, applied 1 mM GABA or with 10 μM Zn^{2+}, and then compared the peak currents with and without Zn^{2+} (Figure 8C). We also applied GABA at the same time interval but without Zn^{2+}, and compared peak currents to quantify current rundown. With coexpression of α1 and β2, α1, β2 and γ2S or α1, β2 and γ2-PTC subunits, currents showed minimum peak amplitude decreases with repetitive GABA applications. The α1β2 receptor peak current amplitude decreased 10%, the α1β2γ2S receptor peak current amplitude did not decrease, and the α1β2γ2-receptor peak current amplitude decreased 17%. However, with Zn^{2+} application, α1β2

receptor peak current amplitude decreased 83.2%, α1β2γ2-PTC receptor peak current amplitude decreased 85.3%, and α1β2γ2S receptor peak current amplitude decreased 9.6%. Zn^{2+} inhibited all three receptor currents with different efficiencies. The α1β2γ2-α1β2γ2-PTC receptors had the same high sensitivity to Zn^{2+} as the α1β2 receptors consistent with formation primarily of surface α1β2 receptors with coexpression of α1, and γ2-PTC subunits.





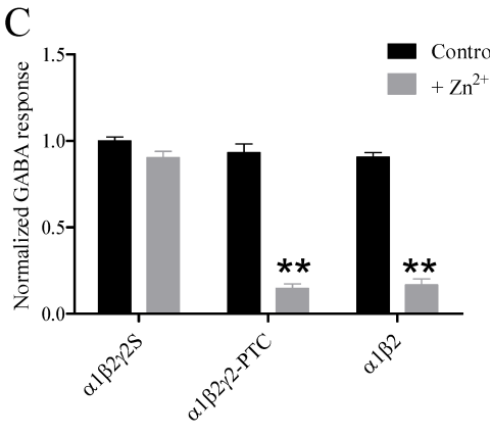


Figure 2.8 GABA-evoked currents recorded from cells coexpressing of α1, β2 and γ2-PTC subunits were similar to those obtained with expression of α1and β2 subunits

A. GABAergic currents were recorded from coexpressed α1β2, α1β2γ2-PTC and α1β2γ2S subunits. The merged picture showed the relative peak amplitude of currents recorded from α1β2γ2-PTC subunits was much smaller than that from α1β2γ2S subunits, but close to those obtained from α1β2 subunits. **B.** Peak current amplitudes from wildtype and mutant receptors were plotted. * = $p < 0.05$, one-way ANOVA with Bonferroni's multiple comparison test. **C.** The currents recorded from coexpressed α1β2γ2-PTC subunits had a Zn^{2+} sensitivity that was similar to that of coexpressed α1β2 subunits. Cells expressing α1β2γ2S, α1β2γ2-PTC or α1β2 subunits were exposed to two 1 mM GABA applications 4 seconds apart or one 1 mM GABA application followed by 10 μM Zn^{2+} washed 4 seconds and 10 μM Zn^{2+} with 1 mM GABA application. The peak currents ratio of each cell was plotted. ** = $p < 0.01$ compared to the control conditions (two-way ANOVA with Bonferroni's multiple comparison test).

The γ2-PTC subunits induced an increase in the ER stress marker BIP

Although γ2-PTC subunit mRNA was decreased by NMD, and the translation product was expressed poorly on the cell membrane, patients carrying one mutant allele had seizures. The mutant γ2-PTC subunit was clearly expressed in the transgenic mouse brain and was stable in HEK 293T cells and produced haploinsufficiency and a dominant negative effect on receptor assembly. Since the γ2-PTC subunit was so stable in transgenic mouse brain, the possibility that the existed mutant subunit had additional functions that might contribute to epilepsy pathogenesis.

The γ2-PTC subunits were stable ER proteins with a sequence similar to a γ2 subunit truncated in the middle of the first transmembrane domain. While the γ2-PTC subunits were stable and not degraded, the subunit may not have the same conformation wildtype γ2 subunits and could induce the unfolded protein response (UPR). The level of the ER chaperone BIP is an indicator of UPR-induced ER stress. We expressed 3 μg of wildtype or mutant γ2-PTC^{HA} subunits in HEK293T cells and then evaluated cellular BIP levels by Western blot (Figure 9A). The BIP band intensity for each condition was normalized to that of pcDNA mock transfected cells (100%) (Figure 9B). BIP levels in untreated and mock transfected cells were not different, but treatment of untransfected cells with tunicamycin, an ER stress inducer, significantly increased BIP levels to 339.3 ± 69.3% (n = 5, $p < 0.01$). Wildtype γ2S^{HA} subunits induced an increase in BIP levels to 143.9 ± 22.4% (n = 5), but it was not significantly different from the mock transfected condition or untreated untransfected cells (not significant). The γ2-PTC^{HA} subunits increased BIP levels to 244.8 ± 31.3% (n = 5), which was significantly more than that of the γ2S^{HA} subunits ($p < 0.05$) or mock transfected cells ($p < 0.01$). The γ2-PTC^{HA} induced BIP more efficiently than the wildtype subunit. The ER-retained γ2(Q390X) subunit has a strong dominant negative effect on $GABA_A$ receptor assembly [191]. The mutant subunit bound to α1 and wildtype γ2^{HA} subunits when coexpressed in the

HEK293T cells, retained them in the ER, and decreased their surface expression. Expressing γ2S(Q390X)^{HA} subunits in HEK293T cells increased the BIP level to 224.6 ± 27.2% (n = 5), which was also significantly higher than the γ2S^{HA} subunits ($p < 0.05$), but had a trend of lower than the γ2-PTC subunits (not significant). We evaluated the of apoptotic cells using Annexin V and found that the expression of γ2-PTC^{HA} subunits not significantly increase cell apoptosis (data not shown). Thus, γ2-PTC^{HA} subunits increased cell stress but did not induce apoptosis in these cells.

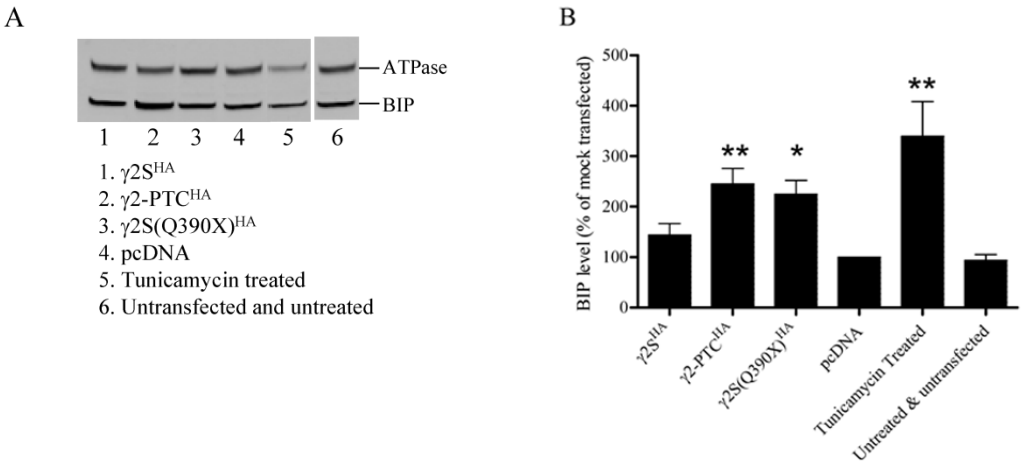


Figure 2.9 The γ2-PTC subunits induced an increase in the ER stress marker BIP.

A. BIP protein levels in γ2^{HA} subunit transfected cells or tunicamycin treated cells were evaluated. HEK293T cells were either transfected with 3 μg of γ2S^{HA}, γ2-PTC^{HA}, or γ2S(Q390X)^{HA} subunit cDNA, or were treated with 1 μM tunicamycin for 3 hours. Total proteins were collected and analyzed with Western blot detecting

detecting ATPase and BIP (n = 5). **B.** The band intensities of BIP and ATPase protein bands were quantified using Odyssey V3.0 software. The BIP intensity of each condition was normalized to ATPase band intensity. The normalized BIP band band intensities were plotted. (**) = $p < 0.01$ and (*) = $p < 0.05$ compared to the normalized BIP level obtained from either pcDNA mock transfected cells or untreated and untransfected cells (one-way ANOVA with Bonferroni's multiple comparison test).

Discussion

The intronic *GABRG2* mutation, IVS6+2T→G, resulted in activation of a cryptic mRNA splice donor site

The *GABRG2* intronic mutation, IVS6+2T→G, mutated the intron 6 splice donor site sequence from GT to GG, thus destroying the function of the site. It was suggested that the most likely pathway for splicing of this mutant mRNA was via exon 5 skipping [198], but the actual splice pattern was not determined. Analysis of mammalian EST sequences revealed that 98.7% of introns contained canonical GU-AG junctions and that 0.56% contained noncanonical GC-AG junctions [260]. The large mutant rabbit β-globin intron with an IVS+2T→G mutation was cleaved at the first step at the correct 5' site reduced efficiency, but the splicing intermediate was not cleaved at 3' site leading to

accumulation of the lariat intermediate [261]. These findings suggested that intron 6 of the mutant *GABRG2(IVS6+2T→G)* gene was unlikely to be normally spliced.

DBASS5 is a database of aberrant 5' splice sites in human disease genes [199] contains 40 mutations at the U of the 5' GU sequence. In this database, the 5' GU sequence was mutated most frequently to GG (35%), GC (35%) or GA (22.5%) Interestingly, 92.5% of the mutations activated a cryptic 5' donor site within about 100 of the wildtype donor site (not shown). When expressed in HEK293T cells, the *GABRG2* mutation, IVS6+2T→G, activated a cryptic 5' donor site 53 bp downstream of the site, consistent with the function of these mutations in the DBASS5 database. While alternative intron splicing is regulated differently among cell types, the core intron machinery is distributed ubiquitously in every cell. To confirm that the splicing pattern found HEK 293T cells *in vitro* is also found in mouse brain *in vivo*, we expressed the human *hGABRG2* BACs driven by its endogenous promoter in C57BL/6J mice. The Tg($hGABRG2^{HA}$) mice expressed the HA-tagged BAC clone RP11-1035I20, and the Tg($hGABRG2^{IVS6+2T\rightarrow G}$) mice expressed the untagged BAC clone carrying the IVS6+2T→G mutation. There is a 20 kbp human chromosome 5 fragment upstream of the *GABRG2* genomic sequence in this BAC clone, which is predicted to contain the

endogenous human γ2 subunit promoter. This BAC clone was recognized by the mouse transcription and translation machineries, resulting in expression of wildtype and mutant human γ2 subunits in the transgenic mice brain. When the BACs were expressed in mouse brain, the mutant intron 6 splicing pattern was the same as the mutant BACs expressed in HEK293T cells.

Pre-mRNA intron splicing is regulated by functional interactions among transcription, splicing and chromatin epigenetic modifications [252, 262]. The *CMV-GABRG2* BACs were driven by a CMV promoter, which could recruit a different set of transcription factors and interact with the splicing machinery differently than with the endogenous *GABRG2* promoter. However, the *GABRG2* BAC and the *GABRG2(IVS6+2T→G)* BAC driven by the endogenous promoter and the CMV promoter-driven BACs had the same intron splicing pattern. Thus, the CMV promoter and the endogenous promoter had the same effect on *GABRG2* gene intron splicing.

The intronic *GABRG2* mutation, IVS6+2T→G, resulted in partial intron 6 retention and a frame shift resulting in a PTC in exon 7 that activated NMD

The mature mutant mRNA retained a 53 bp intron 6 fragment that resulted in an open reading frame shift in exon 7 and generated a PTC in exon 7. Thus, the mutant mRNA was NMD susceptible and we confirmed this by demonstrating that the mutant mRNA was rescued partially by abolishing NMD function. NMD-susceptible mRNAs have lower translational efficiency, and protein translated from NMD-susceptible mRNA is often not stable, probably because such proteins are truncated [201, 263]. Our study suggested that the IVS6+2T→G mutation could significantly decrease mutant γ2 subunit mRNA levels due to NMD, suggesting that the disease may be, at least in part, caused by *GABRG2* haplo-insufficiency.

Transcription of the mutant mRNA resulted in production of a stable truncated protein, the γ2-PTC subunit

Although γ2-PTC subunit mRNAs were subject to degradation by NMD, they not necessarily completely degraded since different cell types have different NMD efficiency [200]. For example, we demonstrated that 39.1% of α1(S326fs328X) subunit

mRNA survived NMD in HEK293T cells, 17% survived in HeLa cells, and 24% in cortical neuronal cell culture [201]. In cell types with less NMD efficiency than HEK293T cells, the amount of mutant transcript could be higher than 40%, and mRNA degraded by NMD could be translated to a stable protein. The mutant *GABRG2(IVS6+2T→G)* mRNA translation product was shown to contain the N-terminal 217 amino acids of the wildtype γ2 subunit and a novel 29 amino acid peptide tail the (the γ2-PTC subunit) composed of retained intron 6 sequence and frame shifted exon 7 sequence at the C-terminus of the mutant protein. The γ2-PTC subunit is homologous to γ2 subunit truncated in the middle of TM1. The sequence homology between the first amino acid of wildtype γ2 subunit and γ2-PTC subunit is 88.2%. Surprisingly, the γ2-γ2-PTC subunit was a stable intracellular protein in HEK293T cells, transfected rat neurons and Tg($hGABRG2^{IVS6+2T\rightarrow G}$) mouse brain, and while the γ2-PTC subunit total was comparable to wildtype γ2S or γ2L subunits in HEK293T cells, its surface level was significantly lower than the wildtype subunits.

The γ2-PTC subunit has a structure similar to AChBPs but has different functions

The γ2-PTC subunit contains the N-terminus of the γ2 subunit, and the sequence identity between γ2-PTC subunit and AChBPs was high (21-29%) [254]. Thus the γ2-PTC subunit is homologous to AChBPs. AChBPs form homopentamers in glial cells [250, 251, 254], and the crystal structure of AChBP homopentamers resembles the N-terminus of assembled cys loop receptors. When wildtype γ2 subunits were coexpressed with α1 and β2 subunits in HEK293T cells or adult rat brain, they oligomerized with α1 and β2 subunits to form α1β2γ2 heteropentamers, but not γ2 homopentamers or α1β2 heteropentamers [183, 257], suggesting that the binding efficiency of γ2 subunits to α1 and β2 subunits is higher than it is between γ2 subunits. Given the sequence similarity to AChBPs, the γ2-PTC subunit might also be able to oligomerize with α1 and β2 subunits and might even be assembled into heteropentameric receptors. Our studies showed that γ2-PTC subunits did oligomerize with α1 and β2 subunits; however, they did not produce heteropentamers that were secreted or trafficked to the cell membrane. Instead, they were retained in the ER and had a dominant negative effect on surface trafficking of α1β2γ2 receptors. Thus, although γ2-PTC subunits and AChBPs are highly homologous, these two proteins clearly have very different functions.

The *GABRG2(IVS6+2T→G)* mutation could induce epilepsy by both γ2 subunit haplo-insufficiency and γ2-PTC subunit dominant negative functions

We demonstrated *in vitro* in transfected HEK293T cells and *in vivo* in mice that mutant mRNA was degraded partially by NMD, and that the mRNA that was not was translated to the stable γ2-PTC subunit. The γ2-PTC subunit was primarily retained in the ER and only minimally expressed on cell membranes. $GABA_A$ receptors containing γ2 subunits are predominately synaptic receptors that mediate phasic synaptic neurotransmission, and the γ2 subunit is required for synaptic $GABA_A$ receptor The IVS6+2T→G mutation decreased the γ2 subunit mRNA level and generated a protein that was poorly trafficked to the cell membrane. It should decrease the membrane level of γ2 subunit-containing $GABA_A$ receptors, decrease the amount of synaptic receptors, and impair inhibitory $GABA_A$ receptor currents. Thus, the *GABRG2(IVS6+2T→G)* mutation could produce epilepsy, at least in part, by γ2 subunit haplo-insufficiency. The γ2-PTC subunit oligomerized with α1 and β2 subunits and had a dominant negative effect on surface trafficking of α1β2γ2 receptors. The

GABRG2(IVS6+2T→G) mutation could also produce with epilepsy due to the dominant negative effects of the γ2-PTC subunit.

The *GABRG2(IVS6+2T→G)* mutation could induce epilepsy also by inducing chronic ER stress

The γ2-PTC subunit is retained in the ER and increased the ER stress marker BIP level significantly higher than wildtype γ2S subunits. Another *GABRG2* epilepsy mutation, the autosomal dominant *GABRG2(Q390X)* mutation, is associated with Dravet syndrome [189]. The mutant γ2(Q390X) subunit is also retained in the ER and not expressed on the cell membrane [191]. The γ2(Q390X) subunit also increased BIP level in HEK 293T cells, but to a level slightly less than that the increase produced by the γ2-γ2-PTC subunit. Increased BIP level during UPR induced ER-stress induces both apoptosis and protective responses such as reduced translation, enhanced ER protein-protein-folding capacity, and clearance of misfolded ER proteins [264]. These adaptation and apoptosis responses are designed to help adaptation to the stress or to remove cells, depending on the nature and severity of the stress [265]. The fact that γ2-PTC subunit transfected cells did not induce apoptosis suggested that the γ2-PTC subunit

induced mild, chronic stress in the cell, but the adaptive responses induced by γ2-PTC subunits would affect how cells respond to other stress factors. ER stress responses contribute to the pathogenesis of diseases including diabetes mellitus, cancer and AIDS [265]. Neurodegenerative diseases such as Alzheimer's disease and Huntington's disease are often associated with ER stress responses induced by mutant proteins. Thus, ER responses may contribute to the pathogenic mechanism of both *GABRG2(Q390X)* and *GABRG2(IVS6+2T→G)* mutations.

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Chapter 3

Novel GABRG2 Frame Shift Mutation in Familial Epilepsy: Impaired Cell Surface Expression of αβγ Receptors

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Abstract

Objective: To explore the pathogenic mechanisms underlying generalized epilepsy and febrile seizures plus (GEFS+) in a family with a novel *GABRG2* frameshift mutation.

Methods: *GABRG2* sequencing, expression of the predicted mutant γ2S subunit cDNA, immunoblotting, flow cytometry assay and electrophysiology in HEK293T cells.

Results: Four affected and one unaffected individuals carried a c.1329delC *GABRG2* mutation [γ2S(S443delC)] resulting in a protein with a modified and elongated carboxy-terminal different from the wildtype γ2S subunit. The mutant subunit was translated as a stable and larger protein compared to the wildtype γ2S subunit; it was retained in the ER and not expressed on the cell surface membrane, suggesting a mechanism of haploinsufficiency. Peak GABA-evoked currents recorded from cells cotransfected with mutant γ2S, α1 and β2 subunits were significantly decreased and comparable to α1β2 receptor currents.

Interpretation: The γ2S(S443delC) mutation is the first GABR epilepsy mutation predicted to produce an unnatural stop codon in the 3' UTR and an extended peptide. GEFS+ phenotype observed in this family is likely caused by γ2S subunit loss-of- and possibly to dominant-negative suppression of α1β2γ2 receptors. Most GABRG2

truncation mutations result in GEFS+, but the spectrum of phenotypic severity is wider, ranging from asymptomatic individuals to the Dravet syndrome. Mechanisms the severity of the phenotype are therefore complex and difficult to correlate with its demonstrable functional effects.

