EPILEPSY-ASSOCIATED MUTATIONS IN *GABRG2*: CHARACTERIZATION AND THERAPEUTIC OPPORTUNITIES

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To my beloved parents, Yaojian Huang and Qingcong Lin.

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List of Abbreviations

3’-UTR: three prime untranslated region

AED: antiepileptic drugs

BAC: bacterial artificial chromosome

Cα RMSD: the carbon alpha root mean squared deviation

CAE: childhood absence epilepsy

CFTR: cystic fibrosis transmembrane conductance regulator

CNS: central nervous system

DMD: Duchenne muscular dystrophy

DRG: dorsal root ganglion

EEG: electroencephalography

EPSP: excitatory postsynaptic potential

ER: endoplasmic reticulum

ERAD: ER-associated degradation

FS: febrile seizures

GABA: γ-aminobutyric acid

$GABA_A$Rs: type-A GABA receptors

GABR: $GABA_A$ receptor gene

GAT: GABA transporter

GE: genetic epilepsy

GEFS+: generalized epilepsy with febrile seizures plus

GGE: Genetic generalized epilepsy syndromes

GTCS: generalized tonic-clonic seizures

HDAC: histone deacetylase

HEK: human embryonic kidney

IPSC: inhibitory post-synaptic current

JME: juvenile myoclonic epilepsy

LTP: long term potentiation

MGE: medial ganglionic eminence

NMD: Nonsense-mediated mRNA decay

NMDA: N-Methyl-D-aspartate

PEI: polyethylenimine

PTC: premature translation-termination codon

PTZ: pentylenetetrazol

SWDs: spike-wave-discharges

TTX: tetrodotoxin

VB: Ventrabasal

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Chapter 1 : Introduction

1. Epilepsy

1) Nomenclature and classification

Epilepsy is one of the most common neurologic disorders affecting more than 65 million people worldwide, including around 0.5% of the population in the United States, and one out of 26 people may develop epilepsy during their lifetime. Epilepsy is characterized by recurrent unprovoked seizures, which are caused by overexcited and hypersynchronized neurons in the brain (*1-4*). Epilepsy can increase mortality, is associated with multiple comorbidities including sudden unexplained death in epilepsy (SUDEP) and depression and affects the health and quality of life of patients, thus imposing a burden on both individuals and society (*4-6*).

As clinical symptoms, seizures are the manifestation of epilepsy. Seizures can be focal, originating in local networks that are limited to one hemisphere. Seizures can also be generalized, engaging bilaterally distributed brain networks. Generalized seizures include tonic-clonic, absence, myoclonic, clonic, tonic and atonic seizures (*7, 8*). They are distinct clinic symptoms correlated with different electroencephalography (EEG) patterns and are important for epilepsy syndrome diagnosis, prognosis, and treatment.

According to the revised classification by the International League Against Epilepsy (ILAE) in 2010, epilepsies are classified as genetic, structural-metabolic, and unknown based on their underlying causes (*8, 9*) (Table 1). **Genetic epilepsies,** such as channelopathies, are directly caused by a known or presumed genetic defect(s); **structural/metabolic epilepsies** result secondarily from distinct structural or metabolic conditions; and **unknown epilepsies** refer to those with unknown causes. Although reflecting current advances in epilepsy research, this classification is not perfect. The symptoms could overlap, and not all patients can be easily categorized. The etiologies of many types of epilepsy syndromes are still unknown,

and we are not sure whether there is a common mechanism of pathogenesis for patients diagnosed with the same epilepsy syndrome. In addition, the process of epileptogenesis is not clear.

2) Current treatments

Most patients with epilepsy are well controlled by antiepileptic drugs (AEDs) and enter long-term remission. However, around 20% of epilepsies are refractory, not responding to current treatments (*6, 10, 11*), and many AEDs are associated with side effects (*12, 13*). Furthermore, most current AEDs are anti-seizure, but not anti-epileptogenesis, therapies. Rather than prevent the development of epilepsy, these medications only suppress seizures (*14*). After treatment with an initial AED, the seizure recurrence rate is as high as 30-40%. A significant proportion of seizure-free patients also relapse after the termination of treatment (*6, 15*). Generally speaking, a prior neurologic insult, focal seizures, age of onset below 10 years or above 65 years, and abnormal EEG patterns are associated with poorer outcomes (*6, 15*).

During the process of epileptogenesis, three elements in neuronal circuits could contribute to the overexcitation: modification of membrane properties of neurons that generates intrinsic bursting, reduction of GABAergic inhibition, and enhancement of excitatory neurotransmission. Thus, most clinical useful AEDs target voltage-gated sodium channels, voltage-gated calcium channels, glutamate neurotransmission or/and GABAergic neurotransmission (*16, 17*) (Table 2). For example, carbamazepine, a major AED, can reduce neuronal high-frequency firing by inhibiting voltage-gated sodium channels (*18*). Ethosuximide, a first-line AED for absence seizures, blocks T-type calcium channels (*19*). Phenobarbital and benzodiazepines like diazepam and clonazepam, are potentiators of type-A γ-aminobutyric acid receptors ($GABA_A$Rs) (*20*). Valproate, one of the most common AEDs effective against a broad spectrum of seizures, has been suggested to elevate GABA levels, suppress N-Methyl-D-aspartate (NMDA)-elicited depolarization or block voltage-gated sodium channels (*21*).

While AEDs are the most common option for treating epilepsy, there are other therapeutic strategies. The ketogenic diet, a high-fat diet effectively increasing the seizure threshold in different animal models,

is still used in modern treatment, although its mechanism of action is still not clear (*22*). Vagal nerve stimulation -- electrical stimulation of the vagus nerve -- has been used when multiple medications have failed and surgery is not an option and can decrease the total AED burden for patients (*23*). Brain surgery to remove or disconnect epileptic regions is an effective treatment for selected patients with intractable epilepsy. Considering current treatment options are not satisfactory, new therapeutic methods based on a good understanding of etiology of individual patients will be helpful.

2. $GABA_A$ receptors

GABA, γ-aminobutyric acid, is the main inhibitory neurotransmitter in the central nervous system (CNS). Although only accounting for 20% of all neurons, GABAergic interneurons are widely distributed in the CNS, sending out broad and extensive inhibitory connections to almost every neuron. Type-A GABA receptors ($GABA_AR$s) are ligand-gated ion channels mostly located at postsynaptic membranes that mediate GABA-induced fast inhibitory neurotransmission. Ubiquitously expressed in the brain, $GABA_AR$s are not only important for maintaining excitation/inhibition balance but are also critical in regulating network oscillations.

1) $GABA_AR$ composition

$GABA_AR$s are pentameric receptors that form a Cl^- ion channel (Figure 1-1). They belong to the superfamily of cys-loop ligand gated ion channels. This family also includes nicotinic acetylcholine receptors (nAChRs), 5-hydroxytryptamine type 3 serotonin receptors ($5\text{-}HT_3$Rs), and glycine receptors (GlyRs) in vertebrates (*24, 25*). Five homologous subunits are arranged pseudo-symmetrically, forming a central pore allowing ions to pass through (*26-28*). Due to the low Cl^- ion concentration in most mature CNS neurons, opening of $GABA_AR$ channels induces the influx of Cl^- ions to hyperpolarize the membrane, thus maintaining inhibitory tone. However, during early development, the expression of the Na^+-K^+-$2Cl^-$ cotransporter dominates that of the K^+-Cl^- cotransporter, leading to a high concentration of

intracellular Cl^- ions. Thus $GABA_A$Rs mediate excitatory neurotransmission in immature neurons, which is critical for neuronal growth and synaptogenesis (*29-31*).

Nineteen different $GABA_A$R subunits, α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3, have been cloned (Figure 1-2A). Interestingly, except for δ, π, and ρ subunit genes, most human $GABA_A$R subunit genes are clustered on chromosomes 4, 5, 15, and X, in a common pattern of β-[α]-α-γ (*32*) . Each cluster is comprised by 1 or 2 α subunit, 1 β subunit and 1 γ subunit genes, where a β subunit gene could be replaced by the related θ subunit gene, and a γ subunit gene by the related ε subunit gene. Chromosome 4 contains β1, α2, α4, and γ1 subunit genes; chromosome 5 contains β2, α1, α6, and γ2 subunit genes; chromosome 15 contains α5, β3, and γ3 subunit genes, while chromosome X contains θ, α3, and ε subunit genes. These gene clusters were suggested to arise from an ancestral gene set, expanding the genetic heterogeneity through duplications and chromosomal translocations.

$GABA_A$R subunits share 60-80% amino acid identity within subunit subfamilies and 20-40% among subfamilies (*33*). Similar to other subunits from the cys-loop receptor family, $GABA_A$R subunits share a conserved structure (Figure 1-2B). Each subunit contains a large N terminal extracellular domain, followed by four transmembrane segments (M1-M4) and a small C terminal tail. Subunit interfaces contributed by extracellular domains provide drug binding sites, the M2 segments line the ion channel, and the large cytoplasmic M3-M4 loop is subject to different types of modulation (*25, 28, 34-36*). The heterogeneity is further increased by alternative splicing (*32, 37*), RNA editing (*38, 39*), as well as phosphorylation (*35, 40*).

However, not all subunit combinations can form functional receptors. The majority of postsynaptic $GABA_A$Rs are αβγ type receptors (*41, 42*), with α1β2γ2 receptors being the most abundant receptor subtype and accounting for about 40% of all $GABA_A$Rs (*43*). αβγ receptors are composed of two α, two β and one γ subunits (*44, 45*) in a counterclockwise sequence of γ- β-α-β-α as viewed from the synaptic cleft (*46, 47*) (Figure 1-1 C). Mostly αβγ receptors mediate phasic inhibition responding to the short and

transient synaptic GABA release (*48*). In contrast, αβδ receptors are predominantly located at extrasynaptic membranes (*49, 50*) and are suggested to mediate tonic inhibition by responding to ambient GABA in the extracellular space (*48, 51*). These two modes of inhibition (phasic/tonic) are generally associated with different subcellular localization (synaptic/extrasynaptic), GABA sensitivity (low/high), and kinetic properties (fast/slow desensitization) of these two receptor types (*48, 52*).

2) Expression and function of major $GABA_AR$ subunits

Although not all subunit combinations can form functional receptors, more than twenty different $GABA_AR$ subtypes still exist. The heterogeneity of $GABA_AR$ subunits may be important to guarantee the redundancy. The heterogeneity of $GABA_AR$ subunits also enables modulation of GABAergic neurotransmission involved in different physiologic activities (Table 3).

α subunits: Six different α subunits have been cloned, among which α1 subunits are the mostly widely distributed in adult brain (*53-55*). For example, α1 subunit-containing receptors account for more than 50% of $GABA_ARs$ in rodent cerebellum (*41, 42*) and more than 40% benzodiazepine binding sites in the whole rat brain (*53*). α2 subunits are expressed in many regions including cortex (but limited to outer layers), showing strong expression in olfactory bulb, hippocampus, amygdala, septum, striatum, nucleus accumbens and hypothalamus. α3 subunits are also expressed in the cortex, but mostly in inner layers. α3 subunits are also expressed in the olfactory bulb, amygdala, reticular nucleus of thalamus, but weak in other thalamic regions (*54*). During development, the expression of α2/3 subunits are dominant in most brain regions at embryonic stages, while the expression of α1 subunits starts perinatally (*56, 57*). However, the expression of α1 subunits increases dramatically after birth, while the expression of α2/3 subunits decreases in many brain areas, thus α1 subunits become the most prevalent α subunits of mature brain. The expression of α4 subunits begins at the perinatal stage (*56*). In adult brain, its expression is high in thalamus, striatum and nucleus accumbens, and the molecular layer of the dentate gyrus. α4

subunits are less concentrated in cerebral cortex and CA1 and are low or absent in the cerebellum (*54*). The expression of α5 subunits also begins at embryonic stages and increases after birth, but decreases quickly in the adult cortex and thalamus (*56*), while the expression in the hippocampus stays strong (*54*). Different from other α subunits, the expression of α6 subunits is quite unique and dominant in postnatal granule cells in cerebellum, and its expression is increased during postnatal development (*54, 56*).

Although more than half of $GABA_A$Rs were lost and recorded miniature inhibitory post-synaptic current (IPSC) amplitudes were greatly reduced in α1 subunit knock-out mice, the mice were still viable and fertile (*58, 59*), which could be explained by compensatory mechanisms. The protein, but not mRNA, levels of α3, α4, and α6 subunits were increased in different brain regions (*60-62*). Increased tonic currents mediated by reduced GABA transporter (GAT) activity were also reported in cerebellar granule cells (*63*). Micro-array analysis revealed large transcriptional responses to regulate the excitability and plasticity of neurons, which may offset the α1 subunit deficiency (*64*). However a recent more detailed study reported reduced viability and absence-like seizures in congenic homozygous and heterozygous α1 subunit knock-out mice respectively (*65*), indicating that α1 subunits are still indispensable. Studies on α1(H101R) subunit knock-in mice, where a critical benzodiazepine binding site of the subunit was mutated, showed that α1 subunits might be involved in myorelaxant and motor-impairing effects of diazepam, but not the anxiolytic and ethanol-potentiating effects (*66, 67*).

Highly expressed in limbic system, α2 and α3 subunits are involved in anxiety and depression (*68*). α2 subunit knock-out mice exhibited depressive-like activities (*69*). Contrary to α1(H101R) subunit knock-in mice, sedative, motor-impairing and anticonvulsant effects of diazepam were retained in diazepam-insensitive α2(H101R) and α3(H126R) subunit knock-in mice (*70*). Further behavioral tests using these two mouse lines suggested that α2, but not α3, subunit-containing $GABA_A$Rs mediated the anxiolytic effects of diazepam (*70*), although later pharmacological studies indicated α3 subunits could also be involved in anxiolysis (*71, 72*). Besides, α3 subunit knock-out mice showed remarkably

attenuated sensorimotor-gating mediated through a deficit of GABAergic neurotransmission in dopaminergic neurons, which is a typical phenotype of schizophrenia (*73*).

Highly expressed in dentate gyrus and thalamus, α4 subunits play an important role in tonic inhibitory currents by forming extrasynaptic α4β2δ receptors (*74, 75*). α4β2δ receptors are not sensitive to benzodiazepine modulation, but are sensitive to ethanol potentiation (*76*). α4 subunit knock-out mice were superficially indistinguishable from wildtype mice (*75*). While the tonic currents were absent in dentate granule cells and thalamic relay neurons, the expression of α2 and γ2 subunits was increased in the hippocampus (*77*), which may compensate for the reduced inhibition. Behaviorally, the global α4 subunit knock-out mice were insensitive to ataxic, sedative and analgesic effects of the hypnotic drug gaboxadol (*75*) and were more susceptible to pentylenetetrazol-induced seizures (*78*), but showed normal responses to moderate-to-high doses of ethanol (*78*). Virus-mediated conditional α4 subunit knock-down in rats that reduced expression of α4 subunits in the shell, but not the core, structure of nucleus accumbens caused decreased consumption of low-to-moderate levels of alcohol, suggesting the involvement of α4 subunits in alcohol uptake (*79*).

Enriched in hippocampus, α5 subunit-containing receptors account for around 20% of hippocampal αβγ receptors (*80*) and are located both synaptically and extrasynaptically (*81, 82*). Recordings from the CA1 region in α5 subunit knock-out mice showed reduced IPSC amplitudes, no change of long term potentiation (LTP), but increased paired-pulse facilitation of the field excitatory postsynaptic potential (EPSP). Consistently, α5 subunit knock-out mice had better performance in Morris water maze testing, indicating a role for them in learning and memory (*83*). Similarly, α5(H105R) subunit knock-in mice, where the expression of α5 subunits was reduced by 20%, exhibited facilitated responses in trace fear conditioning test, indicating improved hippocampus-associated learning (*84*).

α6 subunits are highly expressed in cerebellar granule cells, present in about 40% of cerebellar αβγ receptors, while weak expression in substantial nigra, thalamus and inferior colliculi was also reported

(*85*). Posttranslational loss of δ subunits was identified in cerebellar granule cells, indicating a close association. However, α6 subunit knock-out mice had no overt behavioral deficits (*86*).

β subunits: All three β subunit subtypes are widely distributed, especially in the cortex. Their expression is complementary in subcortical regions and cerebellum. Specifically, β2 subunits are highly expressed in most regions of thalamus, except in the reticular nucleus, where β1 and β3 subunits are expressed (*54*). During early development, β3 subunits are relatively abundant in many brain regions, but their expression is decreased in adult brain.

β1 subunit expression begins after birth with weak expression in the adult cortex and thalamus, and strong expression in the hippocampus. No β1 subunit knock-out mice have been characterized. Recently, two β1 subunit knock-in mice were generated by induced or spontaneous mutagenesis and both mouse models, showed increased spontaneous $GABA_AR$ channel openings and increased tonic currents in nucleus accumbens, exhibited increased alcohol consumption, indicating that β1 subunits are related to alcohol abuse (*87*). β2 subunits are the dominant β subunits in adult brain, accounting for more than half of the $GABA_ARs$ (*58*). The expression of β2 subunits could be identified at embryonic stages and increased during development, highly expressed in cortex, thalamus, pallidum and cerebellum. Although receptors containing different types of α subunits were reduced in β2 subunit knock-out mice, the mice did not show major phenotypic abnormalities except higher spontaneous locomotor activities (*58*). The expression of β3 subunits also starts at an early embryonic stage but decreases after P12 in many brain regions and is almost gone in adult thalamus (except the reticular nucleus), while the expression in the hippocampus is still relative high. Interestingly, mice devoid of β3 subunits showed a severe phenotype (*88*), including high neonatal mortality rate, reduced life span, cleft palate, hyperactivity and epileptic seizures. The amount of $GABA_ARs$ was reduced in many brain regions of β3 subunit knock-out mice, while the GABAergic inhibition was almost absent in the reticular nucleus, and thalamocortical oscillations were greatly intensified (*89*).

γ subunits: Out of three γ subunits, the γ2 subunit is most abundant. γ2 subunit expression begins around E17 (*56*). In adult, they are highly expressed throughout the brain, but relatively weakly in the thalamus where the subunit expression is decreased during development. Meanwhile, γ1 subunit expression is limited to some midbrain areas, while γ3 subunit expression is weakly distributed through the brain (*54*).

In γ2 subunit knock-out mice, the majority of benzodiazepine binding sites were gone, but GABA binding sites were not affected (*90*). No complementary up-regulation of other subunits was observed, and GABA-evoked currents from knock-out dorsal root ganglion (DRG) neurons were greatly reduced (*90*). γ2 subunits are also required for maintenance of postsynaptic $GABA_A$Rs (*91*), and αβ receptors were formed in the absence of γ2 subunits (*90*). Most of mice devoid of γ2 subunits died within a few days after birth, with normal embryonic development. Those that survived exhibited severe sensorimotor deficits and died before P18 (*90*), consistent with physiological importance of γ2 subunits. Heterozygous γ2 subunit knock-out mice also showed about a 25% reduction of γ2 subunits, most pronounced in cerebral cortex, hippocampus and thalamus, accompanied by reduced $GABA_A$R clusters and exhibited elevated anxiety (*92*). Loss of one *Gabrg2* allele in forebrain neurons in mice from early embryonic stages will cause reduced hippocampal neurogenesis and anxiety, but not when that allele is lost at P17 (*93*). In addition, absence-like spike-wave-discharges were reported from heterozygous γ2 subunit knock-out mice in DBA background (*94*). Specifically, γ2L and γ2S subunits are two γ2 subunit splicing isoforms that differ by eight amino acids including a serine residue. It was suggested that phosphorylation of this serine residue inhibited the self-trafficking of γ2L subunits and in the contrast γ2S subunits could insert into surface membrane without forming the heteropentamer (*37*). These two isoforms are almost equally expressed in CNS, although the amount may differ in different brain regions during different age (*95-97*) and the ratio was reported to be altered in schizophrenia patients (*98*). However, replacement of one isoform by the other did not have big impact on mouse behavior except for ethanol response (*99, 100*). How these two isoforms affect the physiology and pathology is still a big

unknown. Knock-out mice devoid of γ1 or γ3 subunits have not been reported. γ3 subunits could form weak postsynaptic clusters with α3 subunits, which was increased in homozygous γ2 subunit knock-out mice. Interestingly, although the overexpression of γ3 subunits partially restored GABAergic neurotransmission in γ2 subunit knock-out mice, the mice still showed perinatal or postnatal lethality. In addition, reduced fertility and seizures were observed in $\gamma3^{tg}/\ \gamma2^{0/+}$ mice, indicating the dominant negative effects of γ3 subunits (*101*).

δ subunits: δ subunits are expressed in postnatal cortex, striatum, thalamus, dentate (limited to dentate granule cells in hippocampus) and cerebellar granule cells (*54, 56*). Primarily coupled with α4 or α6 subunits, δ subunits form extrasynaptic αβδ receptors, which have distinct patterns of channel kinetics, drug responses and subcellular localizations compared to αβγ receptors (*102*). Noteworthy, δ subunit-containing receptors are sensitive to the action of low concentration of neurosteroid, steroid that is synthesized in the nervous system and potentiate $GABA_A$ receptors (*103*). Mice devoid of δ subunits showed higher mortality, decreased pentylenetetrazol (PTZ) -induced seizure threshold and non-responsiveness to neuroactive steroids (*104, 105*). GABA binding sites were greatly reduced in δ subunit knock-out mouse brain, and IPSC decay in hippocampus became faster. Learning and memory was normal in knock-out mice, although better spatial learning was reported during puberty in knock-out mice (*106*), and a more recent study showed that female mice had increased trace fear conditioning but not delayed fear conditioning, which could be caused by changed neurosteroid regulation (*107*).

To summarize, $GABA_A$Rs are broadly expressed in the brain and vital in different brain functions. They are targets of many pharmaceutical drugs such as benzodiazepines and barbiturates as well as neurosteroids (*108, 109*). In addition, $GABA_A$Rs are associated with multiple disorders, including anxiety (*70*), depression (*110*), schizophrenia (*73*), alcoholism (*111*), autism (*112, 113*) and epilepsy (*114-116*).

3. Epilepsy-associated mutations in $GABA_AR$s

Although epilepsies could be caused by acquired factors such as brain tumors and injury, genetic factors play an important role, which may account for more than 60% of all cases (*117*). Family aggregation studies showed that for patients with epilepsy, the recurrent risk ratio was around 2.5 in first-degree relatives; twin studies showed that the concordance of epilepsy was much greater in monozygotic twins compared to dizygotic twins; and linkage analysis also revealed phenotype-genotype cosegregation (*118*). While the genetic factors contributing to epilepsy is complicated and multiple genes are usually involved, studies on multiplex pedigrees from families with epilepsy identified specific epilepsy-associated monogenetic mutations in ion channels, such as voltage-gated sodium channels (*119*), voltage-gated potassium channels (*120*), nicotinic acetylcholine receptors (*121*) and $GABA_AR$s (*122, 123*).

The GABAergic system has been implicated in epilepsy. Reduced benzodiazepine binding was observed in the epileptic foci of patients with epilepsy (*124, 125*). Subunit specific changes in surface $GABA_AR$s was revealed in rat models of induced status-epilepticus (*126*). Enhanced tonic inhibition in thalamocortical neurons was identified in several different models of absence epilepsy (*127*). Meanwhile, an increasing number of mutations were identified in $GABA_AR$s from families or individuals with epilepsy. Mutant proteins may activate the cellular surveillance machinery and could impact GABAergic function through quite different ways. A comprehensive understanding of the molecular deficits and epileptogenesis mechanism underlying these mutations would benefit the diagnosis, prognosis and treatment design (Table 4).

1) <u>Epilepsy-associated missense mutations in $GABA_AR$ subunits</u>

Missense mutations, or non-synonymous mutations, are single nucleotide changes causing the replacement of one residue by another residue. The location of the residue in the mutant protein and the similarity between these two residues determines how disruptive the mutation is. Nine epilepsy-associated missense mutations have been identified in $GABA_AR$s and are distributed among $\alpha1$, $\beta3$, $\gamma2$

and δ subunit genes. They decreased $GABA_AR$-mediated inhibition to different extents, impairing receptor biogenesis or decreasing channel function.

GABRA1(A322D): Missense mutations could affect protein folding and lead to degradation of mutant protein (*128*), with the *GABRA1(A322D)* mutation as an example. *GABRA1(A322D)* was identified in a four-generation French Canadian family with juvenile myoclonic epilepsy (JME), and all affected members carried one mutant allele. An alanine residue conserved in the α subunit subfamily was switched to an aspartic acid in the M3 domain of α1 subunits (*129*). This mutation is associated with lower subunit expression, altered channel kinetic properties and smaller amplitude of GABA-evoked currents. Further study suggested that the major defect was faster degradation caused by subunit misfolding. The M3 domain of mutant α1 subunits failed to insert into the membrane, and thus the misfolded mutant protein was presented to endoplasmic reticulum (ER) chaperone molecules, activated ER-associated degradation (ERAD) and was quickly degraded by both lysosome and proteasome pathways (*130-133*). The amount of mutant α1 subunits was dramatically reduced, and most residual mutant α1 subunits were retained in the ER (*131, 134*), which could still assemble with other $GABA_AR$ subunits in the ER, and slightly prevent their trafficking to the surface (*132*). Mutant α1(A322D) subunits that were trafficked to the cell surface were also internalized faster through dynamin-dependent endocytosis (*135*). The histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) was reported to increase the elevated interaction between mutant α1(A322D) subunits and chaperone molecules, increase the transcription, folding, trafficking and function of mutant α1(A322D) subunits, partially through increasing BiP chaperone level by inhibiting HDAC7 (*136*). As heterozygous *Gabra1*$^{+/-}$ knock-out mice experienced spike wave discharges on EEG and absence-like seizures, α1 subunit haploinsufficiency could contribute to the epilepsy syndromes in patients carrying the *GABRA1(A322D)* mutation (*65*).

GABRA1(D219N), GABRG2(R82Q), GABRG2(R177G), GABRB3(P11S), GABRB3(S15F), GABRB3(G32R): The biogenesis of $GABA_A$Rs is complicated and inefficient. Although different intermediate oligomers are formed, only those with the correct pentameric composition can pass quality-control checkpoints and can be inserted into the surface membrane (*137, 138*). The missense mutations *GABRG2(R82Q),GABRG2(R177G),GABRA1(D219N),GABRB3(G32R), GABRB3(P11S),* and *GABRB3(S15F)* disrupted receptor biogenesis and membrane delivery, consequently depressing inhibitory neurotransmission.

The *GABRG2(R82Q)* mutation is one of the first and most extensively studied epilepsy-associated mutations identified in $GABA_A$Rs. It was originally found in a large Australian family with childhood absence epilepsy (CAE) and febrile seizures (FS) (*123*). A highly conserved (among cys-loop receptor subunits) arginine residue located in an N-terminal loop of γ2 subunits was replaced by glutamine. Although different mechanisms including altered channel kinetics (*139, 140*), impaired benzodiazepine binding (*123, 139*), smaller current amplitudes (*141-143*) and less surface expression of γ2 subunits (*142-146*), have been suggested, most evidence was consistent with the R82Q mutation impairing surface expression of $GABA_A$Rs containing γ2 subunits and thus reducing postsynaptic inhibitory currents, a conclusion that was subsequently confirmed in a knock in mouse model (*147*). The R82 residue is located at the positive face of γ2 subunits in a homology model, contributing to the γ(+)/β(-) interface through salt bridge connections. It seems that the conserved region around the R82 residue plays a role in receptor assembly (*143, 144, 146*), and thus, the mutant γ2 subunits were trapped in the ER (*142*). A small amount of mutant γ2 subunits were shown to still form functional receptors on the surface (*141*), although they were reported to be subjected to faster endocytosis (*148*). However, it is still controversial whether the mutant γ2 subunits affect the surface insertion of their assembly partners (*142, 146*). Overexpression of mutant γ2(R82Q) subunits in hippocampal neurons did not affect synaptic IPSPs, which is largely produced by GABA binding to $GABA_A$Rs containing α1 subunits, but was reported to

reduce extrasynaptic tonic currents by preventing surface expression of α5 subunits, suggesting subunit-dependent dominant negative effects (*145*).

Although individual family members carrying the R82Q mutation exhibited different syndromes including FS, CAE and generalized epilepsy with febrile seizures plus (GEFS+), genetic analysis indicated that this mutation alone could account for FS, while an interaction with other gene/genes is required for the CAE phenotype (*149*). This is partially consistent with observations in a knock-in mouse model carrying this mutation (*150*). While the homozygous knock-in mice were not viable, heterozygous knock-in mice displayed spontaneous absence seizures characterized by a 5-8 Hz, high amplitude spike-wave-discharges (SWDs) on EEG, which could be blocked by antiepileptic drug ethosuximide, similar to the typical 3 Hz SWDs recorded from CAE patients (*147*). Decreased surface γ2 subunits and decreased cortical inhibition in the heterozygous knock-in mice (*147*) is also well correlated with the reduced benzodiazepine binding sites (*151*) and increased intracortical excitability (*152*) in human patients. More detailed characterizations comparing mice from different backgrounds suggested that while loss of γ2 subunit function could account for the absence seizure phenotype, the R82Q mutation might be responsible for the FS phenotype (*153*). However, it is still controversial whether the elevated temperature during FS will exacerbate the defects of this mutation (*154, 155*). It is worth mentioning that in a conditional knock-in study, the presence of the R82Q mutation increased seizure susceptibility compared to a hypomorphic allele and exhibited developmental impacts on epileptogenesis (*156*). Thus the R82Q mutation had effects in addition to haploinsufficiency of the *Gabrg2* gene and the defects during development can exacerbate the epilepsy symptom.

GABRG2(R177G), *GABRA1(D219N)* are also missense mutations identified from small pedigrees with GFES+ in γ2 and α1 subunits respectively (*157, 158*). Although they were shown to affect the channel kinetics and the response to benzodiazepines, it seemed that the major defects were during biogenesis and both mutant subunits were partially trapped in the ER, reducing the surface level of subunits and consequently decreasing current amplitudes (*157-159*).

GABRB3(P11S), *GABRB3(S15F)*, and *GABRB3(G32R)* are three CAE-associated missense mutations in β3 subunits that were reported to cause hyperglycosylation of β3 subunits and to significantly decrease $GABA_AR$ current amplitudes (*160*). P11S and S15F are located in exon1a of the GABRB3 gene that encodes part of the signal peptide. Although identified in multiple CAE and autism families (*161, 162*), P11S was also identified in healthy controls (*162*), thus it is still controversial whether P11S is an epilepsy-associated mutation or it is just a risk variant. Differently, G32R is located in the N terminus of mature β3 subunits. Further characterization suggested that G32R was located in the α1(+)/β3(-) subunit interface, and the mutation might affect the salt-bridge formation in the interface. When coexpressed with α and γ subunits in human embryonic kidney (HEK) cells, mutant β3(G32R) subunits increased the surface level of β3 subunits, decreased the surface level of γ2 subunits and decreased channel current amplitudes. Interestingly, the mean open time of single α1β3γ2L receptor channels was also reduced by this mutation (*163*). Generally, *GABRB3(P11S)*, *GABRB3(S15F)*, and *GABRB3(G32R)* mutations slightly decrease the current amplitude of β3 subunit-containing receptors by affecting receptor biogenesis. Since a loss of β3 subunits intensifies thalamocortical oscillations (*89*), these mutations could increase seizure susceptibility by impacting the thalamocortical circuitry.

GABRG2(K328M), GABRD(E117A), GABRD(R220H): The gating process of $GABA_ARs$ is through a series of conformational change and domain interactions (*164*). Thus structural abnormalities impacting channel gating will also alter channel function. The missense mutations *GABRG2(K328M)*, *GABRD(E117A)*, and *GABRD(R220H)* were found to alter properties of both macroscopic and microscopic currents.