Introduction

$GABA_A$ receptors are ligand-gated chloride ion channels and the primary mediators of fast inhibitory synaptic transmission in the central nervous system. They are formed by pentameric assemblies of different subunit subtypes from eight subunit families (α1-α6, β1-β3, γ1-γ3, δ, ε, π, θ and ρ1-ρ3)[44]. Classical anticonvulsants such as benzodiazepines or barbiturates potentiate $GABA_A$ receptor currents[9]. Mutations in $GABA_A$ receptor subunit genes, including *GABRA1, GABRAB3, GABRBD* and *GABRG2*, in different subunit domains, have been associated with generalized epilepsy syndromes and with the genetic epilepsy with febrile seizures plus (GEFS+) spectrum, including Dravet syndrome, in rare families and in sporadic cases with *de novo* mutations[44].

GABRG2 gene mutations and variants associated with epilepsy include three missense mutations in coding sequences[169, 177, 187], three nonsense mutations in

coding sequences[189, 195, 196], and one mutation in an intron splice donor site that was also predicted to cause a protein truncation[198]. Phenotypes associated with missense mutations are relatively mild and include familial childhood absence epilepsy and febrile seizures[177], GEFS+ without Dravet syndrome[169] and febrile seizures[187]. Nonsense mutations in coding sequences have been associated with more severe phenotypes, including Dravet syndrome[189, 195], but also with phenotypes similar to those caused by missense mutations, including GEFS+[196] and childhood absence epilepsy and febrile seizures[198]. There is evidence that epilepsy syndromes associated with protein truncation mutations are caused by a combination of degradation of unstable subunit mRNA and of unstable truncated subunit protein with a dominant negative suppression of the biogenesis of wild type subunits[191, 201]. This combination of effects would result in a considerable loss of inhibition that might explain the most severe phenotypes [191].

We studied a family with mild generalized epilepsy and febrile seizures in which affected individuals carried a novel frame shift mutation of the *GABRG2* gene, resulting a mutant protein that was predicted to lose the last 24 C-terminal amino acids and gain 50 amino acids different from those of the natural variant, with consequent lower hydrophobicity of the C-terminus. This is the first GABR epilepsy mutation predicted to produce an unnatural stop codon in the 3' UTR and to produce an extended subunit

peptide. The subunit mRNA should be stable and should produce γ2S subunits with a disrupted 4th transmembrane domain and an extended C terminal tail. To explore the pathogenic mechanisms underlying this novel mutation, we expressed the predicted γ2S subunit cDNA in HEK293T cells. The mutant γ2S subunit was translated as a stable protein with a larger molecular mass than the wildtype γ2S subunit. It was not detected the cell membrane, and peak GABA evoked currents recorded from cells cotransfected with mutant γ2S subunit and α1 and β2 subunits were significantly decreased. The GABAergic currents recorded from coexpressed α1, β2 and γ2S(S443delC) subunits likely α1β2 receptor currents.

Subjects/Materials and Methods

Patients

We studied a non consanguineous Italian family comprised of 4 affected members and a healthy carrier (Figure 1A). The proband (III:4), a 5 year-old boy, was brought to medical attention at 9 months after a febrile seizure, lasting less than a minute. He experienced 7 subsequent seizures until age 3 years, always during fever. At 19 months, neurological examination was normal, and the Griffiths developmental scale general

quotient was 96. EEG showed normal background activity with rare bursts of epileptiform abnormalities during sleep (Figure 1B). Brain MRI was normal. No treatment was assigned. The proband's sister (III:3) was a healthy 7 year old girl. The proband's 35 year old mother (II:3), had a single febrile and several nonfebrile convulsive seizures starting at 6 months and recurring during infancy, especially in sleep. She was initially resistant to phenobarbital but responded to valproate and remained seizure free from age 5 to 8 while on this drug. At the age of 8, therapy was A single seizure occurred again at age of 16. EEG, at age 20, showed generalized bursts of slows waves. She had normal cognitive abilities and her brain MRI was normal. The proband's 49 year old uncle (II:2), had experienced a few febrile seizures in infancy. His 10 year old son (III:1) had only had a nonfebrile generalized seizure while awake at the of 9. The proband's 70 year old grandmother (I:1) did not recall having ever been told she experienced seizures. The overall family clustering of clinical features is consistent with generalized epilepsy with febrile seizures plus (GEFS+).

After obtaining informed consent we extracted genomic DNA from peripheral blood of affected family members (II:2, II:3, III:1 and III:4) and their healthy relatives (I:1 and II:4). The study was approved by the Commission for Medical Ethics of the Meyer's University Hospital.

GABRG2 mutation analysis

We performed mutation analysis of the *GABRG2* gene in the proband (III:4) and extended the genetic study to available family members (I:1, II:2, II:3, II:4, III:1) (Figure 1A). DNA was extracted from peripheral blood leukocytes using an automated DNA isolation robot (QIASymphony, QUIAGEN GmbH, Hilden, Germany), according to the manufacturer's protocol. The 9 exons covering the coding regions of *GABRG2* (Reference sequence: NM_000816.3) and their respective intron-exon boundaries were amplified by PCR and cycle sequenced using the BigDye Terminator v.1.1 chemistry (LIFE Technologies, Carlsbad, CA, USA). The sequence reactions were analyzed on a 3130XL sequencer (LIFE Technologies, Carlsbad, CA, USA). The identified *GABRG2* alteration was not found in a control population of 190 ethnically matched subjects and was described according to nomenclature using the cDNA sequence NM_000816.3.

Bioinformatics

The TMpred[266] and TMHMM[267, 268] programs predict the membrane-membrane-spanning regions and their orientation using a database of naturally occurring transmembrane proteins. Since the mutant subunit was predicted to lose the last 24 C-C-terminal amino acids and to gain 50 amino acids different from the wildtype subunit,

used TMPred and TMHMM to predict if the mutant subunit was able to cross the membrane as the 4th transmembrane region of the wildtype subunit.

Expression vectors with $GABA_A$ receptor subunits

The coding sequences of human α1, β2, γ2S and γ2L $GABA_A$ receptor subunits from the translation initiation codon ATG to the stop codon were cloned into pcDNA3.1 expression vectors (Invitrogen) as previously described[211]. The cDNA encoding the HA peptide, YPYDVPDYA, was introduced between the 4th and 5th amino acids of mature γ2S and γ2L subunits to create $\gamma2S^{HA}$ and $\gamma2S(S443delC)^{HA}$ subunits, which has been reported to be a functionally silent position[50]. The γ2 subunit 3' polyA site fragments were cloned from RP11-1035I20 (BACPAC Resources; http://bacpac.chori.org).

Cell culture and transfection

Human embryonic kidney cells (HEK 293T) (ATCC, CRL-11268) were at 37°C in humidified 5% CO_2, 95% air and grown in Dulbecco's modified Eagle's (Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were transfected with cDNAs using the FuGENE

6 transfection reagent (Roche Applied Science) at a DNA:Transfection Reagent ratio of 1:3 according to the manufacturer's instructions. The transfected cells were harvested after 36 hrs in culture for the following experimental protocols.

Flow cytometry

Flow cytometry was performed as described previously [269]. Briefly, transfected cells were collected in FACS buffer (1X PBS pH 7.0 with 2% fetal bovine serum) and separated to single cells, then permeablized with CytoPerm (BD Biosciences) for 15 minutes, and washed with CytoWash (BD Biosciences). Permeablized cells were incubated with anti-HA antibodies (clone 16B12, Covance) conjugated to the Alexa-647 fluorophore for an hour, then washed three times and fixed with 2% paraformaldehyde. Flow Cytometry experiments were performed in the Vanderbilt Medical Center Flow Cytometry Shared Resource, which is supported by the Vanderbilt Ingram Cancer Center (P30 CA68485) and the Vanderbilt Digestive Disease Research Center (DK058404). Samples were run on a 5-laser BD LSRII system equipped with 635 nm red diode lasers. For each staining condition, 50,000 cells were analyzed. Nonviable cells were excluded from analysis based on forward and side scatter profiles (not shown) as determined by staining with 7-aminoactinomycin D (Invitrogen). Data were acquired using FACSDiva 6.0 (BD Biosciences) and analyzed off line using FlowJo 7.5 (Treestar, Inc.). The mean

fluorescence intensity of each sample was evaluated, and normalized to the 100% control ($\alpha1\beta2\gamma2S^{HA}$ as noted in the text). The normalized mean fluorescence intensity was represented as a percentage of the 100% control. Data were plotted as mean ± SEM. Pair-wise two-tail *Student* t-tests were used to compare between conditions unless otherwise specified.

Immunoblotting

Cultured HEK 293T cells were lysed in radioimmune precipitation assay (RIPA) buffers (Pierce) and a protease inhibitor cocktail (Sigma Aldrich). Cell lysates were cleaned by centrifugation at 20,000 X *g* for 30 min. The supernatants were subjected to further experiments or directly to SDS-PAGE. Proteins in gels were transferred to Millipore Immobilon® FL PVDF Membrane (Millipore). Non-specific binding on the membrane was blocked with the Odyssey blocking buffer (Li-cor). Monoclonal anti-HA epitope tag antibodies (0.2 μg/ml; clone 16B12, Covance) were used to detect HA epitope-tagged γ2 subunits. Anti-Na+/K+-ATPase antibodies (0.2 μg/ml; clone ab7671, Abcam) were used to check loading variability. Following incubation with primary antibodies, IRDye® secondary antibodies were used at a 1:10,000× dilution (Li-cor) for visualization of specific bands with the Odyssey imaging system (Li-cor).

Electrophysiology

Lifted whole cell recordings were obtained from transfected HEK 293T cells as described previously[170]. Briefly, cells were bathed in an external solution consisting of (in mM): NaCl 142; KCl 8; $MgCl_2$ 6; $CaCl_2$ 1; HEPES 10; glucose 10, pH 7.4, 325 mOsm. Electrodes were fire-polished to resistances of 0.8-1.5 MΩ and filled with an internal solution consisting of (in mM): KCl 153; $MgCl_2$ 1; MgATP 2; HEPES 10; EGTA 5, pH 7.3, 300 mOsm. The combination of internal and external solutions produced a chloride equilibrium potential of ~0 mV. For all recordings cells were voltage clamped at -20 mV. GABA (1 mM) was applied to cells for 4 seconds and cells were then washed with external solution for 40 seconds. Zn^{2+} (10 μM) was then preapplied for 10 seconds followed by coapplication of GABA (1 mM) and Zn^{2+} (10 μM) for 4 seconds. Finally, cells were washed with external solution for 10 seconds followed by application of GABA (1 mM) for 4 seconds. Whole cell currents were low-pass filtered at 2-5 kHz and digitized at 10 kHz, and peak current amplitudes were quantified using the pClamp9 software suite (Axon Instruments).

Results

The c.1329delC deletion in the *GABRG2* gene is predicted to cause an open-reading frame shift and generate a novel domain terminus in γ2 subunits

The proband (III:4) had a heterozygous c.1329delC deletion in the *GABRG2* gene, and the mutation was also present in individuals I:1, II:2, II:3 and III:1 (Figure 1A). The c.1329delC mutation deleted a cytosine nucleotide in the Ser443 codon TCC of the immature γ2S subunit sequence. It was predicted *in silico* to cause open-reading frame shift, resulting in the loss of the natural stop codon and generation of a new stop codon in the *GABRG2* 3' UTR (p.Tyr444MetfsX51). Specifically, the mutant γ2S subunit, that we named S443delC, was predicted to lose the last 24 C-terminal amino acids and gain 50 amino acids differing from those of the natural variant (Figure 2B). The TMPred-calculated hydrophobicity of the mutant subunit showed that the mutant γ2S(S443delC) subunit C-terminus hydrophobicity was somewhat lower compared to the same region of the wildtype γ2S subunit (2A). The TMHMM program did not identify any transmembrane domain in the novel C-terminus (not shown). Thus, the S443delC mutation likely interrupted the wildtype γ2S subunit membrane topology.

The γ2S subunit genomic sequence carries two polyA sites, 800 bp and ~2.4 kbp downstream of the γ2S subunit translation stop codon. We cloned genomic sequence containing either the proximal polyA site or both proximal and distal polyA sites to γ2S subunit cDNA and introduced the S443delC mutation. It is unclear whether or not the extension of the C terminus of the subunit into the 3' UTR would alter polyA site usage. When wildtype or mutant γ2S subunits were expressed in HEK293T cells, all four subunits had the same 3'-UTR, suggesting that the mutation did not interfere with polyA site recognition (Figure 3). The sequence of the 3'-UTR fragment showed that they all utilized the proximal polyA site. The sequence of the mutant γ2S(S443delC) subunit showed that, as predicted, the mutation caused a frame shift in exon 9 and generated a novel 50 amino acids C-terminus. The mutant γ2S(S443delC) subunit premature peptide is 493 amino acids while the wildtype γ2S subunit premature peptide is 467 amino acids, a difference in length of 26 amino acids.

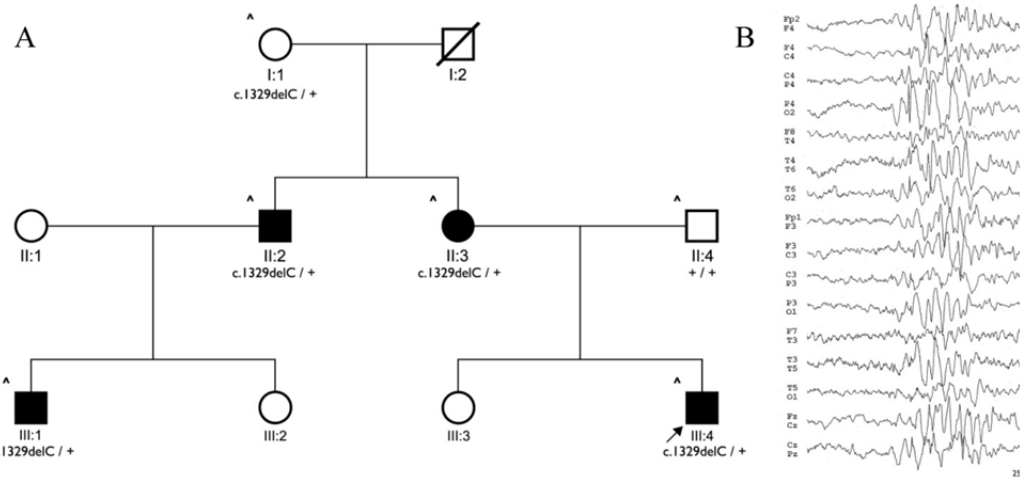

Figure 3.1 *GABRG2(S443delC)* mutation was identified in a GEFS+ family.

A. Pedigree of the family with GEFS+. The arrow points to the proband. B. The EEG presented is from patient III:4 and shows a burst of generalized epileptiform abnormality.

A. γ2S subunit: wildtype sequence

```
  1  MSSPNIWSTG SSVYSTPVFS QKMTVWILLL LSLYPGFTSQ KSDDDYEDYA SNKTWVLTPK
 61  VPEGDVTVIL NNLLEGYDNK LRPDIGVKPT LIHTDMYVNS IGPVNAINME YTIDIFFAQT
121  WYDRRLKFNS TIKVLRLNSN MVGKIWIPDT FFRNSKKADA HWITTPNRML RIWNDGRVLY
181  TLRLTIDAEC QLQLHNFPMD EHSCPLEFSS YGYPREEIVY QWKRSSVEVG DTRSWRLYQF
241  SFVGLRNTTE VVKTTSGDYV VMSVYFDLSR RMGYFTIQTY IPCTLIVVLS WVSFWINKDA
301  VPARTSLGIT TVLTMTTLST IARKSLPKVS YVTAMDLFVS VCFIFVFSAL VEYGTLHYFV
361  SNRKPSKDKD KKKKNPAPTI DIRPRSATIQ MNNATHLQER DEEYGYECLD GKDCASFFCC
421  FEDCRTGAWR HGRIHIRIAK MDSYARIFFP TAFCLFNLVY WVSYLYL*
```

B. γ2S(S443delC) subunit: predicted sequence

```
  1  MSSPNIWSTG SSVYSTPVFS QKMTVWILLL LSLYPGFTSQ KSDDDYEDYA SNKTWVLTPK
 61  VPEGDVTVIL NNLLEGYDNK LRPDIGVKPT LIHTDMYVNS IGPVNAINME YTIDIFFAQT
121  WYDRRLKFNS TIKVLRLNSN MVGKIWIPDT FFRNSKKADA HWITTPNRML RIWNDGRVLY
181  TLRLTIDAEC QLQLHNFPMD EHSCPLEFSS YGYPREEIVY QWKRSSVEVG DTRSWRLYQF
241  SFVGLRNTTE VVKTTSGDYV VMSVYFDLSR RMGYFTIQTY IPCTLIVVLS WVSFWINKDA
301  VPARTSLGIT TVLTMTTLST IARKSLPKVS YVTAMDLFVS VCFIFVFSAL VEYGTLHYFV
361  SNRKPSKDKD KKKKNPAPTI DIRPRSATIQ MNNATHLQER DEEYGYECLD GKDCASFFCC
421  FEDCRTGAWR HGRIHIRIAK MDSMLGSSSP LPSACLIWSI GSPTSTCEEV WVLLIWFLFT
481  ESHGEMSVLS PLK*
```

Figure 3.2 The mutant γ2S(S443delC) subunit sequence.

The peptide sequence of wildtype γ2S (A) and mutant γ2S(S443delc) subunit (B) premature peptide is predicted to have a novel 50 amino acid C-terminus. The underlined sequence in A represents wildtype TM4. The bold red letters represent the novel C-terminus generated by frame shift.

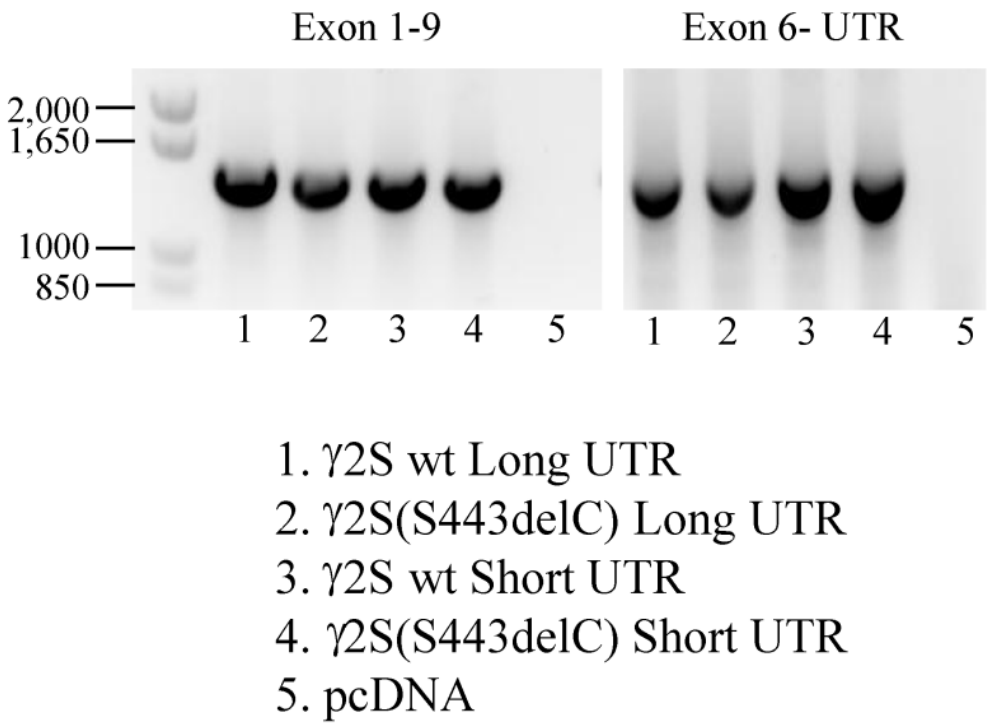

Figure 3.3 The *GABRG2(S443delC)* mutation did not affect polyA site recognition.