The *GABRG2(K328M)* mutation was found in a French family with GEFS+ (*122*). A positively charged lysine residue in the extracellular M2-M3 linker of γ2 subunits, conserved among $GABA_AR$ and glycine receptor subunits, was converted to methionine. The M2-M3 linker where the lysine residue is located has been shown to participate in channel gating (*165*). In response to GABA, the homologous K278 residue in α1 subunits moves closer to the negatively charged D149 residue in loop 7, indicating

that these two regions move closer during the gating process (*166*). Although decreased current amplitude (*122, 167*) was reported using an ultra-fast exchange system (rise time shorter than 400 μs) to mimic the synaptic physiology, our lab found faster deactivation using excised macropatches and shorter single channel mean open time of GABA-evoked currents recorded from mutant α1β3γ2(K328M) receptors (*141*), which will lead to depressed GABAergic inhibition. This observation was confirmed by the finding of faster decay of synaptic IPSCs in γ2(K328M) subunit transfected neurons (*145*). No significant change of protein expression was identified (*145, 168*). Compared to the aforementioned mutations that impaired protein biogenesis, the K328M mutation affected the channel activity of $GABA_AR$ receptors. Reduced volume of negative charge being transferred or the altered temporal kinetics of synaptic inhibition can cause the epilepsy phenotype.

The *GABRD(E117A)*and *GABRD(R220H)* mutations found in GEFS+ and JME families, respectively, are located in the extracellular N-terminal domain of δ subunits and reduced channel current amplitudes (*169*). Although these two mutations also affected subunit trafficking slightly, the major defects were caused by reduced single channel opening time, suggesting the function of mutant receptors is impaired (*170*). As the PTZ-induced seizure threshold was lower in δ subunit knock-out mice (*104, 105*), the dysfunction of mutant δ subunit-containing receptors could contribute to the epilepsy phenotype.

2) <u>Epilepsy-associated nonsense/frame-shift mutations in $GABA_AR$ subunits</u>

Instead of single amino acid alteration, truncation mutations caused by nonsense or frame-shift mutations may generate very different proteins. Nonsense mutations are non-synonymous mutations where a codon coding for an amino acid residue is replaced by a stop codon, causing the translation machinery to pause and generate a truncated protein. Frame-shift mutations are insertions or deletions that often cause a shift of the translation reading frame and usually also generate truncated protein. Depending on the position of the novel stop codon, or premature translation-termination codon (PTC),

mutant truncated protein could exhibit different stability and properties and affect channel function to different extents, ranging from pure loss of function to severe dominant negative effects.

GABRA1(975delC, S326fs328X), GABRG2(Q40X), GABRG2(R136X): Nonsense-mediated mRNA decay (NMD) is a post-transcriptional surveillance mechanism that eliminates abnormal transcripts with PTCs. Generally, PTCs located at least 50-55 nt upstream of the last exon-exon junction will elicit NMD (*171*). The *GABRA1(975delC, S326fs328X)* deletion mutation was identified in one sporadic CAE individual. A deletion in exon 10 of *GABRA1* caused a shift in the reading frame, resulting in a stop codon 74 nt upstream of the last exon-exon junction (*172, 173*). A study using minigene constructs containing intron 10 showed that the mutant α1 subunit mRNA was reduced, which could be reversed by silencing the NMD essential factor UPF1, confirming activation of NMD machinery (*173*). Similarly, *GABRG2(Q40X)* (*174*) and *GABRG2(R136X)* (*175*) are two nonsense mutations located in the second exon of the GABRG2 gene and both PTCs activate the NMD machinery.

Although mutant mRNAs harboring PTCs could be degraded, NMD efficiency is not 100% and varies among different cell types (*176, 177*). A certain amount of truncated protein could still be synthesized from the un-degraded mutant mRNA. The mutant protein generated by *GABRA1(975delC, S326fs328X)*, *GABRG2(Q40X)* and *GABRG2(R136X)* mutations were all trapped in the ER (*175, 178*), and the truncated mutant *GABRA1(975delC, S326fs328X)* subunits were shown to be degraded faster through ERAD (*173*).

GABRG2(Q390X), GABRG2(W429X), GABRG2(S443delC): In contrast, mutant mRNA transcripts harboring PTCs in the last exon do not activate the NMD machinery. *GABRG2(Q390X)* is one such mutation. *Q390X* is a nonsense mutation identified in a family with GEFS+ (*179*), and the proband was diagnosed with the severe myoclonic epilepsy in infancy (Dravet syndrome). This mutation results in a PTC in the large M3-M4 cytoplasmic loop of γ2 subunits, generating a truncated peptide lacking the fourth transmembrane domain and the small C-terminal tail. With "heterozygous" coexpression of mutant γ2 subunits with α1, β2, and wildtype γ2 subunits in HEK cells, the mutant γ2 subunits were

retained in the ER and GABA induced currents were substantially reduced. Mutant γ2 subunits also prevented membrane insertion of wildtype α1, β2 and wildtype γ2 subunits through subunit oligomerization and degraded the partnering α1 subunits by ERAD through ubiquitin-proteasome pathway, producing strong dominant negative effects (*180*). The strong dominant negative effects may come from the surprisingly high stability of mutant γ2(Q390X) subunits, which were subjected to inefficient slow degradation. Accumulated in the ER, the mutant subunit protein formed high-molecular-mass protein complexes, activated ER-resident stress markers, and were degraded through both proteasome and lysosome pathways (*181, 182*).

GABRG2(W429X) is another GEFS+-associated nonsense mutation that is similar to *GABRG2(Q390X)* but with smaller dominant negative effects (*182*). *GABRG2(S443delC)* is a GEFS+-associated frameshift mutation caused by a deletion of cytosine in the last exon, generating a new stop codon in the three prime untranslated region (3'-UTR) . Mutant γ2 subunits of higher molecular weight were generated by this frameshift mutation, although to a lesser amount, and were trapped in the ER (*183*). Haplo-insufficiency of γ2 subunits may account for the major effects.

3) Epilepsy-associated noncoding mutations in $GABA_AR$ subunits

GABRB3(-897T/C), GABRA1(K353delins18X), GABRG2(IVS6+2T->G): Although exonic information encoding the protein sequence is critical for gene function, mutations located in the noncoding regions, including promoter, intron, 5' or 3' untranslated regions could also affect its normal behavior. *GABRB3(-897T/C)*, *GABRA1(K353delins18X)*, and *GABRG2(IVS6+2T->G)* are three such epilepsy-associated mutations identified in noncoding regions of $GABA_AR$ subunit genes.

GABRB3(-897T/C) is a SNP enriched in CAE patients. It is located in the promoter region of exon1a of *GABRB3*, encoding for a fetal splice variant of β3 subunits (*184*). Reporter assay indicated that the -897C haplotype reduced the transcription activity compared to the -897T haplotype, possibly by reducing the binding with neuronal specific activators (*185*).

GABRA1(K353delins18X) is an intronic mutation identified in a GEFS+ family. The extra 25 nt in intron 10 of *GABRA1* is located close to the splice branching point of exon 11, causing intron retention, translating parts of the intronic amino acids and generating a PTC in the M3-M4 loop of α1 subunits. When coexpressed with β2 and γ2 subunits, mutant α1 subunits were retained in the ER and totally abolished GABA evoked currents (*158*).

GABRG2(IVS6+2T→G) is an intronic mutation in the splice donor site of intron 6 of *GABRG2* that segregated with CAE and FS in a small pedigree (*186*). A cryptic splice donor site was activated by the mutation resulting in retention of 53 bp of intron 6 were and causing a frame shift in exon 7 that produced a PTC. NMD was activated to reduce the amount of mutant γ2 subunit transcripts. Meanwhile, the truncated mutant protein generated was retained in the ER and increased ER stress (*187*).

4. Future issues in genetic epilepsies

Characterization of monogenic epilepsy-associated mutations has advanced our understanding of genetic epilepsy. However, genetic epilepsy is complicated, and our current knowledge has only disclosed a small tip of the iceberg. How genetic information causes epilepsy in different individuals and how to design related therapies needs further investigation. While I am discussing issues need to be addressed in future in the following paragraphs, I will show how we characterized three different types of epilepsy-associated mutations found in *GABRG2* gene and how we developed corresponding potential treatments using different model systems in the next three chapters.

1) <u>Genetic epilepsies are complicated disorders.</u>

Most epilepsy-associated $GABA_AR$ mutations discussed above come from studies of epilepsy pedigrees and were enriched in genes coding α1, β3, γ2 and δ subunits. Considering the wide expression pattern of these subunits and the epilepsy related phenotypes observed in respective knock-out studies, it is not surprising that these mutations have big contributions to epilepsy syndromes found in patients

carrying these mutations, if they are not the only contributors. However, although pedigree studies are useful and effective in identifying epilepsy-associated mutations, monogenic epilepsy only accounts for a very small proportion of genetic epilepsy, and many patients with genetically complex forms of epilepsy do not have a family history of disease transmission (*188*). Even for people carrying the same monogenic epilepsy mutation, the disease penetrance is not complete at all and patients exhibit quite diverse symptoms with different severity, further indicating the involvement of risk variants in susceptible genes.

Recently a *GABRA6(R46W)* mutation in α6 subunits was identified in one patient and shown to decrease the receptor current amplitude by impairing gating and assembly (*189, 190*). But whether dysfunction of α6 subunits could affect seizure susceptibility is still unknown. A large ion channel gene exon sequencing project also revealed several new $GABA_AR$ subunit gene variants in patients with epilepsy (*191*). Without support from genetic segregation, it is difficult to speculate their roles in epileptogenesis. In addition, several *de novo* mutations in β1 and β3 subunits were also identified from patients with epileptic encephalopathies by whole exome sequencing (*192*). This sporadic genetic information will not be easily revealed without the development of next-generation-sequencing techniques, and further characterization of these *de novo* mutations may give us better perspectives about genetic epilepsy.

Similar to epilepsy-associated mutations in $GABA_ARs$, many mutations have been identified in other genes especially those encoding ion channels through pedigree studies or candidate gene screening in patients and families with epilepsy (*193, 194*). However, a study comparing the exonic information of ion channel genes between epilepsy patients and a control cohort found that it was impossible to predict outcomes based on the burden of mutations in ion channel genes. With the invention and development of next-generation sequencing techniques, more mutations/variants will be discovered, which may change our understanding of epilepsy diagnosis and prevention (*191, 192*). Thus, how interactions among different risk genes and accumulation of epilepsy-associated variants lead to epilepsy, would be an important and interesting topic worthy of investigation.

2) <u>What can be the next generation AEDs?</u>

As mentioned above, current treatment does not prevent the process of epileptogenesis, and around 20% of epilepsy cases are refractory. Thus, treatments targeting the etiology of epilepsy could be beneficial. Epilepsy research in recent decades has greatly advanced our understanding of why the epilepsy happens and how the epilepsy is generated. Novel targeted therapeutic strategies accompanied by technical advancements could potentially prevent disease onset or slow the progression of epilepsy. Although most new findings have been focused on acquired epilepsies caused by focal insult, underlying principles could be applied to treat genetic epilepsies.

One candidate is gene therapy. Gene interventions using DNA or RNA to replace cellular function are defined as gene therapy (*195*), with adeno-associated virus and lentivirus being the most common gene therapy vectors. They are relative safe, easily manipulated and efficient, and thus have been used to treat CNS disorders in rodent models and are being evaluated by on-going clinical trials. Introducing different genetic information to disease onset region could specifically restore the loss of function, correct the dominant-negative effects, or ameliorate cell damage during/after epileptogenesis. For example, viral delivery of neuroprotective factors including FGF-2 and BDNF (*196*), GDNF (*197*), and Nrf2, a transcription factor orchestrating neuroprotective response, all reduced seizures in mice with temporal lobe epilepsy (TLE) (*198*). Infusion of constructs expressing seizure-inhibiting factors, mostly neuropeptides, could inhibit neuronal activities and attenuate activities of induced seizures (*199-204*). Expression or restoration of inhibitory ion channels including Kv1.1 potassium channels (*205*), $GABA_AR$ α1 subunits, and hyperpolarization-activated cyclic nucleotide-gated (HCN) channel (*206*) have been shown to be both antiseizure and antiepileptogenic in several rodent models of induced seizure. The invention of optogenetic tools further facilitated the control of this method. Optogenetic inhibition of a subset of neurons in the epileptic focus could also attenuate toxin-and stroke-induced seizures (*205, 207*). Although no gene therapies have been studied on models of genetic epilepsies, it will not be surprising if

restoration of mutant gene function or restoration of downstream pathways caused by the mutation, could also suppress seizure activities in genetic epilepsies.

Another candidate is interneuron transplantation. GABAergic interneurons are important elements in inhibiting neuronal firing and orchestrating the network activity in the brain. Loss of interneurons (*208*) or interneuron dysfunction (*113, 209*) has been observed in different models of epilepsy, suggesting that it could be beneficial to replace interneurons in epileptic brain. For example, reduced interneuron excitability was reported in *SCN1a* knock-out mice, a model of severe Dravet syndrome. Grafted embryonic medial ganglionic eminence (MGE) cells could integrate into local circuit of postnatal brain, develop into functional GABAergic interneurons and increase inhibitory neurotransmission (*210*). Transplantation of MGE precursors into cortex of neonatal mice devoid of Kv1.1 potassium channels successfully reduced the seizure duration and frequency (*211*). Compared to MGE precursors, stem cell-derived interneurons are more accessible. Mouse embryonic stem cell-derived GABAergic precursors successfully developed into functional GABAergic neurons and integrated into local circuits after transplantation into the dentate gyrus of mice experiencing TLE (*212*). Transplanted neural stem cells were shown to improve the cognitive deficits in kainic-acid induced TLE mice (*213*). In future, with efforts to better enrich GABAergic precursors, induced interneurons could be tested in models of genetic epilepsies.

Besides gene therapy and interneuron transplantation, focal cooling (*214*) and deep brain stimulation (*215*) are also used in epilepsy treatments. Furthermore, strategies used in treatments of other disorders, could be tried in epilepsy, especially genetic epilepsy. Pharmacological chaperones are small chemical molecules that selectively bind with the target protein, stabilize the structure and facilitate folding. Proteostasis chaperones are also small chemical molecules like enzyme inhibitors targeting the pathways of protein processing. Molecular chaperones are protein molecules like heat shock proteins that assist with the biogenesis of target proteins. All of them have been studied in disorders including neurodegenerative diseases, lysosomal storage diseases and cystic fibrosis, to correct misfolded mutant

proteins (*216-219*). PTC readthrough chemicals like aminoglycosides, which could skip the immature stop codon and resynthesize the mature protein, were also used to correct truncation mutation in multiple disease models (*220*). Although not yet studied in epilepsy-associated mutations, these mutation-specific strategies could be promising.

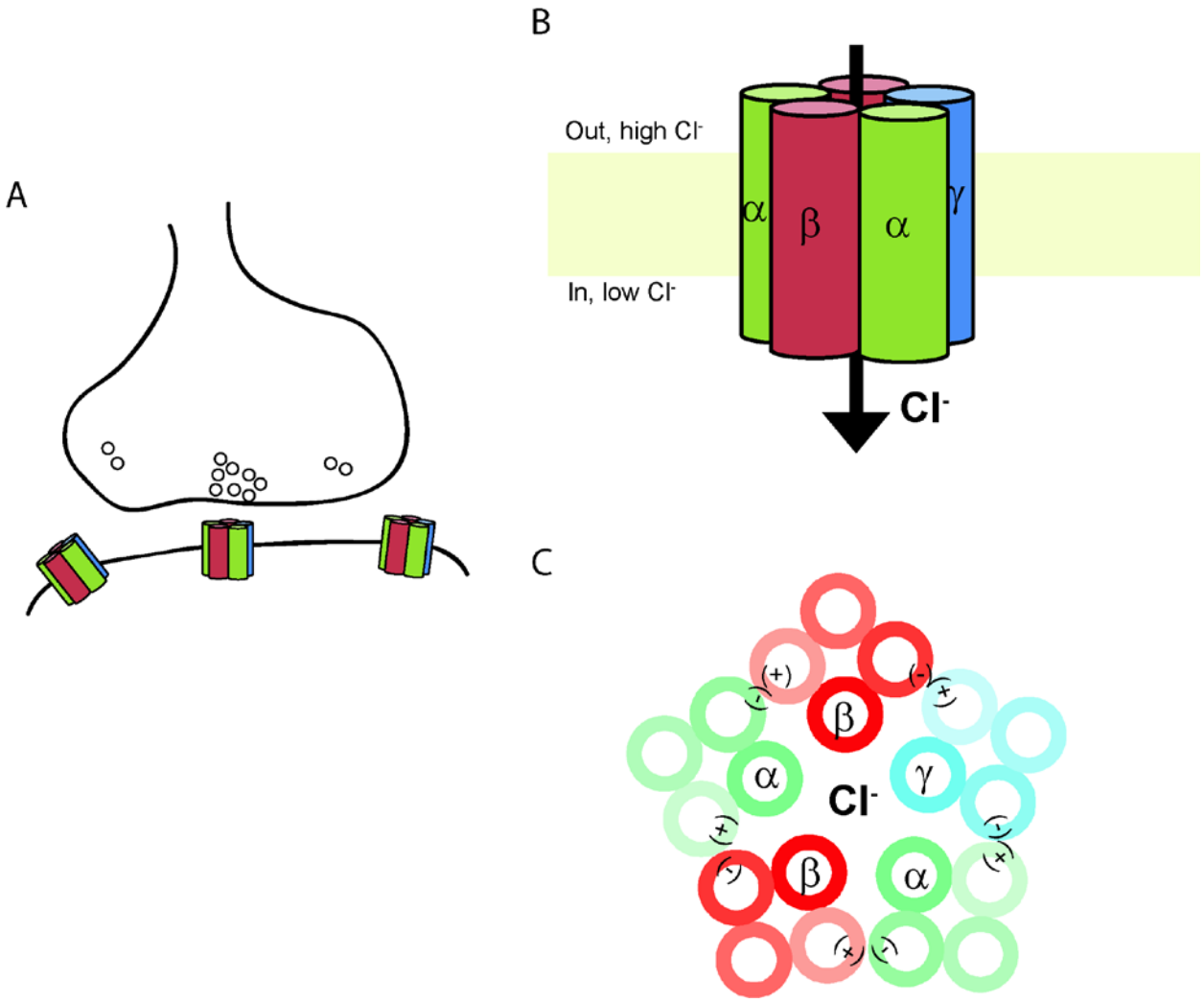

Figure 1-1: Schematics of $GABA_A$ receptors

(A) $GABA_A$ receptors are located in postsynaptic or extrasynaptic membrane. (B) They are pentameric receptors mediating the entry of chloride ions in mature neurons, and the majority of receptors are composed of two α, two β and one γ subunits. (C) Each subunit contains four transmembrane domains, and M2 domains of five subunit line the ion passing pore, forming a pseudo-symmetric receptor in a counter-clock sequence of γ- β- α- β-, if viewed from the synapse cleft. . The extracellular domain of each subunit contains a principle side (+) and a complementary side (-) to contribute to the interface.

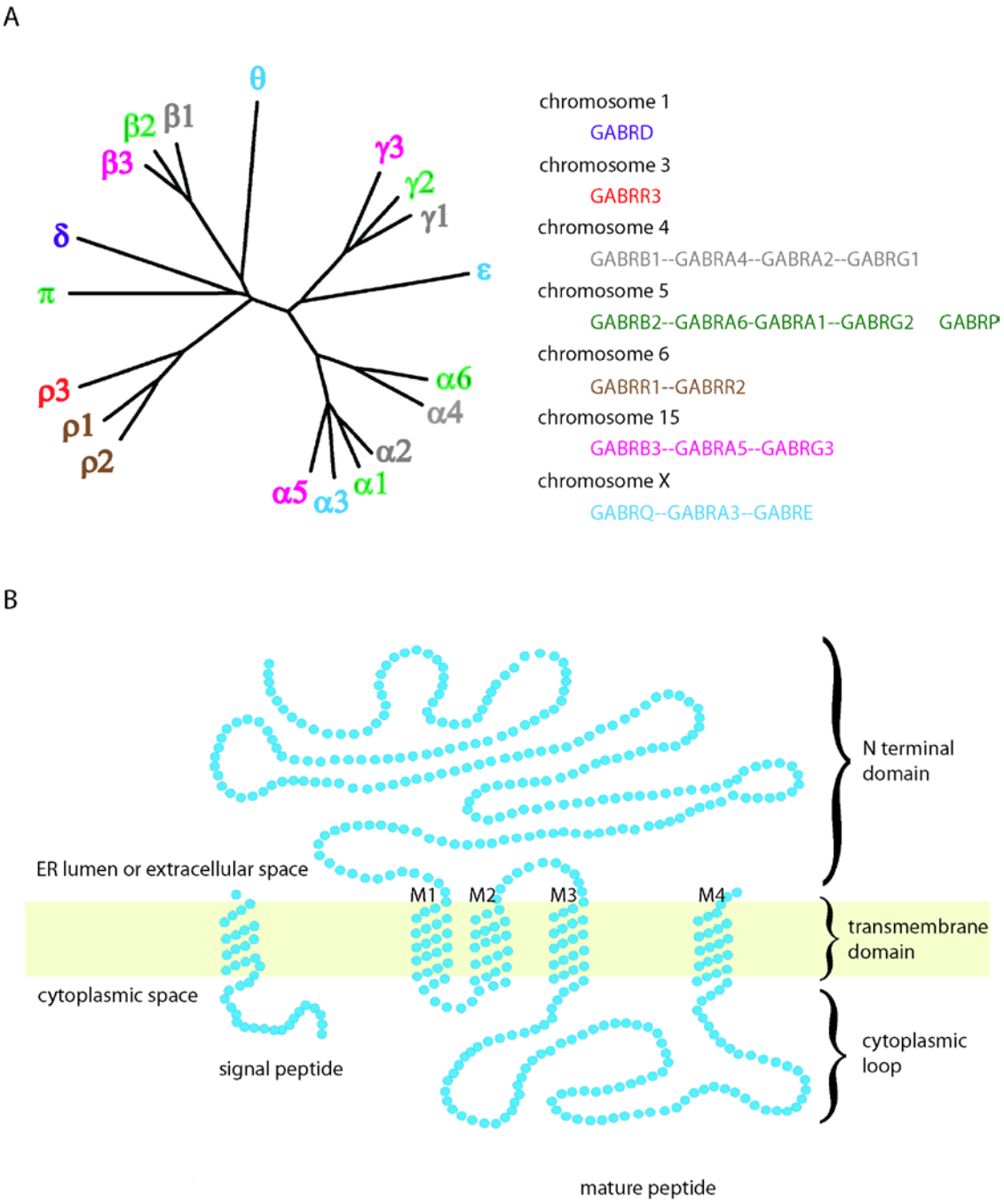

Figure 1-2: $GABA_A$ receptor subunits

(A) Polygenetic tree analysis of 19 human $GABA_AR$ subunits distributed in 7 chromosomes. The dendrogram was generated by Dendroscope based on the alignment generated by ClustalX, using (immature) amino acid sequences obtained from the Uniprot database. (B) Membrane topology of γ2 subunits. Human γ2 subunit of the $GABA_AR$ is composed of 475 amino acids. After the cleavage of the 39 aa long signal peptide, the mature subunits contains one extracellular N terminal domain, followed by four transmembrane domain and a big cytoplasmic M3-M4 domain.

Table 1: Classification of Epilepsy

	Definition(*9*)	Examples of Epilepsy Syndromes
Genetic Epilepsy	"Epilepsy is the direct result of a known or presumed genetic defect(s) in which seizures are the core symptom of the disorder"	Childhood Absence Epilepsy Juvenile Myoclonic Epilepsy Dravet Syndrome Lennox-Gastaut syndrome
Structural/Metabolic Epilepsy	"A distinct structural or metabolic condition or disease has been associated with a substantially increased risk of developing epilepsy in appropriately designed studies"	traumatic brain injury hypoxic-ischemic encephalopathy
Unknown Epilepsy	"The underlying cause is as yet unknown"	

Table 2: Common AEDs

AED	Action Targets	Indications	Side Effects
Benzodiazepines (Diazepam, Clonazepam, Clobazam)	$GABA_AR$ potentiator	Broad use for focal and generalized seizures, including convulsive disorders, Lennox-Gastaut syndrome, and status epilepticus,	Sedative; leads to tolerance
Phenobarbital	$GABA_AR$ potentiator (also work on VDCC, VDPC, and AMPAR)	GTCS, partial seizures and drug-resistant status epilepticus	Skin hypersensitivity
Tiagabine	GAT inhibitor	Partial seizures	
Vigabatrin	irreversible inhibitor of GABA-T	Infantile spasms, complex partial seizures	Vision loss; weight gain
Phenytoin	VDSC blocker	GTCS, partial seizures	Skin hypersensitivity
Carbamazepine	VDSC blocker	GTCS, partial seizures	Skin hypersensitivity
Oxcarbazepine	VDSC blocker	Partial seizures	Skin hypersensitivity
Lamotrigine	VDSC blocker (also an antagonist of AMPAR, also work on VDCC)	GTCS, partial seizures, Lennox-Gastaut syndrome	Skin hypersensitivity
Topiramate	Broad targets including VDSC, VDCC, VDPS, $GABA_AR$, and AMPAR	Broad use for focal and generalized seizures, including GTCS, partial seizures, and Lennox-Gastaut syndrome	somnolence; dizziness; cognitive impairment; speech problems; kidney stones; weight loss
Ethosuximide	T type VDCC blocker	Generalized absence seizure	somnolence; loss of appetite; nausea; vomiting; singultus; depression; psychotic episodes; insomnia; rare aplastic anaemia
Valproate	VDSC blocker T type VDCC blocker Increase GABA by affecting the metabolism	Broad use for focal and generalized seizures, including GTCS, partial seizures and absence seizures	substantial teratogenicity; weight gain
Zonisamide	VDSC blocker T type VDCC blocker	Partial seizure	Sedative
Lacosamide	Enhance slow inactivation of VDSC	partial seizures	
Gabapentin	Not fully understand (may act through GABA synthesis or block VGCC)	GTCS, partial seizure	Weight loss
Levetiracetam	Not fully understand (may act through the synaptic vesicle protein SV2A)	Partial seizures	

(GABA-T: GABA transaminase; GAT: GABA transporter; GTCS: Generalized tonic clonic seizures; VDCC: Voltage dependent calcium channels; VDPC: voltage dependent potassium channels; VDSC: voltage dependent sodium channels) (*16, 17, 221*)

Table 3. Expression pattern in adult rodents and related mouse physiology for major $GABA_AR$ subunits

Subunit Gene	Chromosome (human/mouse)	Expression Pattern	Mouse Physiology
GABRA1 (α1)	**5/11**	Widely distributed in the brain, abundant in cortex, cerebellum and hippocampus	α1 knock-out mice were viable (*58, 59*) , but showed reduced viability and absence-like seizures (*65*); α1(H101R) mice were resistant to the sedative effects of diazepam (*66, 67*)
GABRA2 (α2)	**4/5**	Strong in cortex (out layers), olfactory bulb, hippocampus, amygdala, septum, striatum, accumbens and hypothalamus	α2 knock-out mice exhibited depressive-like activities (*69*); α2(H101R) mice were resistant to the anxiolytic effects of diazepam (*70*)
GABRA3 (α3)	**X/X**	Strong in cortex (inner layers), olfactory bulb, amygdala, reticular nucleus of thalamus	α3 knock-out mice showed remarkably attenuated sensorimotor-gating (*73*)
GABRA4 (α4)	**4/5**	Strong in thalamus, striatum and nucleus accumbens, and the molecular layer of the dentate gyrus, less concentrated in cerebral cortex and CA1, and low or absent in the cerebellum	α4 knock-out mice were more sensitive to pentylenetetrazol-induced seizure (*78*)
GABRA5 (α5)	**15/7**	Strong in hippocampus and weak in most other areas	α5 knock-out mice had better performance in Morris water maze (*83*)
GABRA6 (α6)	**5/11**	Only granule cells in cerebellum	α6 subunit knock-out mice had no overt behavioral deficits (*86*)
GABRB1 (β1)	**4/5**	Weak in cortex and thalamus, but strong in hippocampus	β1(L285R) and β1(P228H) knock-in mice showed increased alcohol assumption (*87*)
GABRB2 (β2)	**5/11**	highly expressed in cortex, thalamus, pallidum and cerebellum	β2 knock-out mice showed higher spontaneous locomotor activities (*58*)
GABRB3 (β3)	**15/7**	Strong in cortex, striatum, hippocampus and cerebellum	β3 knock-out mice showed high mortality rate as neonates, reduced life span, cleft palate, hyperactive and epileptic seizures (*88*)
GABRG2 (γ2)	**5/11**	highly expressed through the brain, but relatively weak in the thalamus	Homozygous γ2 knock-out died within a few days after birth and those survived exhibited severe sensorimotor deficits and died before P18 (*90*); heterozygous γ2 knock-out mice showed elevated anxiety (*92*) and absence seizure (*94*)
GABRD (δ)	**1/4**	cortex, striatum, thalamus, dentate and cerebellar granule cells	δ knock-out mice showed higher mortality, decreased PTZ-induced seizure threshold, and non-responsiveness to neuroactive steroids (*104, 105*)

Table 4. Epilepsy-associated $GABA_AR$ mutations

Subunit Gene	Mutation	Type	Phenotypes	Major molecular deficits	Mechanism
GABRA1	**A322D**	**missense**	**JME**	folding, ERAD	LOF, slight DN effects
GABRA1	**D219N**	**missense**	**GEFS+**	trafficking	LOF
GABRB3	**P11S**	**missense**	**CAE**	trafficking?	LOF
GABRB3	**S15F**	**missense**	**CAE**	trafficking?	LOF
GABRB3	**G32R**	**missense**	**CAE**	trafficking	LOF
GABRG2	**R82Q**	**missense**	**CAE, FS**	trafficking	LOF, slight DN effects
GABRG2	**R177G**	**missense**	**GEFS+**	trafficking	LOF
GABRG2	**K328M**	**missense**	**GEFS+**	channel activity	LOF
GABRD	**E117A**	**missense**	**GEFS+**	channel activity	LOF
GABRD	**R220H**	**missense**	**JME**	channel activity	LOF
GABRA1	**975delC, S326fs328X**	**frame-shift**	**CAE**	NMD, ERAD	LOF
GABRG2	**Q40X**	**truncation**	**DS**	NMD, trafficking	LOF
GABRG2	**R136X**	**truncation**	**GEFS+**	NMD, trafficking	LOF
GABRG2	**Q390X**	**truncation**	**GEFS+**	trafficking, aggregation	LOF, DN effects
GABRG2	**W429X**	**truncation**	**GEFS+**	trafficking	LOF, slight DN effects
GABRG2	**S443delC**	**frame-shift**	**GEFS+**	trafficking	LOF
GABRA1	**K353delins18X**	**intron**	**GEFS+**	trafficking	LOF
GABRB3	**-897T/C**	**promoter**	**CAE**	transcription	LOF
GABRG2	**IVS6+2T->G**	**intron**	**CAE, FS**	NMD, trafficking	LOF

LOF: Loss of function DN: Dominant negative

Chapter 2 : Three Epilepsy-Associated GABRG2 Missense Mutations at the γ+/β- interface Disrupt $GABA_A$ Receptor Assembly and trafficking by Similar Mechanisms but to Different Extents

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1. Abstract

We compared the effects of three missense mutations in the $GABA_A$ receptor γ2 subunit on $GABA_A$ receptor assembly, trafficking and function in HEK293T cells cotransfected with α1, β2, and wildtype or mutant γ2 subunits. The mutations R82Q and P83S were identified in families with GEFS+, and N79S was found in a single patient with generalized tonic-clonic seizures (GTCS). Although all three mutations were located in an N terminal loop that contributes to the γ+/β- subunit-subunit interface, we found that each mutation impaired $GABA_A$ receptor assembly to a different extent. The γ2(R82Q) and γ2(P83S) subunits had reduced α1β2γ2 receptor surface expression due to impaired assembly into pentamers, ER retention and degradation. In contrast, γ2(N79S) subunits were efficiently assembled into $GABA_A$ receptors with only minimally altered receptor trafficking, suggesting that N79S was a rare or susceptibility variant rather than an epilepsy mutation. Increased structural variability at assembly motifs was predicted by R82Q and P83S, but not N79S, substitution, suggesting that R82Q and P83S substitutions were less tolerated. Membrane proteins with missense mutations that impair folding and assembly often can be "rescued" by decreased temperatures. We coexpressed wildtype or mutant γ2 subunits with α1 and β2 subunits and found increased surface and total levels of both wildtype and mutant γ2 subunits after decreasing the incubation temperature to 30 ^{o}C for 24 hours, suggesting that lower temperatures increased $GABA_A$ receptor stability. Thus epilepsy-associated mutations N79S, R82Q and P83S disrupted $GABA_A$ receptor assembly to different extents, an effect that could be potentially rescued by facilitating protein folding and assembly.