The RT-PCR fragments were amplified from total RNAs collected from HEK293T cells transfected with mutant or wildtype γ2S subunit cDNAs carrying either both distal and proximal polyA sites (Long UTR) or only the proximal polyA site (Short UTR). An adaptor primer was added to the end of the UTR during cDNA synthesis. RT-PCR was performed with two sets of primers. The exon 1 forward primer binding site started at the ATG translation start site. The exon 9 reverse primer bound to the complimentary strand of DNA, and the binding site started at the TGA translation termination site of the wildtype γ2S subunit, 1404 bp downstream of the ATG translation start site. The exon 6 forward primer binding site started at the beginning of exon 6 of the γ2S subunit cDNA. The UTR primer was a reverse primer that bound to the adaptor primer at the end of mRNAs downstram of the polyA tail.

The γ2S(S443delC) subunits were stable proteins, but their total level was significantly lower than wildtype γ2S subunits

We cloned the coding sequence of the γ2S(S443delC) subunit cDNA into the pcDNA3.1 vector, introduced an HA-epitope tag at a functionally silent site, and expressed it in HEK293T cells either alone or with α1 and β2 subunits (Figure 4). Western blot showed that when expressed alone, the γ2S(S443delC) subunit was translated to a stable protein in HEK293T cells, but its molecular size was larger than wildtype γ2S subunits (Figure 4A, lane 2, 3). Coexpression with α1 and β2 subunits altered the glycosylation pattern of wildtype γ2S subunits (Figure 4A, lanes 2 and 5, see double bands in lane 5 but not lane 2) but did not affect that of mutant γ2S(S443delC) subunits (Figure 4A, lanes 3 and 6). The epilepsy associated mutation, Q390X, generated a γ2S subunit that was truncated in the second intracellular loop[189]. The γ2S(Q390X) subunit was an intracellular protein that only had endoplasmic reticulum (ER) core glycosylation[191]. The γ2S(Q390X) subunit protein bands were much smaller than wildtype γ2S or mutant γ2S(S443delC) subunit bands (Figure 4A, lane 4). The glycosylation pattern of the γ2S(Q390X) subunit was not affected also by coexpression with α1 and β2 subunits (Figure 4A, lanes 4 and 7). These data suggested that the cellular localization of the γ2S(S443delC) subunits might be the same as that of γ2S(Q390X) subunits.

The Western blot result suggested that the γ2S(S443delC) subunits had decreased protein level with expression of either single subunits or with coexpression with α1 and β2 subunits. We then evaluated the total levels of the γ2S(S443delC) subunits with both single subunit expression or with coexpression with α1 and β2 subunits (Figure 4B). We permeablized the transfected HEK293T cells, stained HA-tagged $\gamma2S^{HA}$ or $\gamma2S(S443delC)^{HA}$ subunits using fluorescence conjugated HA antibodies and evaluated mean fluorescence intensity by flow cytometry. The total level of HA-tag in cells coexpressing α1, β2 and γ2S subunits was set at 100%. When expressed alone, the $\gamma2S^{HA}$ subunit total level was 86.6 ± 1.6%, and with coexpression with α1 and β2 subunits the $\gamma2S^{HA}$ subunit total level was increased (p = 0.01). However, the total level of $\gamma2S(S443delC)^{HA}$ subunits was only 34.6 ± 4.6% when expressed alone as single subunit or 35.7 ± 6.0% when coexpressed with α1 and β2 subunits. The total level of $\gamma2S(S443delC)^{HA}$ subunits was not changed by coexpression with α1 and β2 subunits (p = 0.74), but was significantly lower than the γ2S subunit total level either with single subunit expression or α1, β2 and γ2S subunit coexpression ($p < 0.01$ in both cases).

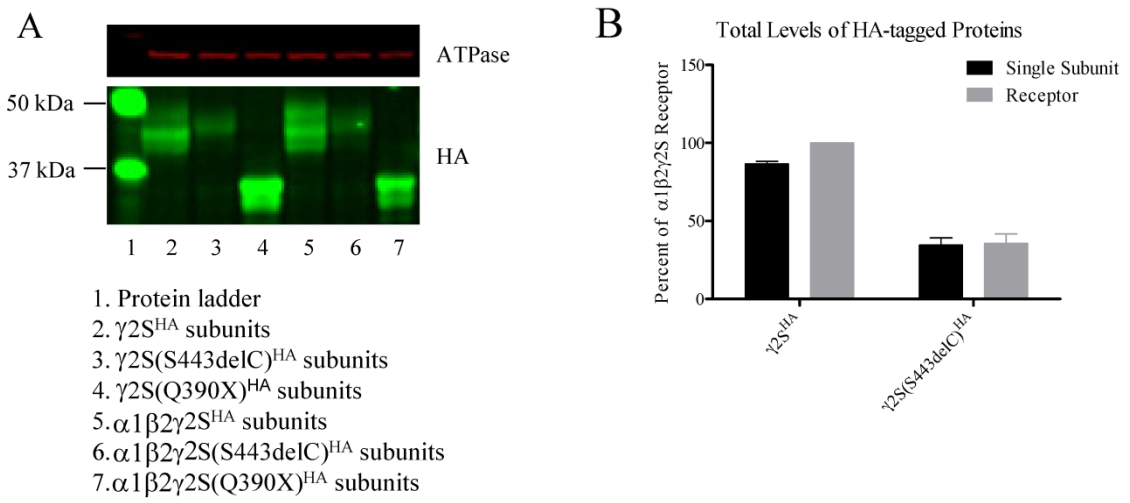

Figure 3.4 The γ2(S443delC)subunits were stable, but their total level was decreased.

A. Western blot was performed on transfected HEK29T cells total cell lysates. The red channel shows the ATPase antibodies signal, and the green channel shows the HA-antibody signal. B. Total HA levels obtained from transfected HEK293T cells were plotted. The wildtype γ2S subunit and mutant γ2S(S443delc) subunit cDNAs were transfected either with pcDNA empty vector or with α1 and β2 subunit cDNAs, and the results were expressed relative to the level obtained for the wild type γ2S subunit coexpressed with α1 and β2 γ2S subunits. The double stars correspond to $p < 0.01$ compared to wildtype α1 β2 and γ2S subunit coexpression.

γ2S(S443delC) subunits were not expressed on the cell membrane

$GABA_A$ receptor subunits are synthesized in the ER where they are assembled to heteropentameric receptors and then trafficked to the cell membrane[50]. Misfolded and unassembled $GABA_A$ receptor subunits are degraded in the ER by the ubiquitin-

ubiquitin-proteasome system[52, 270]. When coexpressed, the truncated γ2S(Q390X) subunits were retained in the ER where they oligomerized with α1 and β2 subunits and decreased surface α1 and β2 subunit levels[191]. Because γ2S(S443delC) subunits might have four transmembrane domains similar to wildtype γ2S subunits, we explored if the γ2S(S443delC) subunits could assemble with α1 and β2 subunits and be expressed on membranes as functional receptors. We coexpressed $\gamma2S^{HA}$ or $\gamma2S(S443delC)^{HA}$ subunits with α1 and β2 subunits in HEK293T cells, stained both permeablized and unpermeablized cells with antibodies against the α1 subunit and the HA tag. The α1 subunits can assemble with β2 subunits to form functional heteropentameric α1β2 receptors that traffic to the cell membrane[56, 183]. Confocal images from cells cotransfected with α1, β2 and γ2S subunits showed the HA-tag signal in both permeablized and unpermeablized cells, suggesting that wildtype γ2S subunits were expressed intracellularly as well as on the cell surface (Figure 5A). The HA signal was colocalized with the α1 subunit signal in both total and surface conditions. With coexpression of α1, β2 and γ2S(S443delC) subunits, HA signal was only detected in permeablized cells (Figure 5B), suggesting that the mutant subunit was retained in the ER and did not form receptors that trafficked to the cell surface.

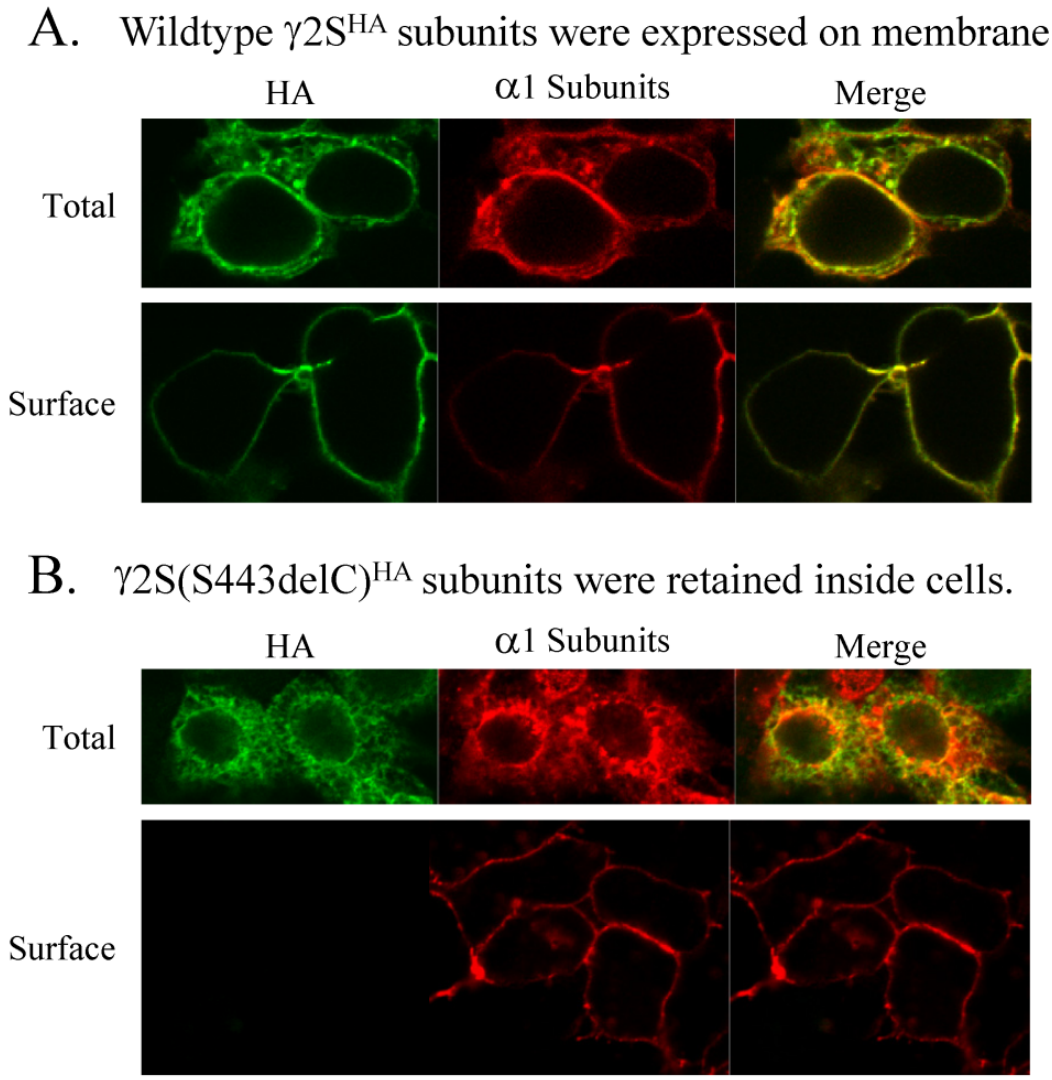

Figure 3.5 The γ2(S443delC)subunits were retained in an intracellular compartment.

Confocal images of wildtype γ2 (A) and mutant γ2S(S443delC) (B) subunits coexpressed with α1 and β2 subunits were obtained. Total signals were evaluated by staining permeablized cells, and surface signals were evaluated by staining paraformaldehyde fixed cells.

The GABA-evoked current recorded from α1β2γ2S(S443delC) receptors was significantly decreased.

We characterized the effect of the γ2S subunit mutation, S443delC, on α1β2γ2S receptor current properties. Whole-cell currents were elicited from lifted HEK293T cells cotransfected with α1 and β2 subunits and wildtype γ2S or mutant γ2S(S443delC) subunits by applying a saturating GABA concentration (1 mM) for 4 sec using the rapid concentration jump technique, and the amplitudes of GABA-evoked currents were determined (Figure 6). Normalized α1β2γ2S(S443delC) receptor current had a different time course compared to wildtype α1β2γ2S receptor current, suggesting different macroscopic current kinetics (Figure 6A). The mutant receptor currents desensitized faster and their residual currents were smaller than those of wild type currents. Mutant receptor peak current was reduced relative to wildtype receptor peak current (Figure 6A). Maximal wildtype peak current was substantially larger (6203 ± 223 pA, n = 6) than that obtained from mutant receptors (466 ± 31.8 pA, n = 3, $p < 0.001$) (Figure 6B). Moreover, in 5 of 8 of the cells expressing mutant receptors, very small currents (26.7 ± 6.81 pA) were evoked by 1 mM GABA (Figure 6C). Wild-type receptors did not show this phenomenon, but γ2 subunit null condition α1β2 receptors

(not shown). All these properties suggested that the GABA-evoked currents recorded from cells coexpressing α1, β2 and γ2S(S443delC) subunits were α1β2 receptor currents.

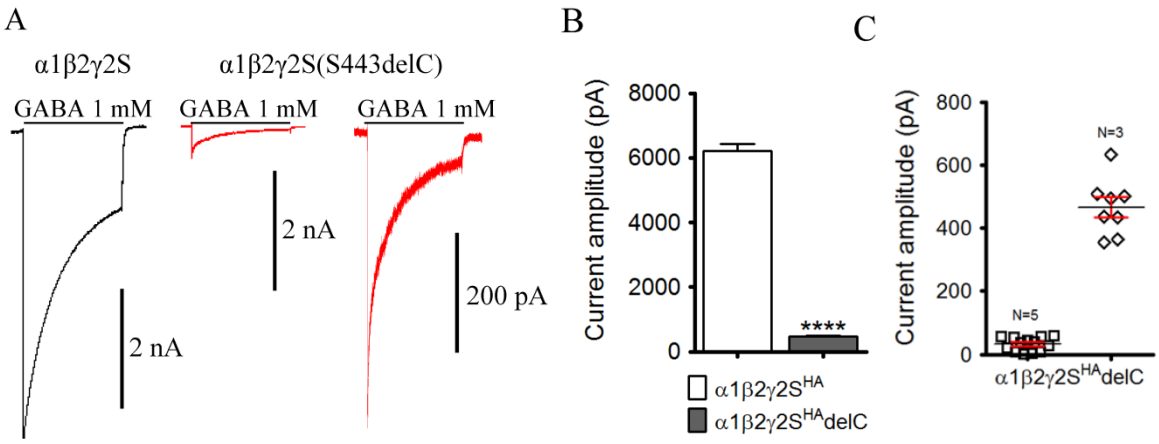

Figure 3.6 The γ2(S443delC)receptors had decreased GABA evoked currents.

A. GABAergic currents were recorded from HEK293T cells coexpressing α1β2γ2S or α1β2γ2S(S443delC) subunits. B. Peak current amplitudes from wildtype and mutant receptors were plotted. C. Cells coexpressing the α1β2γ2S(S443delC) subunits had two different peak currents. Three cells showed an average peak current of 466 ± 31.8 pA, n = 3, while no current was obtained from 5 cells.

Discussion

We identified a novel frame shift mutation, S443delC, in an Italian family with GEFS+. A cytosine nucleotide was deleted from the S443 residue in the last exon of the

GABRG2 gene. The resultant DNA sequence suggested that the mutant allele should be translated to a stable protein with the last 24 amino acids of the wildtype γ2 subunit that contains the 4th transmembrane domain replaced by a novel 50 amino acid C-terminus, which had decreased hydrophobicity. The frame shift mutation shifted the stop codon into the 3' UTR, thus shortening it, but it did not interfere with polyA site recognition. Western blot showed that mutant γ2S(S443delC) subunits were stable proteins, but that their total level was decreased compared to that of similarly expressed wildtype γ2S subunits. When coexpressed with α1 and β2 subunits, γ2S(S443delC) subunits were retained inside of the cell, and GABA-evoked currents from the cells were similar to obtained from α1β2 receptors. Thus, the γ2 subunit mutation, S443delC, might a γ2 subunit null allele and be associated with epilepsy, at least in part, through halplo-halplo-insufficiency.

There have been 16 epilepsy-associated mutations identified in *GABR* subunit genes, 7 of which were identified in the *GABRG2* gene, suggesting its strong association with epilepsy[44]. Although the γ2 subunit is not required for pentameric $GABA_A$ receptor assembly and surface trafficking[50, 56], mutations in the γ2 subunit affected the $GABA_A$ receptor expression, trafficking, and function[44]. The γ2 subunit is important for gephyrin-dependent $GABA_A$ receptor clustering at postsynaptic sites[97]. It also increases $GABA_A$ receptor channel conductance and opening duration[183]. The $\gamma 2^{-/-}$

knockout mice had normal brain structure and body weight, but they died within two of birth[56]. Decreasing γ2 subunit level in heterozygous $\gamma2^{+/-}$ mice[167] or γ2 subunit knockdown mice[168] resulted in decreased $GABA_A$ receptor clustering and abnormal mouse behaviors. However, no studies of possible seizure phenotypes in $\gamma2^{-/-}$ or $\gamma2^{+/-}$ have been reported.

We demonstrated that the γ2(S443delC) subunit is retained in the ER and not expressed on the cell surface membrane, suggesting a mechanism of haplo-insufficiency. However, it might also be associated with epilepsy by dominant negative effects on wildtype $GABA_A$ receptor subunits assembly and membrane trafficking. The epilepsy-associated γ2(R82Q) and γ2(Q390X) subunit mutations also were shown to generate mutant proteins that were retained in the ER[182, 191]. The mutant γ2(R82Q) subunit has decreased oligomerization with partnering subunits and is expressed on the cell membrane at low levels[59, 170]. It decreased surface α1 and β2 subunit levels when they were coexpressed in the HEK293T cells[180, 182], and decreased endogenous α5 subunit surface expression in cultured hippocampal neurons[176]. The γ2(Q390X) subunit was retained in the ER and not expressed on the cell membrane[191]. It bound to α1 and wildtype γ2 subunits when they were coexpressed in the HEK293T cells and decreased the membrane level of these wildtype subunits. However, both γ2(R82Q) and γ2(Q390X) subunits are stable proteins with similar total levels as the wildtype γ2 subunits[182, 193].

The α1(A322D) mutation is associated with juvenile myoclonic epilepsy[209]. The mutation impaired the membrane topology of α1(A322D) subunits so the α1(A322D) subunit was misfolded, retained in the ER, and degraded by proteasomal degradation[53, 210, 211]. The total level of α1(A322D) subunits was around 10% of wildtype α1 subunits with both Western blot and flow cytometry[210, 213]. The γ2(S443delC) subunit has a novel C-terminus that is less hydrophobic than the wildtype C-terminus that was predicted not to fold correctly. The γ2(S443delC) subunit might have decreased total level because of ER retention and increased proteasomal degradation, although that must be confirmed. However, the α1(A322D) subunit associated with wildtype subunits in the ER and reduced wildtype both α1β2γ2 and α3β2γ2 receptor surface expression[213]. It is possible that although the γ2(S443delC) subunit has reduced total levels, it could oligomerize with α and β subunits and decrease wildtype $GABA_A$ receptors surface expression, but must also to explored. The functional consequences of the S443delC mutation could be a combination of haplo-insufficiency and dominant negative effects. The γ2(R82Q) mutation also decreased γ2 subunit-containing $GABA_A$ receptor surface expression, and partnering subunit membrane expression. The mutant $\gamma2^{R82Q/+}$ knock in mice had the same type of seizures as humans bearing the mutation[185]. The effect of γ2(S443delC) subunit could be sufficient to induce epilepsy.