Key words: $GABA_A$ receptors, genetic generalized epilepsy, *GABRG2(N79S)* mutation, *GABRG2(R82Q)* mutation, *GABRG2(P83S)* mutation, loss of function, dominant negative effects, subunit interface, impaired receptor assembly.

2. Introduction

Epilepsy is a common neurological disorder that affects about 1% of the world's population (*223*), and genetic epilepsy (GE) syndromes comprise ~30% of all cases (*224, 225*). Many epilepsy-mutations in affected individuals in families with GEs have been found in ion channels, including $GABA_A$Rs, which are heteropentameric chloride ion channels that mediate the majority of inhibitory neurotransmission in the CNS. The receptor is composed of five subunits, and the predominant synaptic receptors are composed of two α subunits, two β subunits and one γ2 subunit. The most common epilepsy-associated $GABA_A$ receptor gene (*GABR)* is *GABRG2*, and epilepsy mutations in γ2 subunits have been shown to decrease receptor function by altering receptor biogenesis or channel function (*114*). Three *GABRG2* mutations R82Q, P83S and N79S (numbered based on the immature γ2 subunit containing the signal peptide) were reported to be associated with generalized epilepsies and are all located in the same structural loop in the N terminus of γ2 subunits, suggesting that they might impair $GABA_A$ receptor function similarly.

R82Q is one of the best characterized epilepsy-associated *GABRG2* mutations. It was originally found in a large family with GEFS+ (*123, 149*), contributing to childhood absence epilepsy and febrile seizures. A single nucleotide substitution caused a highly conserved arginine residue located within a loop between the α-helix and the β1-sheet (the α-β1 loop) in the extracellular N terminus to be replaced by a glutamine (Figure 2-1 A), resulting in impaired surface expression of γ2 subunits and decreased $GABA_A$R currents (*141-146*). Heterozygous knock-in mice carrying this mutation displayed spontaneous spike-wave discharges and thermal-induced seizures (*150, 153*), consistent with R82Q being an epilepsy-causing mutation. However, whether this mutation has dominant negative effects on other $GABA_A$R

subunits and how it affects subunit-subunit interactions is still controversial (*143, 146*). A recent study showed that while loss of γ2 subunit function could account for the absence seizure phenotype, the R82Q mutation might be responsible for the febrile seizure phenotype (*153*), further suggesting that the R82Q mutation had effects in addition to haploinsufficiency.

Recently, another epilepsy-associated *GABRG2* mutation, P83S, which is also located within the α-β1 loop of the γ2 subunit, was identified in a three generation GEFS+ family (*158*). Although this mutation was found in all affected individuals in this family and was predicted to have damaging effects, it was reported that $GABA_AR$ channel function was not affected by the mutation, and the effects on receptor trafficking were not addressed. How this mutation contributes to epileptogenesis is therefore still uncertain.

Finally, it was reported that a *GABRG2* mutation, N79S, also located in the α-β1 loop of the γ2 subunit, was found in a single patient with generalized tonic-clonic seizures (GTCS) (*226*). The mutation was reported to only modify the steepness of the GABA concentration-response curve (*178*).

All three mutations are located in the N terminal domain of γ2 subunits that forms part of the γ2+/β2- subunit interface (Figure 2-1 B), suggesting that they may produce similar impairments of subunit oligomerization and receptor assembly (*143*). In the present study, we compared the effects of these three epilepsy-associated *GABRG2* mutations on surface expression and function of α1β2γ2 receptors in transfected HEK293T cells and rat cortical neurons and found that they impaired assembly and trafficking of $GABA_A$ receptors by similar mechanisms but to different extents.

3. Materials and Methods

Expression vectors

The coding sequences of human α1, β2 and γ2 $GABA_AR$ subunits were cloned into pcDNA3.1 expression vectors (Invitrogen). All subunit residues were numbered based on the immature peptide. Mutant γ2 subunit constructs were generated using the QuikChange site-directed mutagenesis kit

(Stratagene). An HA or FLAG epitope was inserted at a functionally silent site (between the 4th and 5th residue of the mature peptide) to facilitate our experiments (*137*). Both γ2S and γ2L subunits (*227*), two different splice isoforms, were used. For neuronal transfections, wildtype and mutant γ2L subunits were cloned into pLVX-IRES-ZsGreen vectors (Clontech).

Cell culture and transfection

Human embryonic kidney cells (HEK293T) (ATCC, CRL-11268) were incubated at 37°C in humidified 5% CO_2 incubator and maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with fetal bovine serum (10%, Life technologies), and penicillin/ streptomycin (100 IU/ml, Life technologies). Cells were transfected using the FuGENE 6 transfection reagent (Roche Applied Science) or polyethylenimine (PEI) reagent (40 kD, Polysciences) and harvested 36 hours after transfection. To express wildtype and mutant α1β2γ2 receptors, a total of 3 μg of subunit cDNAs were transfected at a ratio of 1:1:1 into 6 cm dishes for most experiments except for whole cell recording. In experiments studying the effects of low temperature, cells were incubated at 30°C for 24 hours beginning about 16 hours after transfection.

Rat cortical neurons were obtained from E18 embryos as previously described (*187*), incubated at 37°C in 5% CO_2 incubator, and maintained in serum-free Neurobasal medium (Gibco) supplemented with B27 supplement (Gibco), glutamine (Gibco) and penicillin/streptomycin (Gibco, 20 U/ml). Cultured neurons were transfected at DIV5 using Lipofectamine 2000 (Invitrogen). One hour after transfection, culture medium containing DNA/Lipofectamine complex was replaced by fresh medium.

Western Blot, endoglycosidase H (Endo H) digestion, surface biotinylation and immunoprecipitation

After sonication, whole cell lysates of transfected HEK293T cells were collected in modified RIPA buffer (50 mM Tris (pH = 7.4), 150 mM NaCl, 1% NP-40, 0.2% sodium deoxycholate, 1 mM EDTA) and 1% protease inhibitor mixture (Sigma). Collected samples were subjected to gel electrophoresis using NuPAGE® (Invitrogen) 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. Polyclonal anti-γ2 antibodies (Sysy or Millipore) were used to detect $GABA_A$ receptor γ2 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 a 1:10,000 dilution, and the signals were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences). The integrated intensity value of each specific band was calculated using the Odyssey 3.0 software (LI-COR Biosciences).

To remove immature N-linked glycans, cell lysates were incubated with the enzyme Endo H (NEBiolab) at 37°C for 3 hours. Treated samples were then subjected to SDS-PAGE and Western blot.

Surface proteins were collected using surface biotinylation as described before (*228*). 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 and cleaved by sampling buffer (Invitrogen) containing 10% beta-mercaptoethanol.

Protein complexes containing FLAG-tagged $GABA_A$ receptor subunits were extracted in modified RIPA buffer with reduced amounts of detergents (50 mM Tris (pH=7.4), 150 mM NaCl, 1% Triton) and immunoprecipitated using EZview Red Anti-FLAG M2 affinity gel (Sigma) at 4°C overnight, then eluted with 3X FLAG peptide (Sigma).

Immunocytochemistry and confocal microscopy

Cultured cortical neurons were fixed by 4% paraformaldehyde/4% glucose in PBS for 15 min followed by 1h block with 10% BSA in PBS and were supplemented with 0.2% Triton for total staining. Coverslips were then incubated in mouse monoclonal anti-HA antibody (Covance) for 2 h, followed by incubation with Alexa 647-conjugated donkey anti-mouse IgG antibodies.

Confocal images were obtained using a Zeiss LSM 510 META inverted confocal microscope. Images were taken with 8 bit, 512×512 pixel resolution, and an average of four scans was taken to

decrease the background noise. Pinholes were adjusted so that the sample thickness was smaller than 2 μm. Confocal experiments were performed in part through the use of the VUMC Cell Imaging Shared Resource.

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 (*229*). 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), and 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 mean fluorescence value of each experimental condition was subtracted by that of mock-transfected condition and was then normalized to that of the control condition. Flow Cytometry experiments were performed in the VMC Flow Cytometry Shared Resource.

Whole cell voltage-clamp recordings

Whole cell voltage-clamp recordings were performed at room temperature on lifted HEK293T cells 36-48 hrs after transfection with $GABA_A$ receptor subunits as described previously (*230*). 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) using a P2000 laser electrode puller (Sutter Instruments), fire-polished with a microforge (Narishige), 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 – 1.6 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). A rapid exchange system (open tip exchange times ~ 400

μs), composed of a four-barrel square pipette attached to a Warner SF-77B Perfusion Fast-Step (Warner Instruments Corporation) and controlled by Clampex 9.0 software (Axon Instruments) was used to apply GABA to lifted whole cells. The channels were activated by 1 mM GABA for 4 s, followed by an extensive wash for 40 s, and then blocked by 10 μM 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 suite.

Structural modeling and simulation

Three-dimensional models of human $GABA_AR$ subunits were generated using the crystal structure of the *C. elegans* glutamate-gated chloride channel (GluCl) (*231*) as a template (PDB: 3rhw) using DeepView/Swiss-PdbViewer 4.02 (*232*). The initial sequence alignments between human $GABA_A$ receptor subunits and *C. elegans* GluCl subunits were generated with full-length multiple alignments using ClustalW. Then full-length multiple alignments were submitted for automated comparative protein modeling implemented in the program suite incorporated in SWISS-MODEL (http://swissmodel.expasy.org/SWISS-MODEL.html) using human $GABA_A$ receptors sequences as target proteins and the *C. elegans* GluCl sequence as a template structure. To generate pentameric $GABA_A$ receptor homology models, α, β, and γ subunit structural models were assembled in a counter-clockwise β-α-β-α-γ order by superposition onto the *C. elegans* GluCl channel as a template. The resulting models were subsequently energy-optimized using GROMOS96 in default settings within the Swiss-PdbViewer. Side-chain prediction and conformational backbone variability of γ2 subunit mutation were implemented using Rosetta backrub flexible backbone design (*233*) in the program suite incorporated in RosettaBackrub (https://kortemmelab.ucsf.edu/backrub/cgi-bin/rosettaweb.py). Structural models of the best-scoring low-energy backrub structures of wildtype and mutant γ2 subunits were represented.

Data analysis

Numerical data were reported as mean ± S.E. Statistical analysis was performed using GraphPad Prism. Statistically significant differences were taken as $p < 0.05$ using one way ANOVA followed by Dunnet's multiple comparison or by Student's t test.

4. Results

1) The three interface-located γ2 subunit impaired surface levels of α1β2γ2 receptors but to different extents.

The mutations N79S, R82Q, and P83S were located close to each other in the N terminus of γ2 subunits (Figure 2-1A). By comparing sequences of this region we found that the R82 and P83 residues were identical among different $GABA_A$ receptor subunits and other cys-loop receptor subunits (Figure 2-1A, dark grey bars), while the N79 residue was not conserved among the cys-loop receptor subunit families (i.e., acetylcholine receptor α subunit (ACHA), serotonin 3A receptor ($5HT3_A$) subunit and Avermectin-sensitive glutamate-gated chloride channel α subunit (G5EBR3CA)).

We also built three-dimensional pentameric $GABA_A$ receptor homology models based on the crystal structure of the *C. elegans* GluCl channel (Figure 2-1B). We found that this cluster of γ2 subunit mutations was located in the loop between the α-helix and the β1-sheet (the α-β1 loop, purple) at the top of the N-terminal extracellular domain that contributes to the γ+/β- subunit interface in assembled receptors (interface contributed by the principle side of γ subunits and the complementary side of β subunits, as shown in Figure 1-1). The R82 residue (orange) was closer than the P83 residue (green) to the complementary (-) face of β2 subunits, while the N79 residue (light blue) was located behind the interface. Of note it has been shown that the γ2 subunit mutation R82Q disrupts salt bridges at the γ+/β- subunit interface (*146*). Furthermore, *in silico* analysis using Polyphen-2 (*234*) and SIFT (*235*), software programs that predict whether or not protein structure would tolerate mutations based on sequence conservation and local structural features, predicted that that both R82Q and P83S substitutions would not be tolerated and might damage protein structure, while the N79S substitution would be tolerated.

To examine the effects of these three mutations on surface expression of receptors, we cotransfected HEK293T cells with α1, β2, and wildtype or mutant $\gamma2L^{HA}$ or $\gamma2S^{HA}$ subunits at a 1:1:1 α1:β2:γ2 subunit ratio and evaluated surface and total levels of wildtype and mutant $\gamma2^{HA}$ subunits by flow cytometry. For $\gamma2L^{HA}$ subunits containing the R82Q or P83S mutation, we found substantial reductions of surface $\gamma2L^{HA}$ subunit levels and small reductions of total $\gamma2L^{HA}$ subunit levels (Figure 2-2 A top). For coexpressed $\alpha1\beta2\gamma2L(R82Q)^{HA}$ or $\alpha1\beta2\gamma2L(P83S)^{HA}$ subunits, surface HA levels were decreased to 0.14 ± 0.01 ($p < 0.001$, $n = 12$) and 0.26 ± 0.02 ($p < 0.001$, $n = 13$), respectively, relative to that of coexpressed $\alpha1\beta2\gamma2L^{HA}$ subunits (1.00, $n = 17$), while the surface HA level of coexpressed $\alpha1\beta2\gamma2L(N79S)^{HA}$ subunits was not significantly lower (0.92 ± 0.05 , $p > 0.05$, $n = 13$). With coexpression of $\alpha1\beta2\gamma2L(R82Q)^{HA}$ and $\alpha1\beta2\gamma2L(P83S)^{HA}$ subunits, total HA levels were slightly but significantly decreased to 0.79 ± 0.04 ($p < 0.001$, $n = 11$) and 0.84 ± 0.06 ($p < 0.01$, $n = 12$), respectively, relative to that of coexpressed $\alpha1\beta2\gamma2L^{HA}$ subunits (1.00, $n = 16$), while the total HA level of coexpressed $\alpha1\beta2\gamma2L(N79S)^{HA}$ was not affected significantly (0.96 ± 0.05, $p > 0.05$, $n = 12$).

We found similar results with mutant γ2S subunits (Figure 2-2A bottom). For coexpressed $\alpha1\beta2\gamma2S(N79S)^{HA}$, $\alpha1\beta2\gamma2S(R82Q)^{HA}$ and $\alpha1\beta2\gamma2S(P83S)^{HA}$ subunits, surface HA levels were decreased to 0.88 ± 0.01 ($p < 0.001$, $n = 5$), 0.10 ±0.01 ($p < 0.001$, $n = 9$) and 0.11 ± 0.01 ($p < 0.001$, $n = 10$), respectively, compared to that for coexpressed $\alpha1\beta2\gamma2S^{HA}$ subunits (1.00, $n = 11$). Total HA levels of coexpressed $\alpha1\beta2\gamma2S(R82Q)^{HA}$ and $\alpha1\beta2\gamma2S(P83S)^{HA}$ subunits were decreased to 0.76 ± 0.07 ($p < 0.05$, $n = 8$) and 0.68 ± 0.06 ($p < 0.005$, $n = 11$), respectively, relative to that for coexpressed $\alpha1\beta2\gamma2S^{HA}$ subunits (1.00, $n = 11$). The total HA level of coexpression of $\alpha1\beta2\gamma2S(N79S)^{HA}$ subunits was not significantly different (0.92 ± 0.15, $p > 0.05$, $n = 5$).

To control for any artifact produced by the HA epitope-tag, we also coexpressed untagged $\alpha1\beta2\gamma2L$ subunits and examined surface γ2L levels by surface biotinylation (Figure 2-2 B). Similar to the flow cytometry results, compared to wildtype γ2L subunits (1.00, $n = 6$) we found that surface levels of

γ2L(R82Q) and γ2L(P83S) subunits were reduced to 0.17 ± 0.06 ($p < 0.001$, $n = 5$) and 0.24 ± 0.05 ($p < 0.001$, $n = 4$), respectively, and that the surface level of γ2L(N79S) subunits was reduced slightly but significantly to 0.78 ± 0.08 ($p < 0.05$, $n = 5$). Total expression of γ2L(R82Q) and γ2L(P83S) subunits was also decreased to 0.60 ± 0.08 ($p < 0.01$, $n = 8$,) and 0.56 ± 0.10 ($n = 6$, $p < 0.01$), respectively, but total expression of γ2L(N79S) subunits was not reduced significantly (0.86 ± 0.10, $p > 0.05$, $n = 8$).

These results suggested that R82Q, P83S and N79S substitutions were all in the same loop structure contributing to the γ2+/β2- subunit interface and all affected surface levels of receptors, but to different extents. The R82Q and P83S substitutions likely disrupted subunit oligomerization or receptor assembly more severely than the N79S substitution, thus producing a much more substantial decrease of surface γ2 subunits.

2) In neurons, the R82Q and P83S mutations impaired surface trafficking of γ2 subunits, but the N79S mutation had minimal if any effect.

To study the expression pattern of mutant subunits in neurons, we transfected wildtype or mutant $\gamma2L^{HA}$ subunits into rat cortical neurons and labeled the transfected $\gamma2L^{HA}$ subunits using anti-HA antibody (Figure 2-2C). Wildtype and mutant $\gamma2L^{HA}$ subunits were cloned into pLVX-IRES-ZsGreen vectors, and the ZsGreen signal was used to identify transfected neurons. Without cell permeabilization, the surface expression and localization of γ2L subunits could be visualized. For $\gamma2L^{HA}$ and $\gamma2L(N79S)^{HA}$ subunits, the HA signal could be seen outlining the soma and dendrites of ZsGreen positive cells (Figure 2-2 C, left top). In contrast, the surface HA signals for $\gamma2L(R82Q)^{HA}$ and $\gamma2L(P83S)^{HA}$ subunits were almost absent (Figure 2-2 C, left bottom). With cell permeabilization, however, wildtype and all three mutant $\gamma2L^{HA}$ subunits were well detected in both soma and dendrites (Figure 2-2 C, right). Thus, similar to HEK293T cells, $\gamma2L(R82Q)^{HA}$ and $\gamma2L(P83S)^{HA}$ subunit levels were reduced on the cell surface of neurons, but $\gamma2L(N79S)^{HA}$ subunits had surface levels similar to those obtained for wildtype $\gamma2L^{HA}$

subunits. While there was no apparent reduction of surface γ2L(N79S)^{HA} subunits in neurons, this is only a qualitative method that is not suitable for detecting small changes, and thus, we cannot exclude small effects of the N79S mutation on γ2L subunit surface level in neurons.

3) Mutant subunits disrupted $GABA_A$ receptor function and/or changed $GABA_A$ receptor composition.

Because all three mutations decreased surface levels of γ2 subunits, although to different extents, we determined how receptor function was affected using whole cell patch clamp recordings. Wildtype or mutant γ2L subunits were coexpressed with α1 and β2 subunits in HEK293T cells at a 1:1:0.1 α1:β2:γ2L subunit ratio, and macroscopic peak currents were evoked by applying a saturating GABA concentration (1 mM) for 4 s using a rapid exchange system (Figure 2-3A, left traces). Current density of receptors containing γ2(N79S) subunits was only slightly, but significantly, reduced (1003 ± 16.53 pA/pF, n = 10, $p < 0.05$) compared with wildtype receptors (1163 ± 50.95 pA/pF, n = 21). Current densities for receptors containing γ2(R82Q) subunits (394.7 ± 35.95 pA/pF, n = 11, $p < 0.001$) or γ2(P83S) subunits (140.4 ± 16.36 pA/pF, n = 10, $p < 0.001$) were substantially decreased compared with wildtype receptors (Figure 2-3B), consistent with the results described above showing that R82Q and P83S mutations reduced the surface levels of γ2L subunits much more extensively than the N79S mutation.

The reduction in current density produced by the mutations suggested that the mutant subunits may not be effectively assembled into receptor pentamers. $GABA_A$ receptors composed of α1 and β2 subunits can form in the absence of γ2 subunits, and it is possible that the currents recorded in the presence of the mutant γ2 subunits were due, at least in part, to surface α1β2 receptors. While α1β2 receptors can form, they have different physiological and pharmacological properties including increased sensitivity to Zn^{2+} inhibition. To evaluate the possibility of α1β2 receptor formation in the presence of mutant γ2 subunits, we determined the Zn^{2+} sensitivity of currents from receptors formed with coexpression of α1, β2, and wildtype or mutant γ2L subunits. Whole-cell currents evoked by co-application of 1 mM GABA with or

without 10 μM Zn^{2+} were recorded (Figure 2-3A, right traces). The fractional Zn^{2+} inhibition of currents evoked from cells coexpressing α1β2γ2(R82Q) or α1β2γ2(P83S) subunits was significantly higher than inhibition of currents from cells coexpressing α1β2γ2 or α1β2γ2(N79S) subunits (WT: 9 ± 1%, n = 16; N79S: 6 ± 2%, n = 10, $p > 0.05$; R82Q: 49 ± 4%, n = 11, $p < 0.001$; P83S: 84 ± 2%, n = 10, $p < 0.001$) (Figure 2-3B). Because the sensitivity of $GABA_A$ receptor currents to Zn^{2+} inhibition depends on subunit composition, these results suggested that mutant γ2(R82Q) and γ2(P83S) subunits were incompletely incorporated into ternary α1β2γ2L receptors, leading to increased expression of Zn^{2+}-sensitive binary α1β2 receptors and decreased expression of relatively Zn^{2+} insensitive α1β2γ2L receptors on the cell surface, thus resulting in decreased $GABA_A$ receptor currents.

In contrast, although the peak current amplitude was slightly and significantly reduced, cells coexpressing α1β2γ2(N79S) subunits displayed currents that were Zn^{2+} insensitive. Peak currents evoked from α1β2γ2L receptors are much larger than those from α1β2 receptors. Because the N79S mutation only slightly reduced peak current amplitude, it is likely that there was only a small reduction of incorporation of γ2(N79S) subunits into ternary α1β2γ2L(N79S) receptors, and thus the dominant Zn^{2+}-insensitive α1β2γ2L(N79S) receptor currents would have masked any small increase of Zn^{2+}-sensitive α1β2 receptor currents.

4) <u>Mutant γ2L(R82Q) and γ2L(P83S) subunits impaired formation of stable trafficking-competent oligomers with partnering subunits and were retained in the ER and degraded.</u>

During biogenesis of ternary $GABA_A$ receptor pentamers, subunit dimers form but are not trafficked to the cell surface (*236*). Further, it has been demonstrated that γ2L subunits alone and βγ2 subunit complexes did not form trafficking-competent receptors and were trapped in the ER (*44, 137*). As all three mutations are located in the α-β1 loop that contributes to the γ2+/β2- subunit-subunit interface, and surface and total expression of γ2 subunits carrying these mutations were decreased to different levels

(Figure 2-2), we studied how receptor biogenesis was affected. To explore how these mutations affected expression/stability of γ2 subunits in HEK293T cells, we expressed wildtype and mutant $\gamma2L^{HA}$ subunits alone, with only β2 subunits or with both α1 and β2 subunits to study their expression as single subunits, dimeric oligomers and ternary receptors (Figure 2-4A). Whole cell lysates were obtained and Western blots were performed to study the expression pattern of γ2L subunits. We blotted for wildtype and mutant $\gamma2L^{HA}$ subunits using anti-HA antibody, quantified the band intensity, normalized it to that of ATPase, and normalized the HA/ATPase ratio to that obtained with expression of wildtype $\alpha1\beta2\gamma2L^{HA}$ subunits. The expression differences between single subunits, dimeric oligomers and ternary receptors were compared for each wildtype or mutant group.

For wildtype γ2L subunits, total level was greatly increased with coexpression with α1 and β2 subunits compared to expression of γ2L subunits alone or with only β2 subunits (Figure 2-4A, lanes 1, 5, 9), suggesting that coexpression of wildtype α1β2γ2L subunits formed stable oligomers while expression of single subunits and coexpression of γ2L and β2 subunits did not form stable oligomers and were degraded ($n = 4$, $p < 0.01$). Similarly, the total level of γ2L(N79S) subunits was also increased when coexpressed with α1 and β2 subunits, compared to expressed alone or with only β2 subunits (Figure 2-4A, lanes 2, 6, 10) ($n = 4$, $p < 0.01$). In contrast, total levels of γ2L(R82Q) (Figure 2-4A, lanes 3, 7, 11) and γ2L(P83S) (Figure 2-4A, lanes 4, 8, 12) subunits were not significantly different when coexpressed with α1 and β2 subunits ($n = 4$, $p > 0.05$). These results suggested that most γ2L(R82Q) and γ2L(P83S) subunits did not form stable oligomers with α1 and β2 subunits and were likely degraded as single subunits or intermediate oligomers.

Interestingly, we found that when coexpressed with α1 and β2 subunits, γ2L and γ2L(N79S) subunits displayed an additional band with increased molecular mass (Figure 2-4A, lanes 9, 10), which was present but relatively weak for coexpressed γ2L(R82Q) and γ2L(P83S) subunits (Figure 2-4A, lane 11, 12: top band, ~47 kD; bottom band, ~42 kD). During protein biogenesis and trafficking, glycans are attached to

nascent peptides in the ER and then subjected to several rounds of processing. To examine whether this shift of molecular mass reflected different glycosylation patterns, we coexpressed α1 and β2 subunits with wildtype or mutant $\gamma 2L^{HA}$ subunits and digested with Endo H, which cleaves only immature glycans added in the ER but not mature glycans added in the Golgi apparatus (Figure 2-4B). After Endo H digestion, we found that the γ2L and γ2L(N79S) subunits were relatively resistant to Endo H digestion, while in contrast, mutant γ2L(R82Q) and γ2L(P83S) subunits showed high sensitivity to Endo H digestion. The lower molecular weight band (grey arrow), which was the dominant expression pattern of γ2L(R82Q) and γ2L(P83S) subunits, was almost gone after Endo H digestion. We compared the proportion of Endo H sensitive bands (bottom bands after digestion) and found that the Endo H sensitive proportions for γ2L(R82Q) (0.80 ± 0.02, $p < 0.001$) and γ2L(P83S) (0.84 ± 0.05, $p < 0.001$), but not γ2L(N79S) (0.30 ± 0.10, $p > 0.05$), subunits were significantly larger than for wildtype γ2L subunits (0.28 ± 0.06, $n = 4$). Consistent with findings mentioned above (Figure 2-4A), the amount and size of undigested mutant γ2L(R82Q) and γ2L(P83S) subunits were different from the undigested wildtype γ2L and γ2L(N79S) subunits. The lower molecular mass and smaller amount of undigested mutant γ2L(R82Q) and γ2L(P83S) subunits indicated they were immature and nonstable. As none of these three residues are located near the predicted glycosylation sites, it is unlikely that the mutation itself affect the glycosylation patterns. Taken together, the mature glycosylation pattern and increased expression levels of γ2L and γ2L(N79S) subunits demonstrated that the majority of them formed stable trafficking-competent receptors with α1 and β2 subunits, which were successfully delivered to the Golgi apparatus and cell surface. In contrast, the immature glycosylation pattern and decreased total levels of γ2L(R82Q) and γ2L(P83S) subunits suggested that although some of them could still form stable trafficking-competent receptors when coexpressed with partnering subunits, most of them did not and were trapped in the ER and degraded like single subunits or dimeric oligomers.

5) γ2L(R82Q) and γ2L(P83S) subunits were incorporated into pentamers inefficiently.

It has been shown that the extracellular N termini of different $GABA_A$ receptor subunits interact during receptor assembly (*236*). We found that the mutations were located near to or contributing to the γ2+/β2- subunit interface (Figure 2-1B, left panel), and thus we wondered whether the assembly of γ2(N79S), γ2(R82Q), and γ2(P83S) subunits into trafficking-competent receptors was interrupted. Previous studies reported that the α-β1 loop structure on the plus interface of γ2 subunits (γ2+) directly interacts with the minus interface of β2 subunits (β2-), and that the R82Q mutation impaired the interaction of γ2 and β2 subunits mediated by this α-β1 loop (*143*). To better understand how these mutations affected the subunit-subunit oligomerization during receptor assembly, we coexpressed α1 and $\beta2^{FLAG}$ subunits with wildtype or mutant $\gamma2L^{HA}$ subunits, pulled down β2 subunits and their subunit binding partners using anti-FLAG beads and blotted associated γ2L subunits using anti-HA antibody (Figure 2-5A). The amount of $\gamma2L(N79S)^{HA}$ subunit associated with $\beta2^{FLAG}$ subunits was not significantly reduced compared to associated wildtype $\gamma2L^{HA}$ subunits (0.94 ± 0.05, $n = 6$, $p > 0.05$) (Figure 2-5B). In contrast, the amounts of γ2L(R82Q) and γ2L(P83S) subunits associated with β2 subunits were substantially reduced, indicating that fewer γ2L subunits were assembled into αβγ pentamers (R82Q: 0.47 ± 0.05, $n = 6$, $p < 0.001$; P83S: 0.71 ± 0.05, $n = 6$, $p < 0.001$, respectively) (Figure 2-5B).

It is possible that the reduced association was caused by the reduced amount of mutant γ2L(R82Q) and γ2L(P83S) subunits. However, we found that while the main bands of γ2L(R82Q) and γ2L(P83S) subunits in the whole cell lysate had lower molecular mass than wildtype γ2L and γ2L(N79S) subunits, consistent with immature glycosylation (Figure 2-4 and 2-5A bottom, Input), most of the γ2L(R82Q) and γ2L(P83S) subunits associated with β2 subunits were of the same higher molecular mass as the wildtype γ2L and γ2L(N79S) subunits (Figure 2-5A top, IP). Taking these results with previous findings (Figure 2-4), we suggest that when forming α1β2γ2 receptors, mutant γ2(R82Q) and γ2(P83S) subunits

inefficiently assembled into pentamers to form mature receptors and unassembled subunits were trapped in the ER and degraded. Nonetheless, a small portion of the mutant γ2L(R82Q) and γ2L(P83S) subunits were still incorporated into stable pentamers that were trafficked beyond the ER and reached the cell surface. Thus, the reduced amount of mutant γ2L(R82Q) and γ2L(P83S) subunits is the result rather than the cause of reduced subunit/subunit interaction. In contrast, the γ2L(N79S) subunits were much more efficiently assembled into receptors and trafficked to the cell surface, consistent with the finding that this mutation only produced a small reduction of surface level (Figures 2-2A, B;2-3A, B).

6) <u>Mutant subunits impaired trafficking of partnering subunits and/or changed receptor composition.</u>

Our results above demonstrated that processing and assembly of αβγ receptors were reduced, but not abolished totally, by the presence of either γ2(R82Q) and γ2(P83S) subunits and to a much lesser extent by the γ2(N79S) subunit. Increased Zn^{2+} sensitivity of receptors containing either γ2(R82Q) and γ2(P83S) subunits also suggested a changed receptor stoichiometry. However, as mutant subunits were still expressed, they could form unstable trafficking-incompetent intermediate oligomers with partnering subunits in the ER, which could impede their assembly and trafficking. To further investigate which type of $GABA_A$ receptors were trafficked to the surface and if mutant γ2L subunits had a dominant negative effect by decreasing the trafficking of partnering α and β subunits, we coexpressed α1 and β2 subunits with wildtype or mutant $\gamma2L^{HA}$ subunits and evaluated surface and total expression of α1 and β2 subunits by flow cytometry (Figure 2-6).