This report confirms that most *GABRG2* receptor truncation mutations result in a combination of generalized and febrile seizures, also recognized as GEFS+ spectrum[1]. However, it is becoming increasingly obvious that the spectrum of phenotypic severity is inexplicably wide, ranging from asymptomatic individuals (see individual I:1 in this report and the Q40X mutation carrier7) to patients with the Dravet syndrome[1,7]. Mechanisms influencing the severity of the phenotype associated with a given mutation are therefore complex and difficult to correlate with its demonstrable functional effects. Phenotypic severity is likely modulated by the individual genetic background through different and possibly multiple mechanisms, including the response to ER stress[7].

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Chapter 4

The Dravet Syndrome-Associated *GABRG2* Nonsense Mutation, Q40X, Activated NMD and Generated a Truncated Subunit That was Partially Rescued by aminoglycoside-Induced Stop Codon Read-through

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Abstract

The *GABRG2* nonsense mutation, Q40X, is associated with the severe epilepsy syndrome, Dravet syndrome, and is predicted to generate a premature translation-translation-termination codon (PTC) in the *GABRG2* transcription product, $GABA_A$ receptor γ2 subunit mRNA. We determined the effects of the mutation on γ2 subunit mRNA and protein synthesis and degradation, as well as on α1β2γ2 $GABA_A$ receptor assembly, trafficking and surface expression in HEK cells. Using bacterial artificial chromosome (BAC) constructs, we found that γ2(Q40X) subunit mRNA was degraded nonsense mediated mRNA decay (NMD). The undegraded mutant mRNA was translated to a truncated peptide, likely the signal peptide, which was further cleaved. We also that mutant γ2(Q40X) subunits did not assemble into functional receptors, thus GABA-evoked current amplitudes. The *GABRG2(Q40X)* mutation is one of several epilepsy-associated nonsense mutations that have the potential to be rescued by reading through the PTC, thus restoring full-length protein translation. We investigated use of aminoglycoside, gentamicin, to rescue translation of intact mutant subunits by inducing mRNA read-through. In the presence of gentamicin, synthesis of full length γ2 subunits was partially restored, and surface biotinylation and whole cell recording experiments

suggested that rescued γ2 subunits could corporate into functional, surface $GABA_A$ receptors, indicating a possible direction for future therapy.

Key words: $GABA_A$ receptors, epilepsy, γ2 subunit, *GABRG2(Q40X)* mutation, loss of function, gentamicin

Highlights

- Dravet syndrome-associated mutation, *GABRG2(Q40X),* decreased γ2 subunit mRNA levels.
- Undegraded γ2(Q40X) subunit mRNA was expressed as a truncated γ2 subunit and a novel proteolytic band.
- Mutant γ2(Q40X) subunits were not assembled to functional $GABA_A$ receptors.
- Aminoglycosides partially rescued wildtype γ2 subunit expression from mutant mRNA.
- Rescued γ2 subunits had the same expression and function as wildtype γ2 subunits.

Introduction

Epilepsy is a common neurological disorder that affects about 1% of the world's population [271]. Epilepsy syndromes are usually either symptomatic and due to a known brain injury or idiopathic and not due to brain injury. Idiopathic genetic epilepsy syndromes (IGES) comprise ~30% of all cases and can vary in severity from the mild juvenile absence epilepsy syndrome to the severe Dravet syndrome [272, 273]. While many IGES are benign, Dravet syndrome is not. It is associated with myoclonic and generalized tonic-clonic seizures that begin at an early age, frequent episodes of status epilepticus and progressive intellectual decline, and it is resistant to a wide range of antiepileptic drugs. Many epilepsy-associated mutations are in ion channel genes, and about one half of Dravet syndrome patients have nonsense mutations in ion channel genes that create premature translation-termination codon (PTCs), and thus, truncated subunit proteins [274]. *GABRG2(Q40X)* is a nonsense mutation located in $GABA_A$ receptor $\gamma 2$ subunits that has been associated with Dravet syndrome [275].

$GABA_A$ receptors are heteropentameric chloride ion channels that mediate the majority of inhibitory neurotransmission in the CNS. The receptor complex is composed of five subunits from nineteen different genes, and the main synaptic receptors are

composed of two α subunits, two β subunits and one γ2 subunit. Out of the fifteen *GABR* epilepsy-associated mutations or variants, seven are in *GABRG2*, and these mutations been shown to decrease channel function by altering receptor biogenesis or channel function [276]. The *GABRG2(Q40X)* mutation was shown to impair $GABA_A$ receptor channel function and to form granules in neurons [275]. However, the effects of this mutation on $GABA_A$ receptor function are unknown.

Current therapies for the devastating epilepsies produced by truncation mutations are symptomatic and relatively ineffective. One potential treatment would be to rescue the nonsense mutation by drug-induced read-through. Aminoglycosides such as G-418 and gentamicin partially restore the expression and function of full-length proteins by inducing PTC read-through [200, 277]. A drug designed to specifically induce ribosomes to read through stop codons generated by PTCs (Ataluren®) is currently under Phase 3 clinical trial to treat cystic fibrosis patients carrying PTCs in the *CFTR* gene, further confirming the clinical feasibility of this strategy [278, 279]. Because the dramatic loss of function produced by subunit truncation mutations likely contributes to the pathogenesis of Dravet syndrome, the read-through strategy presents a potential approach to treat epilepsies associated with PTCs.

To explore the effects of the *GABRG2(Q40X)* mutation, we studied the transcription of wildtype and mutant *GABRG2* mRNA, the translation of γ2 and

subunit protein and the properties of $GABA_A$ receptors that were assembled with coexpression of α1, β2 and γ2 or γ2(Q40X) subunits in HEK 293T cells. We found that the Q40X mutation engaged the cellular quality control machinery to activate nonsense mediated mRNA decay (NMD) to decrease mutant mRNA levels and produced a signal peptide that was not incorporated into functional receptors. Restoring expression of the full-length wildtype γ2 subunit by read-through should be able to rescue the truncation caused by the Q40X mutation. To evaluate the plausibility of aminoglycoside-aminoglycoside-induced read-through of an epilepsy-associated PTC, we determined whether gentamicin could rescue mutant γ2(Q40X) subunits. We demonstrated that gentamicin partially restored the expression full-length γ2 subunits, and that the rescued subunits assembled with α1β2 subunits to form functional α1β2γ2 $GABA_A$ receptors.

Materials and Methods

Expression vectors

The coding sequences of human α1, β2 and γ2S $GABA_A$ receptor subunits were cloned into pcDNA3.1 expression vectors (Invitrogen) as previously described [211]. All subunit residues were numbered based on the immature peptide. The γ2S(Q40X) and

γ2S(Q40X,TGA) subunit constructs were generated using the QuikChange site-directed mutagenesis kit (Stratagene). An HA epitope was inserted at a functionally silent site (between the 4th and 5th residue of the mature peptide of both wildtype and mutant γ2S subunit) to facilitate our experiments [50]. To detect the truncated protein generated by the mutation, we also inserted an HA epitope at the N terminus of the unprocessed while an FLAG epitope was inserted between the 4th and 5th residue of the mature using overlapping PCR.

The *GABRG2* BAC construct containing the Q40X mutation was generated using the BAC clone number RP11-1035I20 (BACPAC Resources; http://bacpac.chori.org), which contains the wildtype human *GABRG2* gene genomic sequence. The human chromosome sequence upstream of GABRG2 translation start site was replaced with a CMV promoter, and the mutation was introduced by *galK* facilitated BAC recombineering [239]. A reporter gene containing an SV40 early promoter-driven eGFP was integrated to BACs using Cre (NEB) recombination [238]. Thus, both wildtype and mutant *GABRG2* BACs contained the CMV promoter-driven *GABRG2* gene and an eGFP reporter gene driven by the SV40 early promoter.

Cell culture and transfection

Human embryonic kidney cells (HEK 293T) (ATCC, CRL-11268) were incubated at 37°C in humidified 5% CO_2, 95% air and grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were transfected using the FuGENE 6 transfection reagent (Roche Applied Science) at a DNA:Transfection Reagent ratio of 1:3 according to the manufacturer's instructions. Eighteen to 20 hours after transfection, gentamicin (50 mg/ml, GIBCO) was added to the culture dish.

The NMD essential factor UPF1 or SMG6 was knocked down using siRNAs to block the NMD machinery. SilencerSelect® pre-designed and validated siRNA (Ambion, siRNA ID s11926) was transfected to cells using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's manual. Twenty-four hours later cells were transfected again with the wildtype or mutant BAC constructs and harvested two days later for RT-PCR.

RNA extraction, RT-PCR and Taqman real-time qPCR

Total RNAs from transfected HEK 293T cells were extracted by using the PerfectPure RNA Cultured Cell kit (5Prime) following the manufacturer's protocol and

then reverse transcribed to cDNA using the Taqman MicroRNA Reverse Transcription (Applied Biosystems). The transcribed cDNA was used then as the PCR template to identify γ2 subunit transcripts using a forward primer located in exon 6 and a reverse primer located in exon 7. Taqman® probes detecting human *GABRG2* gene mRNA, human *GAPDH* gene, 18S rRNA, or eGFP (part number 4331348 [Custom Taqman Gene Expression Assay Service]) were used to quantify the amount of transcribed cDNA. sample was triplicated in each individual experiment, and the average threshold cycle value for each sample was calculated by the Sequence Detection System v2.3 Standard Edition (Applied Biosystems). The average Ct values of *GABRG2* gene mRNA were normalized to the endogenous human *GAPDH*, 18S rDNA or eGFP amount, to compare the relative RNA abundance.

Western Blot, PNGase F digestion and surface biotinylation

After sonication, the whole cell lysates of transfected HEK cells were collected in modified RIPA buffer (Pierce) and 1% protease inhibitor mixture (Sigma). Collected samples were subjected to gel electrophoresis using NuPAGE® (Invitrogen) or TGX (BioRad) precast gel and then transferred to PVDF-FL membranes (Millipore).

Monoclonal anti-HA antibody (Covance or Cell signaling) and monoclonal anti-FLAG antibody (Sigma) were used to detect the epitope tag in γ2S subunits. 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 at 1:10,000 dilution, and the signals were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences). The integrated intensity value (IDV) of each specific band was calculated using the Odyssey 3.0 software (LI-COR Biosciences).

To remove all N-linked glycan, cell lysates were incubated with the PNGase F enzyme (NEBiolab) at 37°C for 3 hours following manufacturer's manual. Treated samples were then subjected to SDS-PAGE and Western blot.

Surface proteins were collected using surface biotinylation as described before [61]. Transfected cells were biotinylated using the membrane-impermeable reagent sulf-HNS-SS-biotin (1 mg/ml, Thermo Scientific) at 4°C for 1 h. Cells were lysed after being quenched with 0.1 M glycine. The biotin-labeled plasma membrane proteins were pulled down by High Binding Capacity NeutrAvidin beads (Thermo Scientific Pierce) after centrifugation.

Flow cytometry

High throughput flow cytometry was performed to investigate the surface expression of $GABA_A$ receptor subunits. Transfected cells were collected in phosphate-buffered saline containing 2% fetal bovine serum and 0.05% sodium azide as described before [269]. Cell samples were incubated with an Alexa fluorophore (Invitrogen)-conjugated monoclonal anti-α1 antibody (Millipore), monoclonal anti-β2/β3 antibody (Millipore) or monoclonal anti-HA antibody (Covance), then fixed by 2% paraformaldehyde. The fluorescence signals were read on a BD Biosciences FACSCalibur system. Nonviable cells were excluded from study based on the previously determined forward and side scatter profiles. The fluorescence index of each experimental condition was subtracted by the fluorescence index of mock-transfect condition and then normalized to that of the control condition. Flow Cytometry experiments were performed in the VMC Flow Cytometry Shared Resource, which is supported by the Vanderbilt Ingram Cancer Center (P30 CA68485) and the Vanderbilt Digestive Disease Research Center (DK058404).

Whole cell electrophysiology

Whole cell voltage-clamp recordings were performed at room temperature on HEK293T cells 24-72 hrs after transfection with $GABA_A$ receptor subunits as described previously [280]. Successfully transfected cells were identified by the presence of GFP fluorescence (see Cell culture and transfection, above). Cells were bathed in an external solution containing 142 mM NaCl, 1 mM $CaCl_2$, 8 mM KCl, 6 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4, ~325 mOsM). Recording electrodes were pulled from thin-walled borosilicate capillary glass (World Precision Instruments, Sarasota, FL) using a P2000 laser electrode puller (Sutter Instruments, San Rafael, CA), fire-polished with a microforge (Narishige, East Meadow, NY), and filled with an internal solution containing 153 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM EGTA, 2 mM Mg^{2+}-ATP (pH 7.3, ~300 mOsm). All patch electrodes had a resistance of 1–2 MΩ. The combination of internal and external solutions yielded a chloride reversal potential of ~ 0 mV, and cells were voltage-clamped at -20 mV using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). A rapid exchange system (open tip exchange times ~ 400 μs), composed of a four-barrel square pipette attached to a Perfusion Fast-Step (Warner Instruments Corporation, Hamden, CT) and controlled by Clampex 9.0 (Axon Instruments), was used to apply GABA to lifted whole cells. The channels were by 1 mM GABA for 4 s, followed by an extensive wash for 40 s, then blocked by 10 mM

Zn^{2+} for 10 s. GABA (1 mM) was then applied for 4 s in the presence of 10 μM Zn^{2+}. Peak current amplitudes after the Zn^{2+} application were normalized to those before the Zn^{2+} application to calculate the sensitivity to Zn^{2+} blockade. All currents were low-pass filtered at 2 kHz, digitized at 5-10 kHz, and analyzed using the pCLAMP 9 software

Data analysis

Numerical data were reported as mean ± S.E. Statistical differences were determined by one way analysis of variance or by pair wise *Student's* t-test.

Results

The γ2S subunit mutation, Q40X, decreased γ2S subunit transcripts

The nonsense mutation Q40X generated a PTC in the second exon of *GABRG2* (Figure 1A). Since nonsense mutations located at least 50-55 nt upstream of an exon-exon-exon junction activate NMD to degrade susceptible transcripts [281], the mutant γ2S(Q40X) subunit mRNA level should be decreased. NMD efficiency is an inherent property of cells and varies among cell types [282]. In HEK 293T cells, the mRNA level of an NMD-competent construct was degraded by about 60% [201]. To determine whether mutant *GABRG2(Q40X)* mRNA was degraded by the NMD machinery, we expressed mutant or wildtype CMV promoter-driven *GABRG2* BACs in HEK293T cells with siRNAs against the NMD essential factor UPF1 or negative control siRNAs. Total RNA was extracted from transfected cells 36 hours after transfection, and mRNAs were reverse transcribed to cDNA. RT-PCR using primers flanking *GABRG2* 5' exon 6 and 3' exon 7 amplified a fragment from both wildtype and mutant BAC transfected cells 1B). Sequencing showed that the mutant BAC transcript contained the γ2S subunit containing a PTC at codon 40. The γ2S subunit mRNA levels were then quantified using real-time PCR with a probe targeting GABRG2 5' exon 4 and 3' exon 5 border and normalized to GFP mRNA levels for each condition. The transcript levels from cells

treated with siRNA against UPF1 were compared to those from cells treated with control siRNA. The γ2S subunit mRNA level in cells transfected with wildtype *GABRG2* BACs was not changed by UPF1 siRNA (1.04 ± 0.07 fold, n = 4 after UPF1 knock down) 1C). The mutant γ2S(Q40X) subunit mRNA level in cells transfected with mutant *GABRG2(Q40X)* BACs, however, was increased by UPF1 siRNA (1.99 ± 0.35 fold, n = $p < 0.05$) (Figure 1C). Thus, blocking NMD rescued the mutant γ2S(Q40X) subunit, but did not alter wildtype γ2S subunit mRNA levels. A similar trend was observed in cells transfected with siRNAs against the NMD essential factor SMG6 (data not shown).

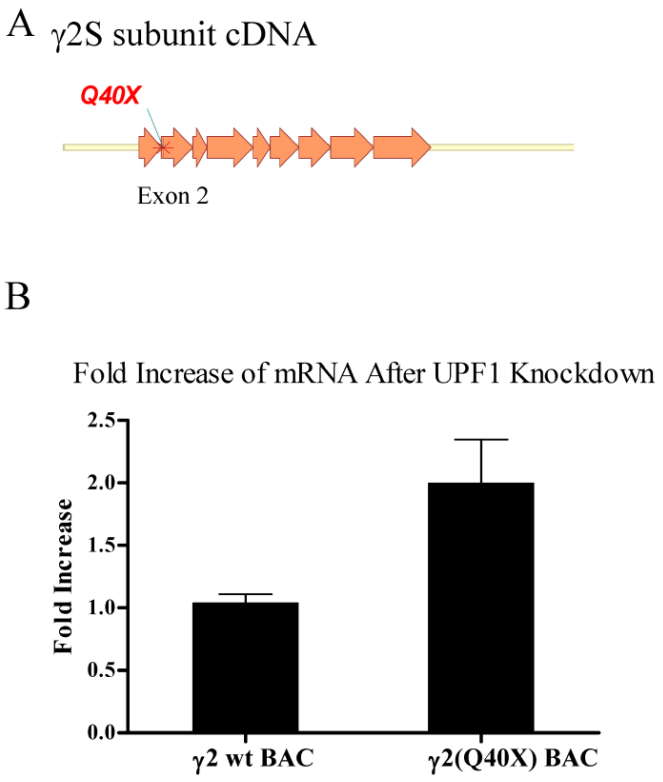


Figure 4.1 Mutant mRNA was degraded by NMD.

A. A schematic representation of the genomic structure of *GABRG2*. Vertical brown arrows represent exons composing γ2S subunit cDNA. The Q40X mutation is located in exon 2. **B.** The γ2S transcript was identified in mutant *GABRG2(Q40X)* BAC transfected cells using RT-PCR. HEK 293T cells were treated with siRNA against the NMD factor UPF1 or with nonspecific siRNA and were then transfected with wildtype or mutant *GABRG2* BAC. A forward primer located in exon 6 and a reverse primer located in exon 7 of the γ2S subunit cDNA were used to amplify reverse transcribed cDNA from transfected cells. **C.** The transcript level of the mutant *GABRG2(Q40X)* BAC was increased by NMD knock down.

The γ2S subunit mutation, Q40X, generated a truncated peptide

We next studied the protein generated by the mutant γ2S(Q40X) cDNA, since not all mutant mRNA was degraded by NMD. The Q40X nonsense mutation is located in the 40^{th} residue of the immature γ2S subunit, which is the first residue of the predicted subunit [283]. Thus, this mutation is predicted to generate a truncated protein encoding the 39 aa γ2 subunit signal peptide. To explore this prediction, we inserted an HA-tag at the N terminus of the immature γ2S subunit cDNA and a FLAG-tag between the 4^{th} and residue of the mature γ2S subunit cDNA, generating a double tagged SP^{HA}-$\gamma2S^{FLAG}$ subunit (Figure 2A). Signal peptides are composed typically of a positively charged 'N domain', a hydrophobic 'H domain' and a slightly polar 'C domain' [284, 285]. The additional HA tag at the N terminus of the immature γ2S subunit did not significantly the hydrophobicity pattern of the signal peptide calculated *in silico* using the ProtScale

sofware [286] (Figure 2B). Insertion of an epitope in the N domain should not change signal peptide topology or function [287, 288].