In the absence of wildtype γ2L subunits, the α1 subunit surface level was slightly increased (1.21 ± 0.05, n = 17, $p < 0.001$), and the β2 subunit surface level was greatly increased (2.82 ± 0.21, n = 14, $p < 0.001$) relative to their surface levels in the presence of γ2L subunits (Figure 2-6A), compatible with a change of receptor stoichiometry from 2α2β1γ to 2α3β (*46, 237*). Surface α1 subunit levels were slightly, but not significantly, reduced with coexpression of α1 and β2 subunits with either γ2L(N79S) (0.92 ±

0.05, n =13, $p > 0.05$) or γ2L(R82Q) (0.92 ± 0.04, n = 11, $p > 0.05$) subunits and only slightly, but significantly, reduced with γ2L(P83S) subunits (0.85 ± 0.03, n = 12, $p < 0.05$) (Figure 2-6A). Surface β2 levels were not increased with coexpression of γ2L(N79S) (0.86 ± 0.07, n = 11, $p > 0.05$). Surface β2 levels were significantly increased with coexpression of γ2L(R82Q) subunits (1.52 ± 0.13, n = 9, $p < 0.05$) and shoed a trend of increase with coexpression of γ2L(P83S) subunits although the trend is not significant (1.38 ± 0.07, n = 11, $p > 0.05$) (Figure 2-6A). Total levels of α1 and β2 subunits also exhibited similar trends (Figure 2-6A). The changed surface receptor stoichiometry in the presence of R82Q or P83S mutations suggested increased expression of surface α1β2 receptors, consistent with the increased Zn^{2+} sensitivity of GABA evoked currents (Figure 2-3), but the increase was lower than that obtained with total removal of the γ2L subunit (α1β2 subunit coexpression condition). The slight reduction rather than an increase of surface α1 subunits as well as the small increase of surface β2 subunits when coexpressed with mutant γ2L(R82Q) or γ2L(P83S) subunits indicated these mutant subunits might have dominant negative effects to suppress assembly of α1 and β2 subunits.

In contrast to γ2L subunits, γ2S subunits can be trafficked to the surface by themselves (*37*). Thus, an excess of γ2S subunits might impede trafficking of partnering subunits less than γ2L subunits did. To identify any dominant negative effects of mutant γ2S subunits, we determined surface and total levels of α1 and β2 subunits coexpressed with wildtype or mutant $\gamma2S^{HA}$ subunits. In the absence of γ2S subunits, the α1 subunit surface level was not changed (0.92 ± 0.05, n = 11, $p > 0.05$), but the β2 subunit surface level was increased (2.09 ± 0.17, n = 11, $p < 0.001$), compared to surface levels in the presence of wildtype γ2S subunits, also suggesting assembly of αβ receptors (Figure 2-6B). Interestingly, in the presence of the mutations, α1 subunit surface levels were all significantly decreased (N79S, 0.76 ± 0.06, n = 5, $p < 0.001$; R82Q, 0.54 ± 0.02, n = 8, $p < 0.001$; P83S, 0.57 ± 0.03, n =11, $p < 0.001$) compared to wildtype receptors, while β2 subunit surface levels were not increased (N79S, 0.77 ± 0.03, n = 5, $p > 0.05$; R82Q, 0.94 ± 0.04, n = 8, $p > 0.05$; P83S, 0.88 ± 0.08, n = 11, $p > 0.05$). Total levels of α1 and β2

subunits also exhibited similar trends (Figure 2-6B). The β2 subunits could not be trafficked to the surface without α1 subunits. Because we found a significant decrease of surface α1 subunits without an increase of surface β2 subunits when coexpressing mutant γ2S subunits, it is likely that the mutations caused a decrease of αβγ receptors that was offset by an increase of αβ receptors. Taken together, these results demonstrated that both γ2 subunit R82Q and P83S mutations caused disrupted pentameric receptor processing or trafficking, not only due to inefficient receptor assembly but also due to trapping partnering subunits in the ER hindering their assembly and trafficking; whereas the N79S mutation had similar, but much smaller, effects on receptor assembly and trafficking.

7) <u>Lower temperature increased surface and total levels of wildtype and mutant γ2L subunits</u>

Membrane proteins with missense mutations that impair trafficking have been shown to have their function "rescued" at lower temperatures, presumably due to slowed protein processing that facilitates subunit folding and receptor assembly and/or slowed subunit degradation or receptor internalization (*238-241*). We thus explored the effects of decreased temperature on the total and surface expression of wildtype and mutant receptors. We coexpressed wildtype or mutant $\gamma2L^{HA}$ subunits with α1 and β2 subunits in HEK293T cells and determined total expression of wildtype and mutant $\gamma2L^{HA}$ subunits after incubation at 37°C or 30°C for 24 h by Western blot using anti-HA antibody (Figure 2-7A). We quantified the band intensity, normalized it to that of ATPase, and normalized the HA/ATPase ratio for mutant $\gamma2L^{HA}$ subunits to that obtained with expression of α1, β2 and wildtype $\gamma2L^{HA}$ subunits. The expression difference between 37°C and 30°C was compared for each wildtype or mutant condition. We found substantially increased total wildtype and mutant $\gamma2L^{HA}$ subunit levels when incubated at 30°C for 24 h compared to those at 37°C (WT: (37°C: 1.00, 30°C: 2.08 ± 0.28, $p < 0.05$); N79S: (37°C: 0.91 ± 0.12, 30°C: 1.55 ± 0.17, $p < 0.05$); R82Q: (37°C: 0.37 ± 0.06, 30°C: 0.54 ± 0.08, $p < 0.001$); P83S: (37°C: 0.36 ± 0.06, 30°C: 0.57 ± 0.03, $p < 0.05$); $n = 5$), indicating that the stability of both wildtype and mutant $\gamma2L^{HA}$ subunits was increased at a lower temperature.

We then determined whether surface levels of receptor subunits were also increased by reduced temperature (Figure 2-7B). We coexpressed $\alpha1\beta2\gamma2L^{HA}$ subunits in HEK293T cells and examined the surface levels of α1 subunits and wildtype and mutant $\gamma2L^{HA}$ subunits after a 24 h incubation at 37°C or 30°C by surface biotinylation using anti-α1 and anti-γ2 antibodies. We normalized the surface expression levels at 30°C to those at 37°C. Interestingly, we found a moderate, but not significant, increase of surface α1 subunit levels with all conditions (WT: 1.33 ± 0.25; N79S: 1.63 ± 0.32; R82Q: 1.40 ± 0.24; P83S: 1.36 ± 0.29; $n = 4$, $p > 0.05$), but a significant large increase of surface γ2L subunit levels (WT: 2.16 ± 0.34; N79S: 2.26 ± 0.20; R82Q: 1.86 ± 0.16; P83S: 1.93 ± 0.21; $n = 4$, $p < 0.05$). These results suggest that the biogenesis of wildtype and mutant γ2L subunits was facilitated by lower temperatures. Although the increase of surface α1 subunit levels was not significant, it is possible that at a lower temperature the rate of $\alpha1\beta2\gamma2L$ receptor assembly is relatively slow compared to that of γ2L homopentamer assembly, leading to a large increase of surface γ2L homopentamers and a small increase of surface $\alpha1\beta2\gamma2L$ receptors.

5. Discussion

1) The R82Q and P83S mutations were located in the α-β1 loop at the γ(+)/β2(-) subunit-subunit interface and disrupted receptor assembly and trafficking.

Biogenesis of cys-loop receptors is complex and inefficient (*138*). After the synthesis of single subunits, intermediate dimers form, but only pentamers with correct subunit folding and assembly will pass the ER quality control, be further trafficked to and processed by the Golgi apparatus and then be trafficked to the cell surface (*44, 236*). Inappropriately folded and unassembled subunits are quickly degraded (*138*). Our data suggested that the γ2 subunit mutations R82Q and P83S decreased to similar extents the efficiency of pentamer formation. Mutant γ2 subunits that were not incorporated into pentamers were trapped in the ER and likely degraded. Due to the inefficient assembly of mutant γ2

subunit-containing pentameric receptors, receptors with a different stoichiometry (α1β2 dimeric receptors) were able to be assembled. We previously reported two other mutations within structural loops contributing to interface interactions with similar fates: the β3 subunit mutation G32R located at the γ+/β- subunit interface (*163*), and the γ2 subunit mutation R177G located at the α+/γ- interface (*157*). Both *GABRB3(G32R)* and *GABRG2(R177G)* mutations were shown to decrease surface levels of mutant γ2L subunits and increase surface levels of αβ heteropentamers and/or β3 homopentamers, indicating a common molecular mechanism shared by this group of mutations.

Our findings in this study were contrary to those in a previous study, which reported that receptor function as well as Zn^{2+} sensitivity of $GABA_A$ receptors containing mutant γ2(P83S) subunits were normal (*158*). This conflict may have been due to the different ratios of subunit cDNAs used for transfection. In contrast to a 1:1:2 α1:β2:γ2 cDNA ratio used in their study, we used a 1:1:0.1 cDNA ratio. Unpublished data from our laboratory has shown that GABA-evoked peak currents in HEK cells transfected with 1:1:0.1 (α1:β2:γ2) ratio were equal to those obtained with a 1:1:1 ratio, although the surface level of γ2 subunits was much lower. Thus, our data indicated that transfection ratios greater than 1:1: 1 are oversaturating for whole cell recordings, which could mask the deficits caused by mutations.

Our study explored how the R82Q and P83S mutations affected receptor biogenesis. We found that the surface level of mutant subunits was greatly decreased (Figure 2-2), while the total level was also slightly, but significantly, decreased (Figure 2-2, 4). This was similar to in the findings with homozygous knock-in mice carrying the R82Q mutation (*150*). While we did not compare the rates of subunit synthesis or degradation, we have previously demonstrated that two other epilepsy-associated $GABA_A$ receptor subunit missense mutations *GABRA1(A322D)* and *GABRG2(Q390X)* altered the degradation rates, but not the synthesis rates, of the subunits (*130, 181*). Recently we found that another subunit-interface-located missense mutation *GABRG2(R177G)* increased the degradation rate of mutant subunits (*159*). Thus, stability rather than synthesis of $GABA_A$ receptor subunits was regulated by the ER quality

control machinery. We also explored how the R82Q and P83S mutations affected assembly of α1, β2 and γ2 subunits into receptors. It has been reported that the γ2 subunit mutation R82Q disrupted the binding of β2 subunits and a GST-fused γ2 subunit peptide N-terminal fragment containing this mutation (*143*). However, another group reported that the mutation did not decrease β3γ2 subunit association (*146*). In the current study, we found a substantial decrease in β2γ2 subunit association when coexpressing α1β2γ2(R82Q) or α1β2γ2(P83S) subunits. Compared to wildtype γ2 subunits, only a small proportion of mutant γ2(R82Q) and γ2(P83S) subunits were assembled into pentamers, trafficked to the Golgi apparatus and inserted into the surface membrane, while the majority of them were trapped in the ER and degraded. Meanwhile, the mutant γ2(R82Q) and γ2(P83S) subunits still formed unstable intermediate oligomers with partnering subunits (data not shown), thus also impeding their assembly and trafficking (Figure 2-6). This is consistent with our structural simulation (discussed below) showing that R82Q and P83S mutations caused substantial structural rearrangements in several distinct domains involved in receptor assembly. The finding that some mutant γ2(R82Q) and γ2(P83S) subunits were still successfully incorporated into pentamers is also in agreement with the finding that small currents with normal kinetic properties were formed with coexpression of α1β2γ2(R82Q) subunits (*141*) and with a recent report showing that some mutant γ2(R82Q) subunits reached the cell surface and triggered endocytosis of the receptor (*148*). The discrepancy with the previous study could have been caused by use of different subtypes of β subunits or by the immunoprecipitation process. In the previous study (*146*), different amounts of wildtype and mutant γ2 subunits were pulled down, while many mutant γ2 subunits that were not incorporated into pentamers were already degraded. In our study, we tried to pull down the same amount of β2 subunits, including γ2 subunits that were both associated and not associated, representing αβγ and αβ pentamers respectively. It is possible that the decreased association was caused by the paucity of mutant γ2 subunits. Nevertheless, in that case, the dominant form of γ2 subunits associated with β2 subunits should be the immature pattern rather than the mature pattern observed (Figure 2-5A). Thus we

believe that the primary defect in receptor biogenesis was during subunit assembly, but we could not exclude some contributions from other steps.

Although also located in the γ2 subunit α-β1 loop, the N79S mutation had only small effects on receptor assembly. Different from R82Q and P83S mutations associated with epilepsy families, the N79S mutation was identified only in one patient (*226*). Similar to a recent finding demonstrating that γ2(N79S) subunits had no effects on $GABA_A$ receptor function except to modify the steepness of the GABA concentration-response curve (*178*), we found that the N79S mutation produced significant but minimal reduction of receptor assembly. We identified a small reduction of surface γ2(N79S) subunits by flow cytometry and surface biotinylation and decreased peak current amplitudes through whole cell recordings in HEK cells. We also found a small reduction of surface α1 subunits, indicating that the N79S mutation also slightly affected the assembly and trafficking of partnering subunits. We did not quantify the fluorescence intensity of immunostaining in neurons because it was not sensitive to small changes. As the defects were minimal and the majority of γ2(N79S) subunits assembled into stable pentamers that were trafficked beyond the ER and expressed on the surface as functional α1β2γ2 receptors, the γ2(N79S) mutation had only small effects on receptor biogenesis. Our model of receptor structure predicted that the N79 residue does not face the γ2+/β2- subunit interface but rather is adjacent to the interface (Figure 2-1B), and thus the N79S mutation may disrupt to a lesser extent the subunit interaction required to form trafficking-competent pentamers. These findings suggest that while *GABRG2(N79S)* decreases receptor surface expression and peak whole cell current amplitude, the magnitudes of effects are small and unlikely to be the major disease-causing factor. Considering that *GABRG2(N79S)* was identified in only one patient without evidence of co-segregation with an epilepsy syndrome or sporadic occurrence and is absent in the NHLBI exome variant server (http://evs.gs.washington.edu/EVS/), we suggest that rather than being an epilepsy associated mutation, *GABRG2(N79S)* might be a relatively benign rare variant or might increase seizure susceptibility and that other unidentified mutations or variants may be responsible

for the GTCSs experienced by the patient. However, for the consistency, we will continue to refer to *GABRG2(N79S)* as a "mutation" in this paper.

2) <u>Structural simulation predicts that mutation-induced changes in protein structure impaired subunit oligomerization.</u>

Not all missense mutations will significantly affect protein structure and function, and a considerable number of mutations are well tolerated for protein folding. Proper folding and trafficking of $GABA_A$ receptors requires specific sequences and structural motifs within subunits that contribute to selective oligomerization among their γ+/β-, β+/α-, and α+/γ- interfaces. For example, it has been reported that γ2 subunit residues 130-143 in between the β2-β3 and β3-β4 loops interacted directly with α1 subunit residues, γ2 subunit residues 122-131 at the beginning of β2-β3 loop interacted with the β3 subunit (*242, 243*) and γ2 subunit residues 106-121 in the β1-β2 loop and β2 sheet were important for formation of the α1+/γ2- subunit interface (*244*) (Figure 2-8C). Many of these sequences lie in homologous regions of α, β, and γ subunits (Figure 2-1B, homologous assembly motifs in α, β, and γ subunits were shown in red, dark blue and yellow loops, respectively). Structural rearrangements of assembly motifs could strongly impair association of partnering subunits, formation of correct subunit/subunit interfaces, oligomerization of pentameric receptors, and receptor trafficking to the cell surface.

We propose that even though the N79S, R82Q and P83S mutations in the γ2 subunit are located at or near the γ2+/β2- subunit/subunit interface in the same general subunit domain, they might have different impacts on receptor structure, and thus impair the assembly and trafficking of partnering subunits quite differently. Using flexible backbone simulations we characterized structural conformational changes (Figure 2-8A) produced by introducing an N79S, R82Q or P83S mutation in γ2 subunits (Figure 2-8, box inserts). We found that all mutations were predicted to cause structural rearrangements to neighboring residues that were within 7Å from their respective mutation site. Greater structural variability was observed by the presence of alternative secondary backbone conformations at specific structural loops

(Figure 2-8A; wildtype in gray, mutation-associated alternative backbones in other colors, and Figure 2-8C), and disordered side-chains of residues surrounding the mutation sites at the α-β1 loop were also observed (Figure 2-8B).