We expressed wildtype γ2S^{HA}, mutant SP^{HA}-γ2S(Q40X)^{FLAG} or wildtype SP^{HA}-γ2S^{FLAG} subunits in HEK293T cells and ran Western blots for HA- or FLAG-tagged proteins (Figure 2C). In cells transfected with γ2S^{HA} subunits, a large band was detected by anti-HA antibody at about 45 kDa, and as expected, no signal was detected by anti-FLAG antibody (Figure 2C, lane 2). In cells transfected with SP^{HA}-γ2S^{FLAG} subunits, a large band just below 50 kDa was detected by anti-FLAG antibody and a small band below 10 kDa was detected by anti-HA antibody (Figure 2C, lane 4). The size of the higher molecular mass FLAG-band was consistent with mature, glycosylated γ2S subunits [191], and the size of the lower molecular mass HA-band was consistent with the predicted signal peptide. In contrast, in cells transfected with mutant SP^{HA}-γ2S(Q40X)^{FLAG} subunits, no FLAG-specific signal was detected (Figure 2C, lane 3), indicating that synthesis of full length γ2S subunits was abolished by the *GABRG2(Q40X)* mutation. Interestingly, two different small peptides below 10 kDa were detected by anti-HA antibody in the mutant SP^{HA}-γ2S(Q40X)^{FLAG} subunit transfected cells (Figure 2C, lane 3), which may have been caused by a further cleavage of the signal peptide by signal peptide peptidase [289].

In addition to the small signal peptide, a clear, but faint, band with a higher molecular mass was also detected from SP^{HA}-γ2S^{FLAG} transfected cells using an anti-HA antibody. Its molecular mass was similar to that of immature γ2S subunits containing signal peptides. To determine molecular masses of γ2S and SP^{HA}-γ2S^{FLAG} subunits more accurately, we removed all of their glycans by PNGase F digestion (Figure 2D). After glycan removal, the size of HA-tagged γ2S^{HA} subunits was shifted from about 45 KDa to about 37 kDa, consistent with a mature, glycosylated subunit. In contrast, the size of HA-tagged SP^{HA}-γ2^{FLAG} subunits was unchanged by glycan removal and remained at about 42 kDa, consistent with an immature, unglycosylated subunit. The 5 kDa difference in molecular mass of the two subunits after PNGase F treatment was consistent with the molecular mass of the signal peptide. Thus, SP^{HA}-γ2S^{FLAG} subunits produced an HA-tagged immature subunit in addition to the HA-tagged signal peptide and FLAG-tagged mature subunit. Mutant SP^{HA}-γ2S(Q40X)^{FLAG} subunits, however, only produced an HA-tagged signal peptide that was subjected to further cleavage. These results demonstrated that the γ2S subunit mutation, Q40X, disrupted translation of mature γ2S subunits and generated a truncated protein composed of the signal peptide.

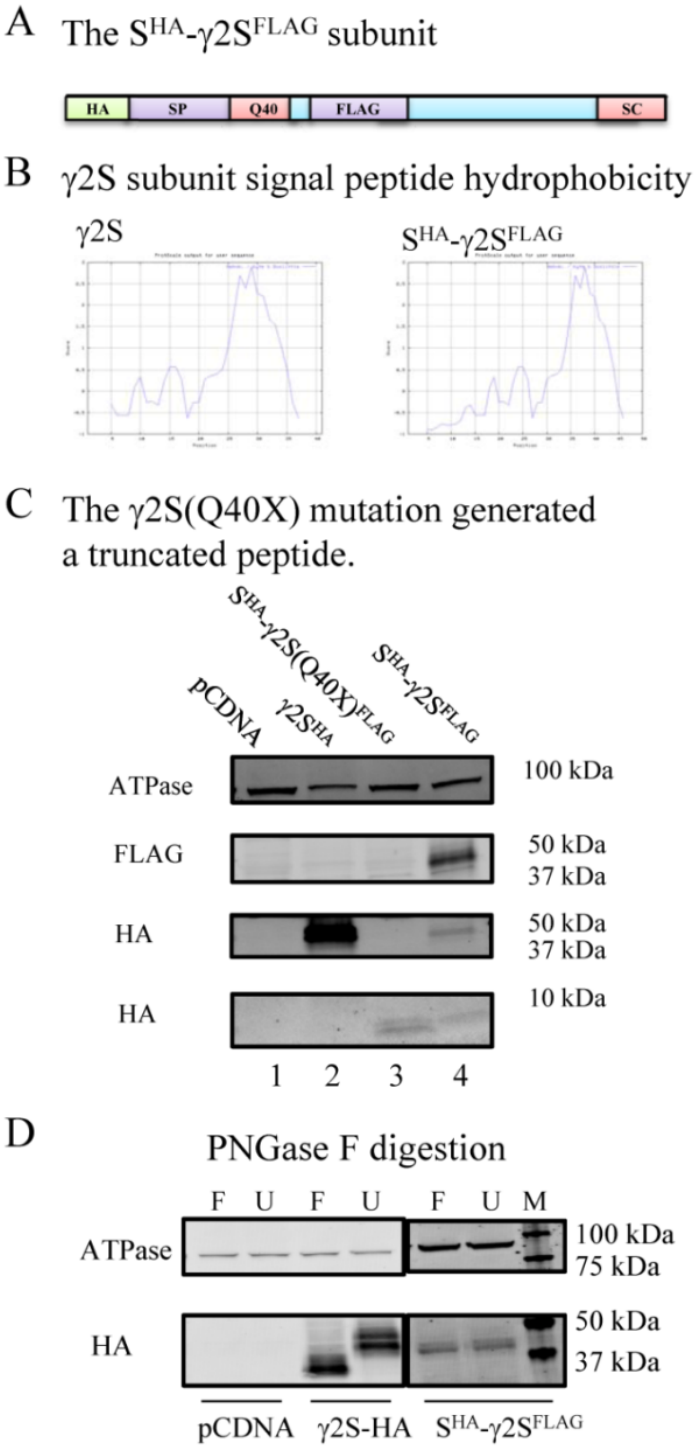


Figure 4.2 The *GABRG2(Q40X)* mutation generated a truncated peptide.

A. To identify the protein generated by the *GABRG2(Q40X)* mutation, an HA tag was inserted into the N terminus and a FLAG tag was inserted between the 4th and 5th residue of the mature γ2S subunit protein to produce the wildtype SP^{HA}-$γ2S^{FLAG}$ or mutant SP^{HA}-γ2S$(Q40X)^{FLAG}$ subunit. SP: signal peptide. SC: stop codon. **B.** The hydrophobicity patterns of γ2S and SP^{HA}-$γ2S^{FLAG}$ subunit signal peptides were calculated using the online PScale program. The Y-axis represents scores calculated based on the hydrophobicity scale of different amino acids; the X-axis represents the numbering of each residue in the signal peptide sequence. **C.** The γ2(Q40X) subunit mutation generated a truncated peptide. HEK 293T cells were transfected with wildtype $γ2S^{HA}$, wildtype SP^{HA}-$γ2S^{FLAG}$ or mutant SP^{HA}-γ2S$(Q40X)^{FLAG}$ subunits. Cell lysates (10 μg) from wildtype $γ2S^{HA}$ subunit transfected cells and cell lysates (50 μg) from wildtype SP^{HA}-$γ2S^{FLAG}$ or mutant SP^{HA}-γ2S$(Q40X)^{FLAG}$ subunit transfected cells were subjected to Western blot by anti-FLAG and anti-HA antibodies. ATPase level was used as a loading control. **D.** Samples from cells transfected with $γ2S^{HA}$ and SP^{HA}-$γ2S^{FLAG}$ subunits were collected and treated with PNGase F to remove all glycans. F: PNGase F digestion; U: undigested control; M: protein loading marker. Figures are representative of 3 different experiments.

The γ2S subunit mutation, Q40X, disrupted the membrane insertion of γ2S subunits and changed the composition of $GABA_A$ receptors

To explore the effects of the *GABRG2(Q40X)* mutation on receptor assembly and channel function, we created HA-tagged γ2S$(Q40X)^{HA}$ subunits with the HA-tag inserted between the 4^{th} and 5^{th} residue of the mature γ2S(Q40X) subunits. We then cotransfected HEK 293T cells with α1, β2 and $γ2S^{HA}$ or γ2S$(Q40X)^{HA}$ subunits. Surface levels of different $GABA_A$ receptor subunits were detected by flow cytometry (Figure 3A). The fluorescence indices of each subunit under different experimental conditions were normalized to those obtained with cotransfection of α1β2$γ2S^{HA}$ subunits. Cotransfection

of either α1β2 or α1β2γ2 subunits can produce functional $GABA_A$ receptors on the cell surface [50, 290]. Binary αβ receptors are likely composed of two α and three β subunits while ternary αβγ receptors are likely composed of two α, two β and one γ subunits [291, 292]. Our flow cytometry analysis revealed a significant relative increase of surface β2 subunit levels with cotransfection of α1β2 subunits compared to cotransfection of $\alpha1\beta2\gamma2S^{HA}$ subunits (α1β2: 2.14 ± 0.23; $\alpha1\beta2\gamma2S^{HA}$: 1.00; n = 7) with no change in the relative amount of surface α1 subunits (α1β2: 0.92 ± 0.05; $\alpha1\beta2\gamma2S^{HA}$: 1.00; n = 7) 3A). In the presence of the Q40X mutation, no surface γ2S(Q40X) ^{HA} signal was by anti-HA antibody (Figure 3A), consistent with finding that synthesis of the full-length γ2S(Q40X) subunits was disrupted by the mutation (Figure 2C). With cotransfection of $\alpha1\beta2\gamma2S(Q40X)^{HA}$ subunits, surface α1 subunit levels were similar to those obtained with cotransfection of α1β2 and $\alpha1\beta2\gamma2S^{HA}$ subunits (α1β2: 0.92 ± 0.05; $\alpha1\beta2\gamma2S^{HA}$: 1.00; $\alpha1\beta2\gamma2S(Q40X)^{HA}$: 0.91 ± 0.03; n = 7). However, with cotransfection of $\alpha1\beta2\gamma2S(Q40X)^{HA}$ subunits, surface β2 levels were increased significantly compared to those obtained with cotransfection of $\alpha1\beta2\gamma2S^{HA}$ subunits, reaching the levels of α1β2 receptors ($\alpha1\beta2\gamma2S(Q40X)^{HA}$: 1.99 ± 0.20; n = 7; $p < 0.05$) (Figure 3A). We also evaluated the total cell expression of the receptor subunits (Figure 3B). The total levels α1 and β2 subunits with cotransfection of $\alpha1\beta2\gamma2S(Q40X)^{HA}$ subunits were also similar those obtained with cotransfection of α1β2 subunits. These data indicated that mutant

γ2S(Q40X) subunits did not incorporate into surface receptors, and thus $GABA_A$ assembled in the presence of mutant γ2S(Q40X) subunits were binary αβ receptors.

To determine how mutant γ2S(Q40X) subunits affected $GABA_A$ receptor we used a rapid exchange system to apply 1 mM GABA for 4s to lifted HEK293T cells coexpressing α1β2, α1β2γ2S^{HA}, or α1β2γ2S(Q40X)^{HA} subunits (Figure 3C). Peak amplitude recorded from cells coexpressing α1β2 subunits was 1351 ± 158 pA (n = 9), approximately 67% smaller than that recorded from cells coexpressing α1β2γ2S^{HA} subunits (4106 ± 156 pA, n = 15, $p < 0.001$) (Figure 3C, left traces), a difference with previously reported data [290, 293, 294]. Peak current amplitude from cells coexpressing α1β2γ2S(Q40X)^{HA} subunits was also decreased (1778 ± 232 pA, n = 18) to about 57%, significantly smaller than that recorded from cells coexpressing α1β2γ2S^{HA} subunits ($p < 0.001$), but not different from that obtained from cells coexpressing only α1β2 subunits ($p > 0.05$). Furthermore, currents recorded from cells containing α1β2γ2S(Q40X)^{HA} subunits were substantially more sensitive to Zn^{2+} inhibition than currents recorded from cells containing α1β2γ2S^{HA} subunits. Currents evoked by 1 mM GABA from cells coexpressing α1β2, α1β2γ2S^{HA} or α1β2γ2S(Q40X)^{HA} subunits were inhibited to different extents by coapplication of 10 μM Zn^{2+} chloride (Figure 3C, right traces). The fractional Zn^{2+} inhibition of currents evoked from cells coexpressing α1β2γ2S(Q40X)^{HA} subunits was significantly higher than those evoked from cells

coexpressing α1β2γ2S^{HA} subunits (93 ± 1%, n = 18; 9 ± 2%, n = 15, respectively, p < 0.001) but similar to those evoked from cells containing α1β2 subunits (94 ± 1%, n = 17, > 0.05). Because the sensitivity of $GABA_A$ receptors to Zn^{2+} inhibition depends on subunit composition, these results also suggested that mutant γ2S(Q40X) subunits were incorporated into ternary α1β2γ2S(Q40X) receptors, thus leading to expression primarily of binary α1β2 receptors on the cell surface.

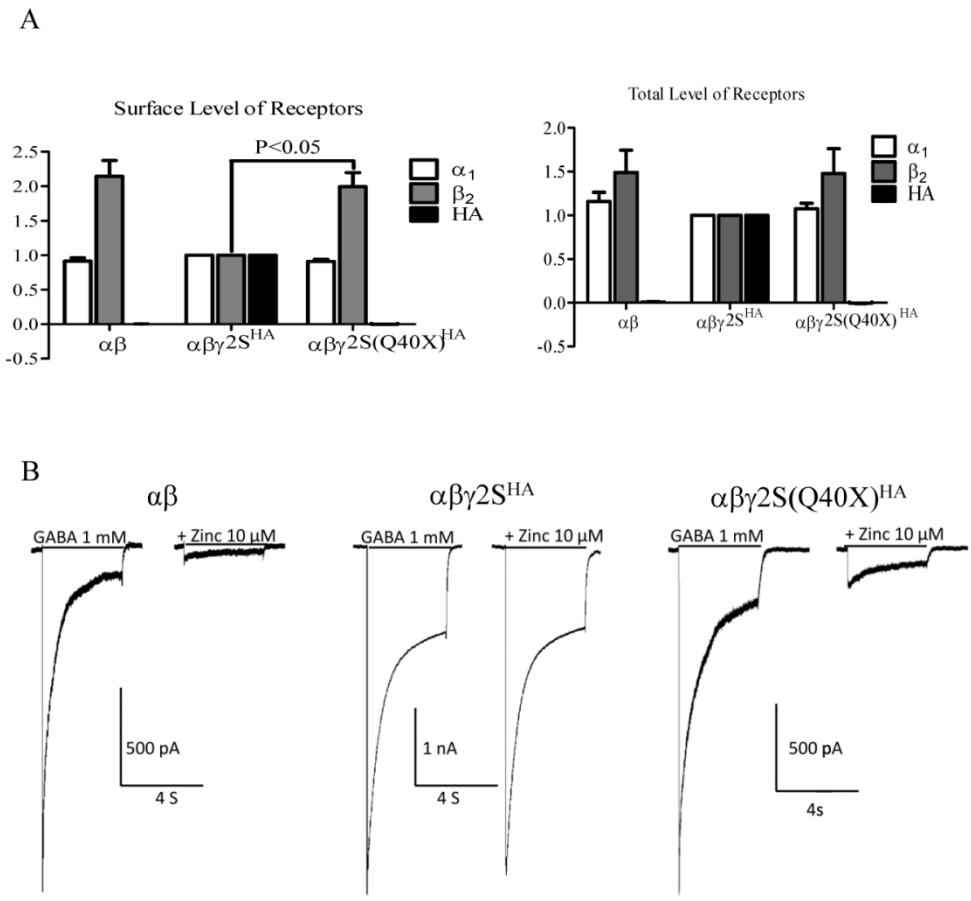


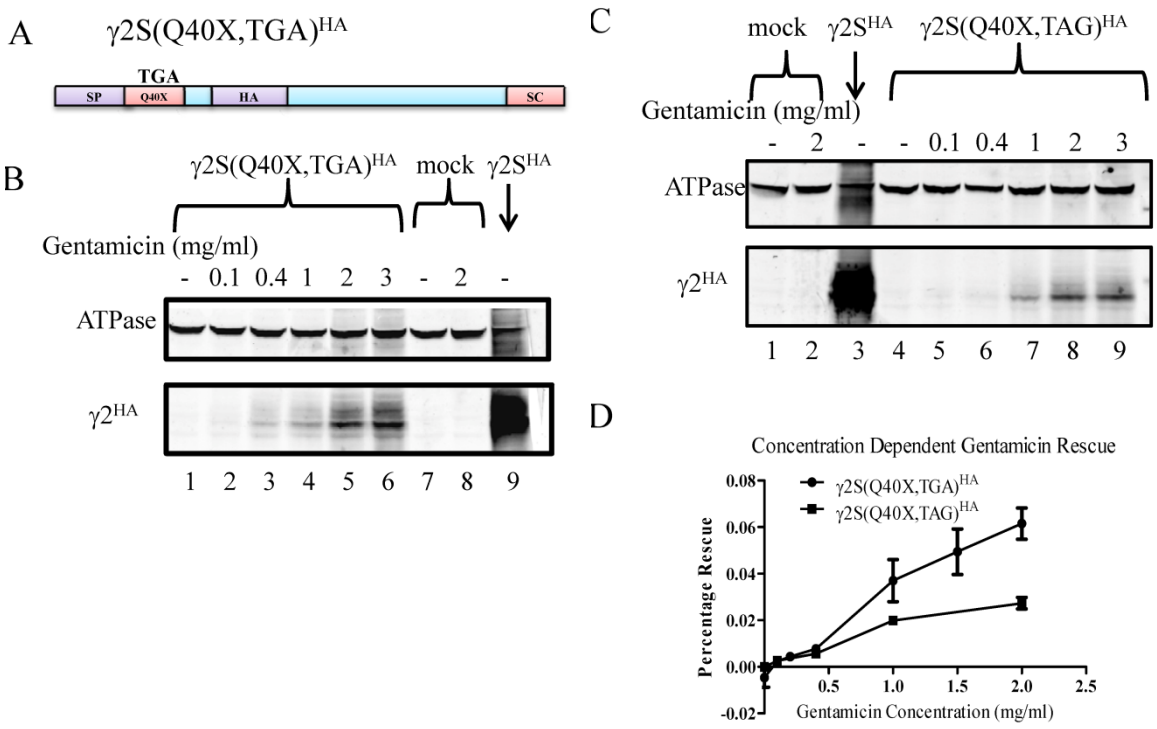
Figure 4.3 The mutant γ2S(Q40X) subunit was not expressed on the cell surface.

A. Mutant α1β2γ2S(Q40X)^{HA} subunits were coexpressed in HEK293T cells. Surface and total level of each subunit were evaluated through flow cytometry. The fluorescence indices of each subunit under different experimental conditions were normalized to those obtained with cotransfection of α1β2γ2S^{HA} subunits (n = 7, mean ± SEM). Group differences were analyzed by the one way ANOVA test. **B.** Sample traces of whole cell recordings from cells expressing α1β2γ2S^{HA} or α1β2γ2S(Q40X)^{HA} subunits. $GABA_A$ receptor-mediated currents evoked by 1 mM GABA were recorded. After a 4.0 sec wash, the currents were recorded again with coapplication of 10 μM Zn^{2+}

Full-length γ2S(Q40X) subunits were partially rescued by gentamicin-induced stop codon read-through.

The Q40X mutation generated a PTC in *GABRG2* and failure to produce full-length γ2S subunits likely contributes to its epilepsy pathogenesis. Aminoglycosides, such as G-418 and gentamicin, can promote partial read-through of PTCs, thus partially rescuing the synthesis of functional, full-length subunits [295, 296]. Therefore, we determined to what extent gentamicin could rescue the *GABRG2(Q40X)* mutation. The read-through efficiency of gentamicin depends on the nature of the stop codon as well as the surrounding nucleotides, with the TGA stop codon being most efficiently bypassed [297]. To maximize read-through efficiency, we replaced the original TAG stop codon with the TGA stop codon (Figure 4A) and then transfected γ2S(Q40X,TGA)^{HA} subunit cDNA into HEK cells. Eighteen hours after transfection, varying concentrations of gentamicin were added to culture media. Forty-eight hours later, the transfected cell

samples were collected and amounts of full length γ2S^{HA} subunit translated from the mutant γ2S(Q40X,TGA)^{HA} subunit mRNA was evaluated by Western blot with anti-HA antibody (Figure 4B) .



Fgure 4.4 Gentamicin partially restored expression of full length γ2S subunits by read-through of γ2S(Q40X) subunit mRNA.

A. The original TAG stop codon was replaced by the TGA stop codon to maximize read-through efficiency. **B. and C.** Cells were transfected with γ2S(Q40X,TGA)^{HA} (B) or γ2S(Q40X)^{HA} (C) subunits and treated with different concentrations of gentamicin for 48 hours. Cell lysates (10 µg) from wildtype γ2S^{HA}

subunit transfected cells were loaded, while cell lysates (50 μg) from mutant γ2S(Q40X,TGA)^{HA} or γ2S(Q40X)^{HA} subunits transfected cells were loaded. **D.** Band intensity of the γ2S^{HA} subunit was normalized to the ATPase signal and plotted against gentamicin concentration (n = 7 and 5 respectively, mean ± SEM).

In the absence of gentamicin treatment, mature, full-length, HA-tagged γ2S subunits were detected from wildtype transfected cells (Figure 4B, lane 9), but mature, full-length, HA-tagged γ2S(Q40X,TGA)^{HA} subunits were not detected from mutant transfected cells (Figure 4B, lane 1). After addition of gentamicin, we were able to detect an HA-tagged protein band of the same size as the wildtype γ2S^{HA} subunit in cells transfected with γ2S(Q40X,TGA)^{HA} subunits (Figure 4B, lanes 2-6). No HA signal was detected from mock transfected cells in the presence or absence of gentamicin (Figure lanes 7-8), indicating that the rescue was specific and that expression of full length γ2S subunits was partially restored from γ2S(Q40X,TGA)^{HA} transfected cells. Compared to non-treated wildtype γ2S^{HA} subunit transfected cells, the rescue efficiency of γ2S(Q40X,TGA)^{HA} subunits was gentamicin concentration-dependent (Figure 4D, filled circles), reaching as high as 6.2 ± 0.7% at a concentration of 2 mg/ml gentamicin (n = 7), which is comparable to previous reports [297, 298]. We also evaluated the read-through efficiency of γ2S(Q40X)^{HA} subunits whose mRNA contained the native TAG stop codon. We found that a smaller, but still substantial, amount of full-length γ2S^{HA} subunit (2.5 ±

0.2%, n = 5) was rescued (Figure 4C) in a gentamicin concentration-dependent fashion (Figure 4D, filled squares).

Gentamicin-rescued γ2S subunits were trafficked to the cell surface.

A functional γ2S subunit will oligomerize with partnering α and β subunits to form pentameric αβγ2S receptors that are trafficked to the cell surface. To determine whether the γ2S subunits rescued by gentamicin were functional, we evaluated their surface expression. We cotransfected HEK 293T cells with $\alpha1\beta2\gamma2S(Q40X,TGA)^{HA}$ subunits, and after forty-eight hours of gentamicin treatment (1 mg/ml), surface protein was collected through surface biotinylation and blotted by anti-HA antibody. We found that after gentamicin treatment a small, but significant, amount of HA-signal was detected on the cell surface with a molecular mass similar to that of wildtype $\gamma2S^{HA}$ subunits (Figure 5A, lane 2 versus 4). HA-signal was not found in non-biotinylated samples, indicating that the detected HA-signal was not caused by artifact introduced during experiments (Figure 5A, lane 3).

To exclude the possibility that the HA-signal we detected through surface biotinylation was due to membrane destruction after gentamicin treatment, we also for the cytoplasmic marker GAPDH. Then we compared the HA/GAPDH ratio between

total samples and surface samples. Although a little GAPDH signal was found in surface samples, it was much lower than that obtained from total samples. After gentamicin treatment, the HA/GAPDH ratio of surface samples from mutant transfected cells was more than 200 times higher compared to the HA/GAPDH ratio of total samples (data not shown). This result indicated that the HA signal detected through surface biotinylation was not caused by cytoplasmic contamination and that the rescued γ2S subunits were expressed on the cell surface.

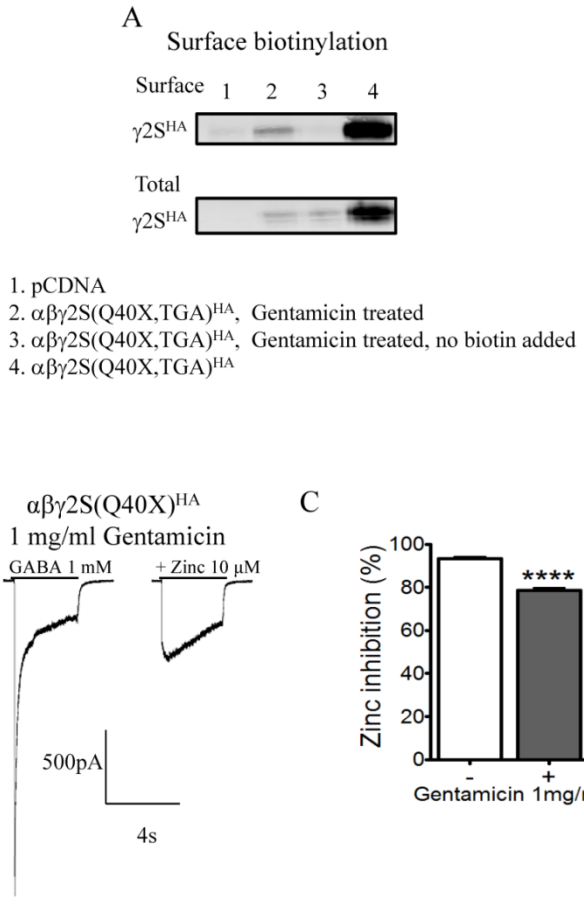


Figure 4.5 Gentamicin increased surface expression of mutant γ2(Q40X) subunits and decreased Zn^{2+} sensitivity of mutant receptor currents.

A. HEK 293T cells were cotransfected with $\alpha1\beta2\gamma2S(Q40X,TGA)^{HA}$ subunits. Cells were then treated with 1 mg/ml of gentamicin for 48 hours, then surface protein samples were collected through surface biotinylation and blotted by anti-HA, anti-ATPase and anti-GAPDH antibody. Cell lysates (0.5 mg and 1 mg) from cells expressing wildtype $\gamma2S^{HA}$ and mutant $\gamma2S(Q40X,TGA)^{HA}$ subunits were used to collect the surface fraction. Cell lysates (10 μg and 50 μg) from wildtype $\gamma2S^{HA}$ and mutant $\gamma2S(Q40X,TGA)^{HA}$ subunits transfected cells were loaded as total fraction. Samples not coated with biotin were also collected as controls. B. HEK 293T cells were cotransfected with $\alpha1\beta2\gamma2S(Q40X,TGA)^{HA}$ subunits. Cells were treated then with 1 mg/ml of gentamicin for 24 hours, and whole cell currents in response to 1 mM GABA then were recorded. The current amplitudes recorded in the presence of 10 μM Zn^{2+} were normalized to those recorded in the absence of Zn^{2+}. C. The percentage of current amplitudes inhibited by Zn^{2+} was compared to that obtained from cells untreated with gentamicin ($n = 19$, $p < 0.001$).

Gentamicin-rescued γ2S subunits were functional

We then evaluated assembly of $\alpha1\beta2\gamma2S(Q40X)$ receptors after gentamicin treatment by studying Zn^{2+} sensitivity of GABA-evoked currents to distinguish αβ from αβγ receptor currents. In the absence of gentamicin, currents recorded from cells containing $\alpha1\beta2\gamma2S(Q40X,TGA)^{HA}$ subunits were substantially sensitive to (Figure 3B). In contrast, after 24 h gentamicin treatment, the fractional Zn^{2+} inhibition of currents recorded from treated cells containing $\alpha1\beta2\gamma2S(Q40X,TGA)^{HA}$ subunits was significantly smaller than those recorded from untreated cells (Figure 5B) (79 ± 1%, $n = 19$; $p < 0.001$). This appearance of Zn^{2+} insensitive currents indicates the existence of

receptors on the cell surface. Taken together, these results suggest that gentamicin read-through of some of the γ2S(Q40X) subunit transcripts to produce full length γ2S subunits, and that the rescued full length γ2S subunits were assembled with α1 and β2 subunits to form functional receptors on the cell surface.

Discussion

The *GABRG2* mutation, Q40X, may induce epilepsy through haploinsufficiency

The *GABRG2(Q40X)* mutation was identified from heterozygous dizygotic twin sisters with Dravet Syndrome [275]. We investigated the effects of this mutation on the assembly, trafficking and function of receptors in HEK cells cotransfected with α1β2γ2S(Q40X) subunits. Q40X is a mutation that produces a PTC in exon 2 of genomic DNA. Using BAC constructs containing this mutation, we found that mutant γ2S subunit mRNA levels were increased significantly after we knocked down the NMD factor UPF1 or SMG6, indicating that the mutant mRNA was degraded by NMD. NMD is a cellular surveillance mechanism that reduces expression of truncated products by degrading nonsense mutation-containing mRNA during translation [298]. It was shown that NMD could reduce the level of a PTC-containing transcript to 20% in the brain,

although the regional specificity was not addressed [299]. If NMD destroys the mutant mRNA completely, heterozygous patients carrying one mutant *GABRG2(Q40X)* allele would suffer from *GABRG2* haploinsufficiency. However, not all mutant transcripts will be degraded, and NMD efficiency was shown to vary among different cell types [282]. Thus, we also characterized the mutant protein generated by this mutation. Q40 is the residue of the predicted mature γ2 subunit. Therefore, production of a truncated protein composed only of the signal peptide would be predicted. To investigate this small peptide, we generated the double tagged SP^{HA}-γ2S(Q40X)^{FLAG} subunits. We found that synthesis of full-length γ2 subunit protein was abolished by this mutation and production of the signal peptide was increased. Surprisingly, the signal peptide generated by SP^{HA}-SP^{HA}-γ2S(Q40X)^{FLAG} subunits was further cleaved (Figure 2B), probably through signal peptide peptidase [289, 297]. Our strategy successfully demonstrated the signal peptide processing products of γ2 subunits, providing a method to study other signal peptide related mutations. Our strategy also revealed an additional outcome of the Q40X mutation. It is possible that the signal peptide peptidase cleavage site was better exposed in the truncated γ2(Q40X) subunits, resulting in further cleavage. Although quite limited, a few studies have indicated that in addition to membrane targeting, signal peptide fragments could interact with signaling molecules [300] or be processed as antigenic

epitopes [47]. Whether or not the novel cleavage pattern of the γ2(Q40X) subunit signal peptide contributes to the epilepsy pathogenesis requires more detailed study.

To further explore how the truncated γ2(Q40X) subunits affected receptor assembly, we compared $GABA_A$ receptors formed by coexpression of α1β2γ2S or α1β2γ2S(Q40X) subunits. Both flow cytometry and whole cell recordings showed that mutant γ2(Q40X) subunits did not incorporate into functional ternary α1β2γ2S(Q40X) receptors. Instead, binary α1β2 receptors were formed that conducted much smaller currents. Therefore, *GABRG2(Q40X)* is likely a non-functional allele, and this mutation could cause haploinsufficiency of γ2 subunits in patients. γ2 subunits are widely distributed in the brain [148], and homozygous γ2 knockout mice died within a few days after birth [56]. Although seizures have not been reported from heterozygous $\gamma2^{+/-}$ knockout mice, heterozygous $\gamma2^{R82Q/+}$ knock-in mice carrying one mutant *GABRG2* allele developed absence epilepsy [185]. Hence, loss of one functional *GABRG2* allele in patients carrying the *GABRG2(Q40X)* mutation is likely responsible for development of the epilepsy phenotype.

The expression and function of mutant γ2(Q40X) subunits were partially rescued by gentamicin in vitro

Out of the seven epilepsy-associated mutations identified in *GABRG2*, four generated PTCs [276], and out of mutations identified from Dravet Syndrome patients, 50% were PTC mutations [274]. Aminoglycosides, including G418 and gentamicin, have been used to promote read-through of PTCs by disturbing stop codon recognition during translation. *In vitro* and *in vivo* in animals and in preclinical studies in humans, successful rescue of the mutant phenotype has been reported for several different disease models [296, 301, 302]. In our study, we observed that full length γ2S subunits were rescued from both γ2S(Q40X, TGA) subunits containing an optimized PTC and γ2S(Q40X) subunits containing the native PTC TAG, suggesting that this strategy could be applied to partially compensate for PTC mutations. Furthermore, the rescued γ2 subunits were trafficked to the cell surface and were incorporated into functional receptors, which is promising for future therapy.

Aminoglycoside-induced read-through has been used primarily in recessive disorders, where protein expression is almost null. However, this therapeutic approach may also work in autosomal dominant disorders [303], including epilepsy. On the one hand, it is possible that a small amount of rescued γ2 subunits during a critical time could benefit patients substantially. GABA acts as a trophic factor during neural

development [304-306], disrupting postsynaptic γ2 subunit clusters decreased presynaptic GABAergic innervation [307]. Study of heterozygous $\gamma2^{R82Q/+}$ mice revealed that $GABA_A$ receptor dysfunction during development increased seizure threshold in [308]. Thus lack of functional $GABA_A$ receptors during development may cause reduction of GABAergic neurons, further contributing to the decreased inhibitory tone in adult brain. If neuronal inhibitory tone could be increased in patients carrying mutations such as Q40X before synaptogenesis is complete, it is possible that only a small amount rescued γ2 subunits could ameliorate the developmental deficits and decreased seizure susceptibility in later life. On the other hand, perhaps full rescue of mutant γ2 subunits is not needed to compensate for the haploinsufficiency. Our *in vitro* data showed that 75% of γ2 subunits were still expressed on the cell surface when only half amount of γ2 cDNA was transfected with α1 and β2 subunit cDNAs at 1:1:0.5 ratio and had about 63% of GABA-evoked current compared to cells expressing α1β2γ2 subunit cDNAs at 1:1:1 ratio [191]. According to the 2:2:1 stoichiometry ratio of αβγ receptors, with expression of αβγ2 subunits mRNA in a 1:1:1 ratio, γ2 subunits may be in excess. *In vivo* studies in heterozygous $\gamma2^{+/-}$ knockout mice also showed 25% reduction of αβγ receptors [167]. If that also holds true in patients carrying a haplo-insufficient *GABRG2* allele such as *GABRG2(Q40X)*, less than 50% of γ2 subunits would be required to restore the normal function of γ2 subunits. Furthermore, mutations like Q390X in γ2 subunits display a

dominant negative effect to impair trafficking of wildtype subunits [191]. Read-through of γ2(Q390X) subunits could not only increase surface γ2 subunits translated from mutant γ2(Q390X) subunits, but also increase trafficking of γ2 subunits translated from wildtype γ2 subunits as well as partnering α and β subunits. Therefore, it would be worthwhile to evaluate read-through of *GABRG2(Q390X)* subunit mRNA.

Long term use of aminoglycosides could cause nephrotoxicity and ototoxicity [309]. With treatment using a high concentration of gentamicin (2 mg/ml), our cells also exhibited lower survival rates (data not shown). Although gentamicin has been tested in patients carrying PTC mutations of cystic fibrosis [310] and Duchenne muscular dystrophy [301], it is necessary to explore other less toxic drugs. PTC124 (Ataluren®) is a nonaminoglycoside read-through compound with superior read-through efficacy and lower toxicity [279, 311]. A phase II prospective trial showed that PTC124 administration reduced abnormalities in cystic fibrosis patients [312]. Compounds with better efficacy and therapeutic window could be identified in future and our work shows a possible direction for epilepsy therapy.

Acknowledgements

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Chapter 5

Conclusion and Future Directions

Summary

The *GABRG2* mutation could induce epilepsy through multiple mechanisms

The goal of this dissertation study is to elucidate the mechanisms of epileptogenesis for novel epilepsy channelopathies associated with the *GABRG2* mutations, including the intronic mutation *GABRG2(IVS6+2T→G)*, deletion mutation *GABRG2(S443delC)* and truncation mutation *GABRG2(Q40X)*, and to propose novel clinical strategies to treat epilepsies associated with mutant $GABA_A$ receptor genes.

1. *GABRG2(IVS6+2T→G)* mutation

The *GABRG2(IVS6+2T→G)* mutation altered the *GABRG2* intron 6 splice donor site sequence from GT to GG. As a result, it was proposed that intron 6 is spliced out either with the donor site from another intron, resulting in exon skipping, or with an alternative donor site downstream of the wildtype site, resulting in cryptic splice donor

activation and partial intron 6 retention in the mutant mature mRNA [198]. We mutant and wildtype minigenes and BACs and expressed them in HEK293T cells and transgenic mouse brain and determined the mutant *GABRG2(IVS6+2T→G)* gene intron splicing pattern. We found that the mutant intron 6 was spliced out between the wildtype acceptor site and a novel donor site 53 bp downstream of the wildtype one, resulting in partial intron 6 retention, which produced a frame shift in exon 7 that generated a PTC. The resultant mutant *GABRG2(IVS6+2T→G)* mRNA was partially degraded by NMD, or translated as the γ2-PTC subunit containing the first 6 *GABRG2* exons and a novel frame shifted 29 amino acid C terminal tail. We also identified γ2 subunit truncated mRNAs when the *GABRG2* gene was expressed in human cells but not in mouse brain. These truncated mRNAs were truncated due to exon skipping from both wildtype and mutant BAC transcripts, and the *GABRG2* mutation, IVS6+2T→G, enhanced expression of the truncated mRNAs. These results demonstrated that the mutation, *IVS6+2T→G,* reduced *GABRG2* transcript level by activation of NMD and enhancing truncated γ2 subunit mRNA prodocution. *It might produce epilepsy by GABRG2 gene haplo-insufficiency*.

The full length *GABRG2(IVS6+2T→G)* BAC transcript translation product, γ2-γ2-PTC subunit, was cloned and expressed in HEK293T cells. The γ2-PTC subunit contains the signal peptide and the N-terminal 217 aa of the wildtype γ2 subunit and a hydrophobic novel C-terminus. It is a stable protein and its total level was comparable to

that obtained from wildtype γ2 subunits. NMD efficiency varies among cell types [313]. The mutant NMD-susceptible $GABA_A$ receptor (GABAR) subunit mRNA level was decreased to 17% of wildtype level in cultured cortical neurons [201]. The mRNA level of an NMD-susceptible mRNA was decreased to 18% in mouse brain, and varied 16% in ovary to 36% in thymus [314]. This variation resulted from different protein levels of the NMD machinery components in each tissue. These studies suggested that about 17-36% of γ2-PTC mRNA should survive NMD, and that its protein level would be about 17-36% of wildtype γ2 subunits.

The sequence identity between the γ2-PTC subunit and the mollusk AChBP was high (21-29%) [254], and thus the γ2-PTC subunit is homologous to the AChBP. However, further studies demonstrated that the γ2-PTC subunit and AChBPs have protein functions. AChBPs form homopentamers in glial cells, which have crystal structure that resembles the N-terminus of assembled cys loop receptors [250, 251, 254]. AChBP homopentamers are released to synaptic gaps in an ACh-dependent manner and bind with ACh to terminate ACh neurotransmission. The γ2-PTC subunits did oligomerize with partnering subunits, but they were not assembled to pentameric that were secreted or trafficked to the cell membrane. Instead, they were retained in the ER, bound to αβ subunits, and had dominant negative effects on GABAR surface trafficking. Although it supported the hypothesis that the *GABRG2(IVS6+2T→G)*

mutation induced epilepsy by *GABRG2* gene haplo-insufficiency because the γ2-PTC subunit was not expressed on cell membrane, the *GABRG2(IVS6+2T→G)* mutation could also induce epilepsy by γ2-PTC subunit dominant negative effects because the γ2-PTC subunit induced ER-stress and retained αβ subunits in the ER. More than 80% of the α1(A322D) subunit is degraded in the ER [53], but it still oligomerized with wildtype subunits and decreased surface α1β2γ2 and α3β2γ2 receptor levels, preferentially those α3β2γ2 receptors [213]. The α1(A322D) mutation is associated with JME [210]. Because the γ2-PTC subunit total level is comparable to wildtype γ2 subunits, if 16-36% the γ2-PTC mRNA escaped NMD, the γ2-PTC subunit level would be 16-36% of subunits. *The γ2-PTC subunit could induce GABAR disinhibition through dominant negative effects similar to the α1(A322D) subunit*.

An ER-stress response could also contribute to GABRG2(IVS6+2T→G) mutation pathogenesis because the γ2-PTC subunit increased the ER stress marker BIP level significantly higher than wildtype γ2S subunits. Increased BIP level during unfolded protein response (UPR) induced ER-stress induces both apoptosis and protective such as reduced translation, enhanced ER protein-folding capacity, and clearance of misfolded ER proteins [264]. These adaptation and apoptosis responses are designed to adapt to the stress or removal of the affected cells, depending on the nature and severity the stress [265]. ER stress responses contribute to the pathogenesis of diseases including

diabetes mellitus, cancer and AIDS. Neurodegenerative diseases such as Alzheimer's disease and Huntington's disease are often associated with ER stress responses induced mutant proteins. Another *GABRG2* epilepsy mutation, the AD *GABRG2(Q390X)* mutation, is associated with Dravet syndrome [189], and the γ2(Q390X) subunit is also retained in the ER and not expressed on cell membrane [191]. The γ2(Q390X) subunit increased BIP level in HEK 293T cells, but to a level slightly less than that the increase produced by the γ2-PTC subunit. The fact that γ2-PTC subunit-transfected cells did not have apoptosis suggested that the γ2-PTC subunit induced mild stress in the cell, but the adaptive responses induced by γ2-PTC subunits would affect how cells respond to other stress factors. If ER stress responses contributed to its pathogenic mechanism, the *GABRG2(IVS6+2T→G)* mutation might be more efficient in inducing epilepsy than the *GABRG2(Q390X)* mutation. Furthermore, patients bearing the *GABRG2(Q390X)* mutation had epilepsy ranging from mild febrile seizures to severe Dravet Syndrome, and sibling that has the wildtype γ2 subunit gene had myoclonic astatic epilepsy, suggesting that the disease phenotype of the mutant γ2(Q390X) subunit might be affected by genes [189]. Although the *GABRG2(IVS6+2T→G)* and *GABRG2(Q390X)* mutations were associated with different epilepsies, they may possibly induce epilepsy though a common pathway. However, the γ2(Q390X) subunit had slow degradation and formed SDS-resistant, high-molecular-mass complexes or aggregates in multiple cell types,

including neurons [193], but we did not detected any of such complexes formed by γ2-subunits (not shown). If slow protein degradation and aggregates contributed to *GABRG2(Q390X)* mutation pathogenesis, *GABRG2(IVS6+2T→G)* mutation would not have such effects.

In summary, the *GABRG2(IVS6+2T→G)* mutation affected *GABRG2* gene mRNA intron splicing, decreased mutant mRNA level by NMD and enhanced alternative intron splicing, generated a γ2-PTC subunit that was a stable protein retained in the ER, induced ER-stress through unfolded protein response (UPR), and had dominant negative effects on GABAR membrane trafficking. It associated with epilepsy through both haplo-insufficiency and dominant negative effects.

2. *GABRG2(S443delC)* mutation

The *GABRG2(S443delC)* mutation, associated with GEFS+, deleted a cytosine nucleotide from the S443 residue in the last exon of the *GABRG2* gene. We cloned the mutant mRNA into the pcDNA vector and expressed it in HEK293T cells. RT-PCR results suggested that the mutant allele was translated to a stable protein with the last 24 amino acids of the wildtype γ2 subunit containing the 4th transmembrane domain by a novel 50 amino acid C-terminus. Bioinformatics analysis suggested that the novel C-terminus had decreased hydrophobicity compared to the wildtype C-terminus. The frame shift mutation shifted the stop codon into the 3' UTR, thus shortening it, but it did

not interfere with polyA site recognition. The mutant γ2S(S443delC) subunit was a protein and appeared on a Western gel as a band with larger molecular mass than the wildtype γ2S subunit band, but with a total level that was decreased to ~35% of wildtype γ2S subunit under similar expression conditions. When coexpressed with α1 and β2 subunits, γ2(S443delC) subunits were retained in the ER and not expressed on the cell surface membrane, and GABA-evoked currents from the cells were similar to those obtained from α1β2 receptors. Another frame shift mutation in the α1 subunit, α1(S326fs328X), was subcloned into an intron8 minigene, and mutant minigene mRNA was partially decreased by NMD. Depending on NMD efficiency, 16-39% of mutant mRNA level was translated to mutant α1(S326fs328X) subunit, which is truncated in the middle of the third transmembrane domain [201]. The α1(S326fs328X) subunit was retained in the ER and degraded by ER-associated degradation. In cells with higher efficiency, the α1(S326fs328X) subunit was not detected in minigene transfected cells [201]. The α1(S326fs328X) mutation would induce epilepsy by *GABRA1* gene haplo-haplo-insufficiency [201]. *Similar to the α1 subunit mutation, S326fs328X, the γ2 subunit mutation, S443delC, might generate a γ2 subunit null allele and be associated with epilepsy, at least in part, through halplo-insufficiency.*

However, the γ2(S443delC) subunit might also be associated with epilepsy by dominant negative effects on wildtype $GABA_A$ receptor subunit assembly and membrane

trafficking. The epilepsy-associated γ2(R82Q), γ2(Q390X) and α1(A322D) subunit mutations also were shown to generate mutant proteins that were retained in the ER [182, 191, 210]. Both γ2(R82Q) and γ2(Q390X) subunits were found to be stable proteins with total levels similar to those of wildtype γ2 subunits [182, 191, 193]. These two subunits oligomerized with partnering subunits and efficiently decreased surface α1 and β2 levels when they were coexpressed in HEK293T cells [59, 170, 180, 182]. The subunit also decreased endogenous α5 subunit surface expression in cultured neurons, and impaired tonic GABAergic current [176]. The mutant $\gamma 2^{R82Q/+}$ knock in mice had the same type of seizures as humans bearing the mutation [185].

The α1(A322D) subunit, associated with juvenile myoclonic epilepsy (JME) was misfolded, retained in the ER, and extensively degraded by proteasomal degradation [53, 210, 211]. The total level of α1(A322D) subunits was around 10% of wildtype α1 subunits [210, 213]. However, the α1(A322D) subunit associated with wildtype subunits in the ER and reduced wildtype both α1β2γ2 and α3β2γ2 receptor surface expression, preferentially that of α3β2γ2 receptors [213]. The γ2(S443delC) subunit was predicted not to fold correctly because of the novel C-terminus that is less hydrophobic than the wildtype C-terminus. The very low total level of γ2(S443delC) subunit might be because of increased proteasomal degradation due to ER retention, although that must be confirmed. It is possible that although the γ2(S443delC) subunit has reduced total levels,

it could oligomerize with α and β subunits and decrease wildtype $GABA_A$ receptors surface expression, but must also to explored. The functional consequences of the S443delC mutation could be a combination of haplo-insufficiency and dominant negative effects. The α1(A322D) mutation was associated with juvenile myoclonic epilepsy, and the γ2(R82Q) mutation induced the same type of seizures as human patients in heterozygous mutation knock-in mice. *The dominant negative effect of γ2(S443delC) subunit could also be sufficient to induce epilepsy.*

3. *GABRG2(Q40X)* mutation

The *GABRG2(Q40X)* mutation, associated with Dravet Syndrome [275], significantly decreased γ2(Q40X) mRNA by NMD [298]. The Q40X mutation produced a PTC in exon 2 of *GABRG2* genomic DNA. The mutant γ2S subunit mRNA levels were increased significantly after we knocked down either NMD factor UPF1 or SMG6 in BAC-transfected HEK293T cells. *If NMD destroys the mutant mRNA completely, heterozygous patients carrying one mutant GABRG2(Q40X) allele would suffer from GABRG2 haploinsufficiency.* However, NMD could only reduce the level of a PTC-containing transcript to 20% in the brain [299], or 17% in cultured neurons [201, 282]. Thus, we also characterized the mutant protein generated by this mutation.

The γ2(Q40X) subunit is predicted to produce a truncated protein composed only of the signal peptide. Using double tagged SP^{HA}-γ2S(Q40X)^{FLAG} subunits, we

demonstrated that synthesis of full-length γ2 subunit protein was abolished by this mutation, production of the signal peptide was increased, and surprisingly, the SP^{HA}-SP^{HA}-γ2S(Q40X)^{FLAG} subunit signal peptide had novel peptide cleavage, probably signal peptide peptidase [289, 297]. We are determining if the novel cleavage pattern of the γ2(Q40X) subunit signal peptide contributes to the epilepsy pathogenesis [47, 300], which would demonstrate possible dominant negative function of the γ2(Q40X) mutation.

Further studies supported the hypothesis that *the γ2(Q40X) mutation induced epilepsy through GABRG2 gene haplo-insufficiency.* The mutant γ2(Q40X) subunits did not incorporate into functional ternary α1β2γ2S(Q40X) receptors. GABA-evoked current recorded from coexpressed α1β2γ2S(Q40X) subunits had properties similar to those obtained from binary α1β2 receptors. Although α1β2γ2(R82Q) receptors had normal GABA-evoked current properties but decreased pentameric receptor assembly and surface trafficking [170], heterozygous $\gamma2^{R82Q/+}$ knock-in mice carrying one mutant *GABRG2* allele developed absence epilepsy that resembles patients symptoms [185]. Hence, loss of one functional *GABRG2* allele in patients carrying the *GABRG2(Q40X)* mutation is likely responsible for development of the epilepsy phenotype.

4. Using aminoglycosides to treat epilepsies induced by mutant GABRG2 allele carrying PTC mutations.

We have successfully improved the function of PTC mutation, *GABRG2(Q40X)* mutation, using aminoglycosides gentamicin or G418, neither of which is a conventional anticonvulsant drug. Aminoglycosides have been used in clinical trials to rescue the mutant phenotype by disturbing stop codon recognition during translation and promote read-through of PTCs [296, 301, 302]. We demonstrated that full length γ2S subunits were rescued from γ2S(Q40X) subunits after aminoglycoside treatment. Furthermore, the rescued γ2 subunits were trafficked to the cell surface and incorporated into functional receptors, which is promising for future therapy. *We provided proof of principle evidence that this strategy could be applied to partially compensate for PTC mutations.*

All epilepsy associated $GABA_A$ receptor subunit mutations are AD mutations, including the PTC mutations *GABRG2(Q40X)* and *GABRG2(Q390X)* [44]. Although aminoglycoside treatment only rescued ~10% of functional γ2 subunits, it could be sufficient to decrease patient's seizures. *In amplified RNA (aRNA) studies in dentate granule cells, the amount of γ2 subunit mRNA was comparable to the total amount of α(1-6) mRNAs or the total amount of β(1-3) subunits mRNAs* [315]*; γ1 and γ3 mRNA not detected. Nevertheless, a*ccording to the 2:2:1 stoichiometry of αβγ receptors, with expression of αβγ2 subunits mRNA in a 1:1:1 ratio, γ2 subunits may be in excess. The

subunit is preferentially assembled with αβ subunits to form pentameric receptors and trafficked to cell membranes [183, 191]. Decreasing the dosage of one gene in heterozygous $\gamma 2^{+/-}$ knock-out mice only reduced αβγ receptors by 25% [167]. If that also holds true in patients carrying a haplo-insufficient *GABRG2* allele such as *GABRG2(Q40X)*, less than 50% of the normal γ2 subunit level would be required to the normal function of γ2 subunits. GABAergic neurotransmission is important for both neural development and normal brain function in adults [304-307]. Decreased $GABA_A$ receptor levels during development reduced the number GABAergic neurons and increased seizure threshold in adulthood [308]. Thus, it is possible that a small amount of γ2 subunit rescue during a critical period, such as during or before synaptogenesis, could ameliorate the developmental deficits and decreased seizure susceptibility in patients' life. *This read-through strategy is likely to improve seizure symptoms in patients PTC mutations.*

Furthermore, PTC mutations such as γ2(Q390X) had strong dominant negative effects to impair wildtype $GABA_A$ receptor biogenesis, and induced a very severe form epilepsy [191]. Read-through of γ2(Q390X) subunits could not only increase surface γ2 subunits translated from mutant γ2(Q390X) subunits, but also eliminate the amount of mutant γ2(Q390X) subunits and decrease the disturbance on wildtype $GABA_A$ receptor

biogenesis. Therefore, it would be worthwhile to evaluate the read-through strategy to improve the function of *GABRG2(Q390X)* mutation.

Future Directions

The complexity of *GABRG2* mutation mechanisms of pathogenesis provides a model not only to understand how a point mutation in GABAR subunit gene causes GABAR disinhibition and induces epilepsy in patients, but also how to explore strategies to improve GABAR haplo-insufficiency, membrane trafficking or ER-stress in neurons, and ultimately improve patients quality of life. We have demonstrated successful rescue of the *GABRG2(Q40X)* mutation. However, it is not feasible to obtain wildtype γ2 subunits from γ2-PTC or γ2(S443delC) subunits using aminoglycosides treatment. *To better understand how these mutations affect mouse brain function and find strategies to treat these epilepsies, we would use cultured cells, cultured neurons, and transgenic mice to further study the function of the γ2-PTC subunit, understand how it affects brain function [167, 184], and attempt to improve the function from pathways downstream of mutant $GABA_A$ receptor functions.*

Decreased GABAR surface expression was associated with decreased mIPSC amplitude and frequency in GABAergic neurons [65]. Heterozygous $\gamma 2^{+/-}$ knock-out mice had decreased GABAR postsynaptic clusters [167] and also had decreased neurogenesis in adult mouse brain [184]. We have demonstrated that a human *GABRG2(IVS6+2T→G)* BAC was translated to a γ2-PTC subunit in a transgenic (Tg($hGABRG2^{IVS6+2T\rightarrow G}$) mouse. Although the mRNA transcribed from the mutant gene was susceptible to NMD, about 16-39% of the mutant mRNA could be translated to γ2-PTC subunit because NMD efficiency varies among cell types [201, 314]. The γ2-PTC subunit is a stable ER protein with total level comparable to wildtype γ2 subunits. It had a dominant negative effect on cells because it induced the unfolded protein response and ER-stress and pulled down α1 and β2 subunits at low efficiency. Studies in epilepsy-associated γ2 subunit mutations suggested that the γ2-PTC subunit could decrease GABAR biogenesis [175, 176, 180, 182, 191]. However, it is unknown whether the γ2-PTC subunit could bind to other α or β subunits, or δ subunits which are assembled into predominantly extrasynaptic GABARs, or whether it decreased membrane trafficking of wildtype receptors or affected surface GABAR ion channel function. It is also unknown whether the ER-stress adaptive response in cells expressing γ2-PTC subunit affects GABAR biogenesis.

If γ2-PTC subunits decreased surface GABAR expression, decreased the number postsynaptic GABAR clusters, and reduced synaptogenesis and adult neurogenesis in

mouse brain, it would be possible that the *GABRG2(IVS6+2T→G)* gene expressed in mouse as a transgene (Tg($hGABRG2^{IVS6+2T\rightarrow G}$) mouse) decreases seizure threshold or generates spontaneous seizures in mouse. It is unknown if the γ2-PTC subunit could affect GABAR expression and targeting in neurons, and whether its function was to affect synaptogenesis and neurogenesis in mouse brain. We propose several future studies to address these questions.

Determine if the γ2-PTC subunit decreased GABAR biogenesis in HEK293T cells.

In Chapter 3 we demonstrated that the γ2-PTC subunit oligomerized with α1 and β2 subunits and retained them in the ER. There are 19 GABAR subunit subtypes. Each subtype has a unique temporal and spatial expression pattern [149]. GABAR heterogeneity ensures the functional diversity of GABARs. The JME-associated α1(A322D) subunit is retained in the ER and degraded by 80% [52, 53, 213]. It oligomerized with wildtype subunits in the ER and reduced the surface expression of α1β2γ2 and α3β2γ2 receptors, preferentially α3β2γ2 receptors [213]. When the CAE-CAE-associated γ2(R82Q) subunit was expressed in cultured neurons, it decreased level of endogenous α5 subunits and reduced tonic GABAergic current [176]. These studies suggested that the γ2-PTC subunit might have a differential dominant negative effect on GABAR subunits. HEK293T cells do not have endogenous expression of any

GABAR subunit genes [44]. We would coexpress FLAG-tagged $\gamma2S^{FLAG}$ or $\gamma2\text{-}PTC^{FLAG}$ subunit with HA-tagged α1-α6, β2, β3, wildtype γ2L, γ2S, and δ subunits, pull down FLAG-tagged proteins with FLAG beads, and blot for HA-tagged subunits. The result will demonstrate if the $\gamma2\text{-}PTC^{FLAG}$ subunit can bind to any wildtype subunits other than and β2 subunits.

Then we would determine if the γ2-PTC subunit could alter GABAR ligand binding affinity or decrease the number of GABAR ligand binding sites. We would coexpress 1 μg each of γ2S or γ2-PTC subunit cDNA with α1 and β2 subunit cDNAs in HEK293T cells, collect cell membrane, perform radioligand binding with [^{3}H]Ro 15- and [^{3}H]muscimol, and analyze the binding curve by nonlinear regression [180]. [^{3}H]Ro 15-4513 binds to the benzodiazepine binding site at α/γ subunit interfaces, and [^{3}H]muscimol binds to GABA binding site at α/β interface. Radioligand binding to cells coexpressing α1β2γ2(R82Q) subunits showed that mutant receptors had substantially decreased maximum binding (B_{MAX}) at the benzodiazepine site, but that the amount of GABA binding sites was not changed [180]. The mutant α1β2γ2(R82Q) receptors also had similar binding affinity (K_D) to [^{3}H]Ro 15-4513 and [^{3}H]muscimol as wildtype receptors. Coexpression of α1β2γ2-PTC subunits showed that GABA-evoked current was similar to α1β2 receptor current, but if γ2-PTC subunits decreased surface of α1β2 receptors, GABA binding sites would be decreased also. We would evaluate if

coexpression mutant α1β2γ2-PTC subunits had substantially decreased B_{MAX} for [^{3}H]Ro 15-4513 and [^{3}H]muscimol compared to coexpression of wildtype α1β2γ2S or α1β2 subunits. There are two α and two β subunits in a heteropentameric αβγ or If the γ2-PTC subunit preferentially decreased surface α or β level, it could change the stoichiometry of the surface GABARs, which might change the number of GABA sites in each GABAR or [^{3}H]muscimol binding affinity. There are two identical GABA binding sites at α1/β2 interface in wildtype α1β2 or α1β2γ2S receptors. We would evaluate if the mutant α1β2γ2-PTC coexpression had different shape [^{3}H]muscimol binding curve from coexpressed wildtype α1β2γ2S or α1β2, subunits and if the [^{3}H]muscimol binding affinity (K_D) was different. We would further analyze the [^{3}H]muscimol binding curve of coexpressed α1β2γ2-PTC or α1β2γ2S and α1β2 subunits with a Hill plot. The result will show if the GABAR ligand binding site is changed [316].

There are 19 GABAR subunits expressed in the CNS. The pull down experiment shows whether γ2-PTC subunits bind to wildtype subunits, and the radioligand binding study further elucidates how γ2-PTC subunits affect GABAR assembly and membrane trafficking. We would perform high throughput flow cytometry and electrophysiology studies to explore how the γ2-PTC subunit affects GABAR biogenesis and ion channel function. We would coexpress 0.2 μg each of α1β2 subunits and 0.2 μg of HA-tagged γ2 or δ subunit cDNAs with increasing amounts of untagged γ2S or γ2-PTC subunit cDNA

ranging from 0 μg (wildtype coexpression) to 1.6 μg (8 times more than each wildtype subunit). Total transfected cDNA will be normalized to 3 μg with pcDNA empty vector. Previous experiments determined that GABAR subunit mRNA and protein levels increased in linearly if the transfected cDNA was less than 3 μg. The α and β subunits will be those that are pulled down by γ2-PTC^{FLAG} subunits. Only one type of α or β subunit will be expressed in each experiment. We would immune-stain the permeablized and unpermeablized cells with antibodies detecting α or β subunit, or HA-epitope tag. We would run flow cytometry to evaluate surface and total levels of each subunit in the presence of mutant or wildtype untagged γ2 subunits at increasing amounts. The total level of each subunit will be quantified from permeablized cells, and surface level will be quantified from unpermeablized cells. If the γ2-PTC subunits decrease wildtype subunit surface and/or total levels significantly more than the wildtype γ2 subunit does, it had a dominant negative effect on wildtype GABAR subunit expression and/or membrane trafficking. The high throughput and quantitative flow cytometry technique facilitates quantifying the efficiency of γ2-PTC subunit dominant negative effects. These studies will elucidate how γ2-PTC subunits affect GABAR ER-to-membrane trafficking, which a major regulation mechanism of GABAR biogenesis [64, 270].

The α1β2γ2-PTC subunit had GABA evoked current similar to α1β2 receptors, and the flow cytometry experiment quantified the efficiency of γ2-PTC subunit decreases

in synaptic and extrasynaptic GABAR membrane trafficking. In order to understand how the γ2-PTC subunit affects GABAR function, we would coexpress γ2S or γ2-PTC with α1β2 or an αβ subunit that could be pulled down by γ2-PTC subunit, record GABA evoked current from transfected cells, and evaluate whether γ2-PTC subunits changed current amplitude or kinetics. The mean current amplitude will demonstrate if γ2-PTC subunits decreased the membrane level of αβ subunits, which is evaluated by flow cytometry, and the current kinetics will demonstrate if γ2-PTC subunits affected surface GABAR stoichiometry and pharmacological properties, which are also evaluated by radioligand binding.

The γ2-PTC subunit substantially increased cellular BIP levels and induced ER-ER-stress. It is possible that the γ2-PTC subunit retained α1 and β2 subunits in the ER because these subunits all bind to ER chaperones such as BIP, and ER-stress response enhanced ubiqutin-proteasome degradation and eliminated α1β2 subunits. To evaluate this possibility, we would coexpress 1 μg of wildtype α1β2γ2 or α1β2δ subunit cDNAs HEK293T cells and treat cells with 1 μM tunicamycin for 3 hours, and then evaluate surface and total levels of treated and untreated cells by flow cytometry. A previous demonstrated that tunicamycin treatment increased BIP level significantly higher than expressing γ2-PTC subunit in cells. If the wildtype subunit surface and total levels were not affected by tunicamycin treatment, the γ2-PTC subunit dominant negative function

not result from ER-stress. If they were, we would introduce the R82Q mutation to the γ2-γ2-PTC subunit, which decreases the oligomerization between γ2 and β2 subunits [59], evaluate if the γ2-PTC^{R82Q} subunit could decrease surface and total α1β2 levels.

Determine if the γ2-PTC subunit affected neuronal function.

Studies in HEK293T cells can elucidate the effects of γ2-PTC subunits on GABAR ER-to-membrane trafficking. We would like to extend these studies to biogenesis and trafficking of γ2-PTC subunits in cultured cortical neurons. After GABARs are trafficked to neuronal surface membranes, they diffuse laterally and then become restricted to specific synaptic or extrasynaptic compartments depending upon interaction with cytoskeletal proteins [64, 115, 270]. GABAR mobility at synaptic and extrasynaptic sites is regulated in an activity-dependent manner [317]. These regulatory processes are absent in HEK293T cells.

To determine subcellular localization of γ2-PTC subunits in cultured cortical neurons, we would express IRES-ZsGreen vectors containing HA-tagged γ2S or γ2-PTC subunit cDNA because there is endogenous rat γ2 subunit expression in cultured neurons, stain for endogenous rat α1 subunit and HA-tagged γ2 subunit, and take confocal images green fluorescent cells. Axons and dendrites will be visualized by ZsGreen, which is

synthesized in cell bodies and diffuses in the cytoplasm. Wildtype $\gamma2S^{HA}$ subunits will be assembled into GABARs and expressed on postsynaptic membranes [176] and will have substantial colocalization with $\alpha1$ subunits on both somatic and dendritic sites on ZsGreen-positive neurites. If $\gamma2\text{-PTC}^{HA}$ subunits are still retained in the ER, as was seen in HEK293T cells, HA staining will be somatic staining with little dendritic staining, and its colocalization with $\alpha1$ subunit at dendritic sites will be substantially decreased.

To determine whether $\gamma2\text{-PTC}^{HA}$ subunits decreased the number of postsynaptic GABAR clusters and GABAergic synaptogenesis, we would stain $\gamma2S^{HA}$ or $\gamma2\text{-PTC}^{HA}$ subunit cDNA transfected neurons with antibodies against $\alpha1$ subunit and pre- or post-synaptic markers including vesicular inhibitory amino acid transporter (VIAAT), glutamate decarboxylase 65 (GAD65) and gephyrin, take confocal images, and then quantify the number of $\alpha1$ subunit-containing GABAR clusters and pre- and postsynaptic markers on ZsGreen positive neurites. VIAAT and GAD65 are GABAergic presynaptic markers, and gephyrin localizes at postsynaptic membranes [65]. Decreased levels of postsynaptic $\gamma2$ subunit-containing GABAR clusters were associated with reduced pre- and postsynaptic membrane marker staining [65]. Our study will demonstrate if $\gamma2$-PTC subunits decreased postsynaptic targeting of endogenous $\alpha1$ subunit-containing GABARs, and if this dominant negative effect was sufficient to reduce the level of GABAergic pre- and postsynaptic membrane markers.

Decreased expression of postsynaptic γ2 subunit-containing GABAR clusters reduced GABAergic mIPSC frequency and amplitude [65]. Overexpressing ER-retained γ2(R82Q) subunits in cultured neurons also reduced tonic GABAergic currents [176]. Studies in HEK293T cells will elucidate if γ2-PTC subunits have dominant negative effects on synaptic or perisynaptic GABARs. We would express IRES-ZsGreen vector-containing $\gamma2S^{HA}$ or $\gamma2\text{-}PTC^{HA}$ subunit cDNA in cultured neurons, record GABAergic mIPSCs from ZsGreen positive neurons, and determine if γ2-PTC subunits decreased frequency, amplitude or kinetics of GABAergic mIPSCs [65]. We would also record tonic GABAergic current with prolonged, focal application of 1 μM GABA from ZsGreen positive cells and evaluate the current amplitude normalized to the membrane capacitance of the recorded neuron [176], which will demonstrate if γ2-PTC subunit expression inhibited extrasynaptic GABAergic currents.

These studies will demonstrate if γ2-PTC subunits affected the number of and GABAergic electrophysiological properties in neurons. Induced ER-stress in cultured hippocampal neurons by tunicamycin or thapsigargin increased mEPSCs in an activity-dependent manner but did not alter mIPSC or the number of active synapses suggesting that if γ2-PTC subunits have effects, they would be associated with altered GABAR biogenesis but not induced ER-stress response. This possibility can also be

explored by studying GABAergic synaptogenesis and synaptic/extrasynaptic currents in tunicamycin treated cultured neurons.

Determine if the *hGABRG2(IVS6+2T→G)* transgene affected mouse brain development.

Our studies in HEK293T cells and cultured neurons should elucidate whether and how γ2-PTC subunits interact with wildtype GABAR subunits and affect neuronal function. We would explore how γ2-PTC subunits affect mouse brain development and behavior using transgenic mice overexpressing a wildtype or mutant *GABRG2* BAC. We would study the expression and neuronal colocalization of wildtype hγ2^{HA} subunits in transgenic Tg(*hGABRG2*^{HA}) mice and identify neuronal populations in which GABAR trafficking and targeting are likely to be affected by γ2-PTC subunits. We would determine then the efficiency of NMD to degrade γ2-PTC subunit mRNA in neurons and whether the γ2-PTC subunit affected neuronal development and function in transgenic mice overexpressing the *GABRG2(IVS6+2T→G)* BAC (Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse). However, we generated Tg(*hGABRG2*^{HA}) and Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mice through two different pronuclear injections, so the number of BAC transgenes integrated to each genome is likely to be different for these two transgenic mouse models, as well as the γ2 subunit transcription level. To avoid this pitfall, we would compare Tg(*hGABRG2*^{HA}) or

Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse data obtained from wildtype littermates from the same breeding. Because we did not disturb endogenous wildtype mouse γ2 subunit expression in these two transgenic mouse lines, the result will demonstrate the phenomenon with overexpression of wildtype hγ2^{HA} or mutant γ2-PTC subunits.

Determine the regional expression pattern of hγ2^{HA} and γ2-PTC subunit.

We would first perform *in situ* hybridization using probes binding to endogenous mouse γ2, hγ2^{HA} or hγ2-PTC subunits in Tg(*hGABRG2*^{HA}) and in Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) transgenic mouse brain sections collected at 30-35 days after birth (P30-35), when γ2 mRNA and protein levels are stable [144, 150, 319]. The Tg(*hGABRG2*^{HA}) mouse brain had high level of hγ2^{HA} subunit in cortex, hippocampus, thalamus and cerebellum the as mouse endogenous γ2 subunit protein [149], but hγ2^{HA} mRNA pattern was not studied. The *in situ* hybridization shows the mRNA expression patterns of hγ2^{HA} or hγ2-PTC subunits or endogenous mouse γ2 subunits and confirms if the hγ2^{HA} mRNA expression pattern is the same as the endogenous mouse γ2 mRNA. We would quantify the signal intensities of each subunit in cortex, hippocampus, thalamus and cerebellum, where the subunit has high mRNA expression [145, 146]. The cortex and thalamus are important generalized epilepsy epileptogenesis [5], while decreased γ2 subunit level interfered with hippocampal function including increased anxiety behavior traits and decreased adult

neurogenesis [167, 184]. The γ2-PTC mRNA level is determined by the number of *GABRG2(IVS6+2T→G)* BAC transgenes integrated to the mouse genome, *GABRG2* transcription rates, and NMD efficiency in each cell. Comparing hγ2 and γ2-PTC mRNA signal intensities in these four regions will demonstrate which region has lower NMD efficiency, suggesting higher γ2-PTC subunit expression, and hence higher γ2-PTC dominant negative effects. The γ2 mRNA signal intensities quantified from brain slices will be confirmed by Northern blot. We would dissect P30-35 cortex, hippocampus, thalamus and cerebellum from acute dissected Tg($hGABRG2^{HA}$) or Tg($hGABRG2^{IVS6+2T\rightarrow G}$) mouse brain, extract total RNA, and run Northern blot with used in *in situ* hybridization and quantify the band intensities of human and mouse γ2 mRNAs. Exposing the radio-labeled slides in emulsion will visualize the cellular distribution of hγ2-PTC subunits and further identify the neuronal populations that could have more γ2-PTC dominant negative effects.

The γ2-PTC subunit protein level will be determined by Western blot. We would dissect P30-35 mouse cortex, hippocampus, thalamus and cerebellum from acute Tg($hGABRG2^{IVS6+2T\rightarrow G}$) mouse brain, prepare total tissue lysates, run Western blot with antibodies, and quantify the band intensities of human γ2-PTC and mouse γ2 mRNAs. The γ2 antibodies detect both endogenous mouse γ2 subunit and human γ2-PTC subunit, but the hγ2-PTC band has a smaller molecular mass than the mγ2 band. Western blot

demonstrate the relative levels of hγ2-PTC and mγ2 subunit in these regions, which have the same trend as Northern blot. If there is regional variation in hγ2-PTC subunit protein level, it will improve our understanding of hγ2-PTC subunit dominant negative effect when the hγ2-PTC subunit is expressed at different levels.

The cellular localization of $h\gamma2^{HA}$ subunits will be studied by immunohistochemistry. We would co-immunostain with HA-antibody and either α1 antibody or GABAergic postsynaptic membrane marker gephyrin in Tg(*hGABRG2*^{HA}) mouse brain sections. The $h\gamma2^{HA}$ subunit had strong colocalization with α1 subunit when coexpressed in HEK293T cells. When it was overexpressed in cultured hippocampal neurons, it was expressed at GABAergic synapses the same as endogenous γ2 subunits [176], suggesting that the α1 and $h\gamma2^{HA}$ signal should both be expressed at GABAergic synapses and colocalized with gephyrin. After confirming this, we would co-immunostain Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain sections with α1 and gephyrin antibodies, take confocal images in regions that have different γ2-PTC subunit protein levels, and quantify the colocalization of α1 and gephyrin. The results will suggest if the γ2-PTC subunit dominant negative effect is sufficient to decrease the number of GABAR postsynaptic clusters.

To further confirm if the surface GABARs are decreased in Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain, we would cryosection 20 μm fresh frozen

Tg(*hGABRG2*^{HA}) or Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain sections and perform radioligand binding with [^{35}S]TBPS, [^{3}H]muscimol and [^{3}H]Ro 15-4513. [^{35}S]TBPS binds to extrasynaptic GABARs, [3H]muscimol binds to GABA binding sites, and 15-4513 binds to benzodiazepine sites [166]. The heterozygous □2+/- mouse had decreased [3H]Ro 15-4513 binding but increased [35S]TBPS binding, suggesting that extrasynaptic GABAR levels were increased [166]. Compared to wildtype littermate, the Tg(hGABRG2IVS6+2T→G) mouse should have increased [3H]muscimol and [3H]Ro 15-4513 binding because the hGABRG2(IVS6+2T→G) BAC transgene overexpressed h□2 subunits, which can be assembled into GABARsand trafficked to the neuronal membrane. If □2-PTC subunits decrease membrane GABAR levels, the Tg(hGABRG2IVS6$^{+2T\rightarrow G}$) mouse would have decreased [^{3}H]muscimol and [^{3}H]Ro 15-15-4513 binding. If γ2-PTC subunits pull down extrasynaptic GABAR subunits in HEK293T cells, the [^{35}S]TBPS binding would decrease too. The alteration will be stronger in the regions with higher γ2-PTC protein and vice versa.

Determine if the hγ2-PTC subunit decreased the number of synapses or newborn neurons in Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain

Decreased surface GABAR expression is associated with decreased active synapses in neurons [65, 320, 321], while inhibiting GABAR degradation increases

synapse number [136]. We would quantify the number of synapses in the neurons overexpressing γ2-PTC subunits from Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse brain sections and determine whether γ2-PTC subunits could decrease surface GABAR levels enough to decrease synapse numbers, and whether the decrease would be dependent on γ2-PTC subunit amount. The $\alpha1^{-/-}$ knock-out mice lacking postsynaptic GABARs had significantly decreased synapse density after P11 [320]. We would prepare brain sections from PFA fixed Tg(*hGABRG2*^{HA}) or Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mice at P14 and P28 and immuno-stain sections for HA-tagged $\gamma2^{HA}$ or endogenous mouse α1 subunits with pre- and post-synaptic markers including GAD6, VGAT and gephyrin. Confocal images will demonstrate the regions where $h\gamma2^{HA}$ subunits are overexpressed and assembled to GABARs, and quantification of colocalization the coefficient between α1 subunit in HA-HA-positive synapses with pre- and postsynaptic markers will elucidate if the synapse density is increased or decreased by overexpressing wildtype $h\gamma2^{HA}$ subunits at P14 and P28. Then we would repeat the experiment in Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mouse and take confocal images from the same regions studied in Tg(*hGABRG2*^{HA}) mouse brain and showed strong γ2-PTC total levels in *in situ* hybridization and Western blot. The result will demonstrate if overexpression of γ2-PTC subunits decreases synaptic density in brain.

Adult neurogenesis increases after symptomatic stimulation such as brain seizures, radiation, or neurodegenerative diseases [322]. However, decreased surface GABAR levels in conditional $\gamma2^{+/-}$ mice lacking γ2 subunits in forebrain since early embryonic development is associated with decreased adult hippocampal neurogenesis [184]. The γ2-PTC subunit is expressed the same as the endogenous γ2 subunit. The γ2-γ2-PTC dominant negative effect should also appear early in development [144, 253, If it increased chronic ER-stress enough, adult neurogenesis might be increased as an adaptive response to ER-stress [322]. If it decreased surface GABAR levels sufficiently to mimic the knock-out effect in the heterozygous $\gamma2^{+/-}$ mouse, it might decrease adult neurogenesis. We would explore whether γ2-PTC subunit overexpression is sufficient to affect adult neurogenesis. We would inject BrdU into Tg($hGABRG2^{HA}$) or Tg($hGABRG2^{IVS6+2T\rightarrow G}$) mice at P56 (4 × 80 mg/kg, i.p., at 2 h intervals, in saline at 8 mg/ml, pH 7.4) and harvest brain 28 d later to detect mature neurons differentiated from adult-born BrdU-labeled cells. Alternatively, we would inject a single dose of 200 mg/kg (20 mg/ml) to 8-week-old mice and harvest brain 24 hours later to quantify adult-born replicating/undifferentiated cells. The PFA fixed brain would be dissected to 40 μm and immunestained for BrdU and NeuN. The number of $BrdU^+/NeuN^+$ cells in cortex, thalamus, hippocampus and cerebellum would be compared between transgenic mouse wildtype littermates from the same breeding [184]. Overexpressing $h\gamma2^{HA}$ subunits in

HEK293T cells did not increase ER-stress, suggesting that Tg(*hGABRG2*^{HA}) mice might not have different numberx of adult-generated neurons compared to wildtype littermate mice. However, Tg(*hGABRG2*$^{IVS6+2T\rightarrow G}$) mice might have different numbers of adult-adult-generated neurons, and the alteration might suggest the mechanisms for the mutant BAC-induced phenomenon.

Summary

We proposed several future studies comprehensively evaluating how the γ2-PTC subunit dominant negative effect affects GABAR biogenesis, neuron function, and brain development. These studies will demonstrate whether γ2-PTC subunits could bind to GABAR subunits expressed at synaptic or extrasynaptic sites, how it could affect surface GABAR biogenesis in neurons, and whether these alterations would be sufficient to affect mouse brain development. These findings will facilitate our understanding about the expression and function of the *GABRG2(IVS6+2T→G)* mutation, and how it induces seizures. It will also help us understand the pathogenesis of other epilepsy-associated GABAR subunit mutations, such as *GABRG2(S443delC)* mutations.

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