We also compared the mutation-induced structural differences by analyzing the carbon alpha root mean squared deviation (Cα RMSD) from the wildtype structure caused by each substitution (Figure 2-8C). Cα RMSD provides Cα-Cα comparisons between two structurally aligned models; the larger the Cα RMSD, the more the mutant structure deviates from the wildtype structure. The R82Q mutation had a much larger Cα RMSD than P83S and N79S mutations for any structural loop. With a Cα RMSD of 0.73 ± 0.05 Å for residues D65-G102 in the α-helix, α-β1 loop and β1 sheet, the R82Q substitution had a less preserved native conformation than P83S (Cα RMSD of 0.16 ± 0.02 Å) or N79S (Cα RMSD of 0.14 ± 0.02 Å) substitutions. We previously found that this α-β1 loop domain, which participates in formation of the γ+/β- interface (Klausberger et al., 2000), interacts with the β2 subunit N32 glycosylation site impairing receptor assembly and function (*243, 245*), suggesting that the R82Q mutation disrupted primarily the α-β1-loop-mediated γ+/β- interaction.

All three mutations were predicted to cause rearrangements in other domains also (Figure 2-8C). While both R82Q and P83S mutations primarily produced changes among residues at the γ+/β- interface, they also produced rearrangements in the β7-β8 loop and β8 sheet (Cα RMSD of 0.07 ± 0.005 Å, and Cα RMSD of 0.02 ± 0.005 Å, respectively for residues Y213-Y220) and P83S also produced rearrangements in the β3-β4 loop (Cα RMSD of 0.06 ± 0.01 Å for residues V142-P148). Strikingly, only the P83S mutation had side-chain rearrangements among residues found at both α+/γ- and γ+/β- interfaces (Cα RMSD of 0.19 ± 0.02 Å for residues W121-K127 in the β2 sheet and β2-β3 loop) (Figure 2-8A and C). In addition, the P83S mutation had rearrangements of two conserved tryptophan residues (W121 and W146), which are among residues located at homologous assembly domains described as necessary for the formation of the γ2, α1, and β2 subunits interfaces (*243, 244, 246*). On the other hand, despite the fact that the N79S mutation had the largest Cα RMSD at the β3-β4 loop (Cα RMSD of 0.43 ± 0.04 Å for

residues V142-P148), which formed part of an assembly motif between γ2 and α1 subunits (*243, 244*), this structural rearrangement did not seem as important for the stability of the receptor as it was not accompanied by additional structural changes in other loops nor in the core of the subunit. This could be due to differences in side-chain rearrangements between a buried-polar mutation at site 83 (P83S) and an exposed-polar mutation at site 79 (N79S), resulting in a somewhat smaller pocket for surrounding interactions and formation of buried polar interactions for P83S. It seems that mutations at positions 82 and 83 were less "tolerated" than at position 79, resulting in R82 and P83 contributing the most to interactions at the γ2 interfaces and the core of the subunit. Overall, both R82Q and P83S mutations caused the most disruptive rearrangements at both γ+/β- and α+/γ- interfaces and were less structurally "tolerated" than N79S. These findings are in agreement with our functional data, which demonstrated that R82Q and P83S mutations were less tolerated with marked impairment of receptor function and assembly, and in contrast, the N79S mutation was located two-three residues away and had minimal effects on $GABA_A$ receptor function and assembly.

3) How do the GABRG2(R82Q) and GABRG2(P83S) mutations contribute to epileptogenesis?

In contrast to the γ2 subunit N79S mutation, the P83S mutation was as detrimental to $GABA_A$ receptor function as the R82Q mutation. Heterozygous knock-in mice carrying the R82Q mutation developed absence and febrile seizures, recapitulating patients phenotype (*150*). Compared to a hypomorphic allele, the R82Q mutation increased seizure susceptibility indicating that it had effects in addition to haploinsufficiency (*156*). A recent study also suggested that γ2 subunits haploinsufficiency could account for genesis of absence seizures in $\gamma2^{R82Q/+}$ knock-in mice but not the increased thermal seizure susceptibility, while the R82Q mutation increased thermal seizure susceptibility, independent of genetic background (*153*).

There is controversy concerning whether mutant γ2(R82Q) subunits have dominant negative effects on partnering α1 and β2 subunits (*143, 145, 146, 155*). Here we determined the effects of mutant γ2

subunits on surface expression of α1 and β2 subunits using flow cytometry, which is more quantifiable and sensitive. Surface α1 levels were significantly reduced by all three mutant subunits when coexpressed with β2γ2S subunits, but not when coexpressed with β2γ2L subunits. The dominant negative effects may have been caused by the formation of dimers between mutant γ2 subunits and α1 or β2 subunits, preventing formation of αβ receptors and trapping partnering subunits in the ER. It could also have been caused by internalization of receptors containing these mutations (*148*), which would result in subunit degradation through the endosome/lysosome pathway. We did not observe a significant decrease of surface β2 subunits, probably because there were increased αβ receptors on the cell surface, which would increase surface β2 subunit levels. Compared to γ2L subunits, γ2S subunits can be trafficked to the cell surface in the absence of α and β subunits (*37, 227, 247*), probably by forming trafficking-competent homopentamers. With excess γ2 subunits, unassembled γ2L subunits are retained in the ER, while γ2S subunits assemble into pentamers and are trafficked to the cell surface. As unassembled γ2L subunits can still interact with α1 and β2 subunits, excessive wildtype γ2L subunits could produce dominant negative effects on partnering subunits similar to those caused by trafficking-incompetent mutant subunits. This could have contributed to the failure to find significant dominant-negative effects of mutant γ2L subunits.

In summary, while R82Q and P83S mutations decreased surface α1β2γ2 receptors and increased surface α1β2 receptors, they also decreased the amount of surface receptors through slight dominant negative effects, both of which would be expected to contribute to the epileptogenesis.

4) <u>Implications for future treatments</u>

Trafficking-deficient mutant proteins have been shown to be rescued by lower temperature and molecular or pharmacological chaperones (*238-241, 248, 249*). Here we found that lower incubation temperature (30°C) increased surface and total levels of wildtype and mutant γ2L subunits. Interestingly,

although the surface α1 subunit levels showed a trend of increase, they were insignificantly. This demonstrated that the biogenesis of $GABA_A$ receptor subunits was complex and inefficient. Many misfolded or unassembled subunits were degraded, which were rescued by lower temperature. However, the assembly of $GABA_A$ receptor subunits was still slow at 30°C. Trafficking of mutant γ2L(R82Q) and γ2L(P83S) subunits was not further improved further compared to wildtype γ2L subunits, and the dramatic increase of surface γ2L subunits could be partially caused by increased γ2L homopentamers formed at the low temperature .

Compared to temperature-induced rescue, which affects multiple proteins, specific pharmacological chaperones may be favored. It was reported that $GABA_A$ receptor ligands could promote receptor trafficking as ligand chaperones (*250*). However, we did not find significant chaperone effects of either GABA or diazepam on wildtype or mutant receptors (data not shown). With more thorough drug screening, chemicals with specific chaperone effects on $GABA_A$ receptors may be identified and developed for future treatment of GEs.

6. Acknowledgements

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Flow Cytometry experiments were performed in the VMC Flow Cytometry Shared Resource. The VMC Flow Cytometry Shared Resource is supported by the Vanderbilt Ingram Cancer Center (P30 CA68485) and the Vanderbilt Digestive Disease Research Center (DK058404).

This work was supported by NIH R01 NS 33300 to RLM.

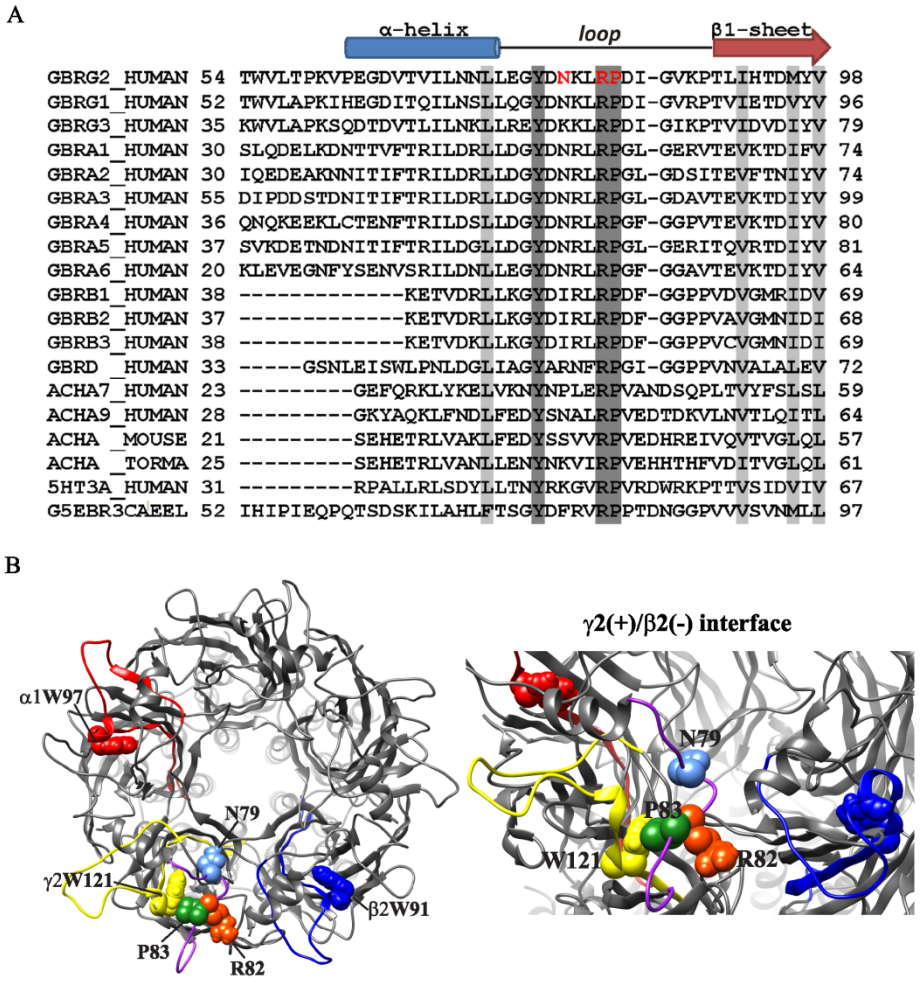

Figure 2-1: Sequence and structural model of N-terminal region containing three mutations

A. Sequences of N-terminal α-helix, α-β1 loop and β1-sheet domains of human α(1-6), β(1-3), γ(1-3) and δ subunits from the $GABA_A$ receptor family were aligned with sequences of the nicotinic acetylcholine receptor α subunit (ACHA(7,9)), 5-hydroxytryptamine 3_A receptor subunit (5HT3A) and glutamate-gated chloride channel GluCl α subunit (G5EBR3). Sites of missense mutations in the γ2 subunit were highlighted in red. In all sequences, identical residues were highlighted in dark gray and conserved residues were highlighted in light gray. The α-helix, α-β1 loop and β1-sheet domains were also represented across subunits above the alignments. **B.** On the left, a structural model of the α1β2γ2 $GABA_A$ receptor, as viewed from the synaptic cleft, was shown. Sites of missense mutations in γ2 subunit, located at the γ2(+)/β2(-) interface, were shown in space-filling representation, i.e., N79 in light blue, R82 in orange, and P83 in green, and the α-β1 loop where these three residues were located was shown in purple. Homologous motifs for α1β2γ2 receptor assembly at the respective complementary (-) interfaces (α1: red; β2: dark blue; γ2: yellow) and conserved tryptophan residues located in these motifs (α1W97, red; β2W91, dark blue; γ2W121, yellow) were also represented. On the right, an enlarged 45 ° side view of the γ+/β- subunit-subunit interface with a close-up of missense mutations in the α-β1 loop was also shown.

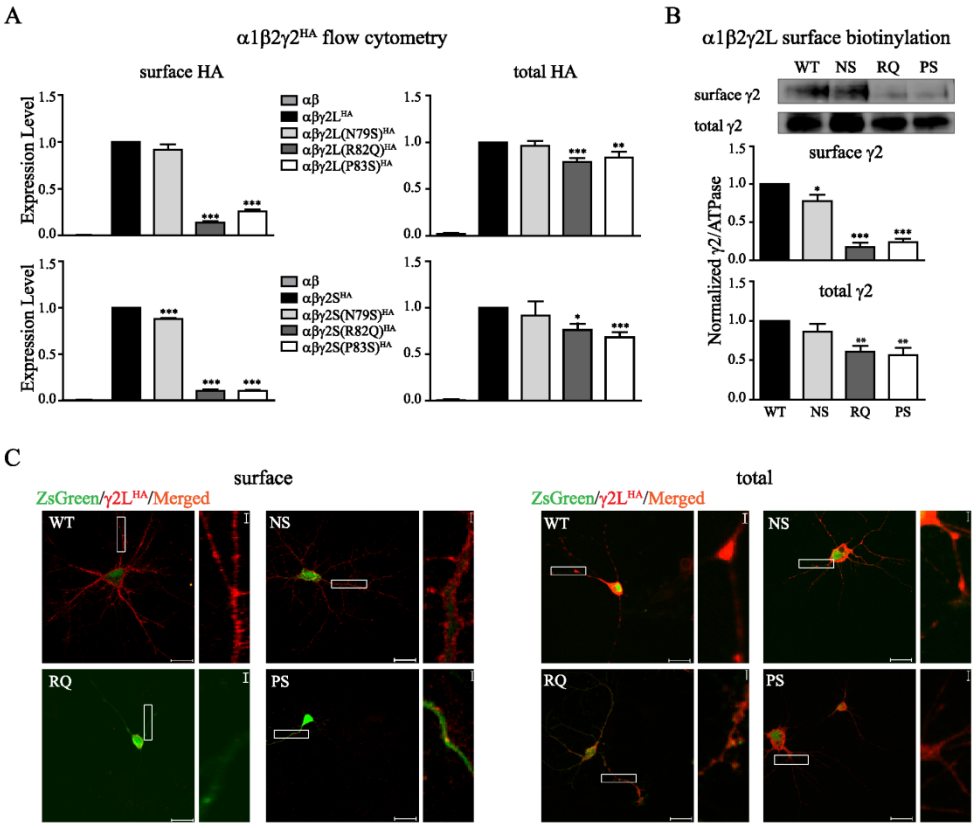

Figure 2-2: Surface expression of mutant γ2 subunits was reduced to different extents

A. α1β2 and $\alpha1\beta2\gamma2^{HA}$ (wildtype or mutant γ2S or γ2L) subunits were coexpressed in HEK293T cells. Surface and total $\gamma2^{HA}$ subunit levels were evaluated through flow cytometry. The mock-subtracted mean fluorescence value of $\gamma2^{HA}$ subunits under different experimental conditions were normalized to those obtained with cotransfection of wildtype $\alpha1\beta2\gamma2^{HA}$ subunits ($n \geq 5$, mean ± SEM). Differences compared to cotransfection of wildtype $\alpha1\beta2\gamma2^{HA}$ subunits were analyzed by the one way ANOVA test followed by Dunnett's multiple comparison test. (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$). **B.** Wildtype or mutant γ2L subunits were coexpressed with α1β2 subunits in HEK293T cells. Surface protein samples were collected through surface biotinylation and blotted by anti-γ2 and anti-ATPase antibodies (not shown). Cell lysates from transfected cells were loaded as the total fraction. Band intensity of the $\gamma2L^{HA}$ subunit was normalized to the ATPase signal ($n \geq 4$, mean ± SEM). Differences compared to cotransfection of wildtype α1β2γ2L subunits were analyzed by the one way ANOVA test followed by Dunnett's multiple comparison test. (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$). **C.** Wildtype or mutant $\gamma2L^{HA}$ subunits were expressed in rat cortical neurons in pLVX-IRES-ZsGreen vectors and stained by anti-HA antibody. Surface (without permeabilization) and total (with permeabilization) staining patterns were revealed by confocal imaging. Scale bar = 20 μm. Inset scale bar = 2 μm.

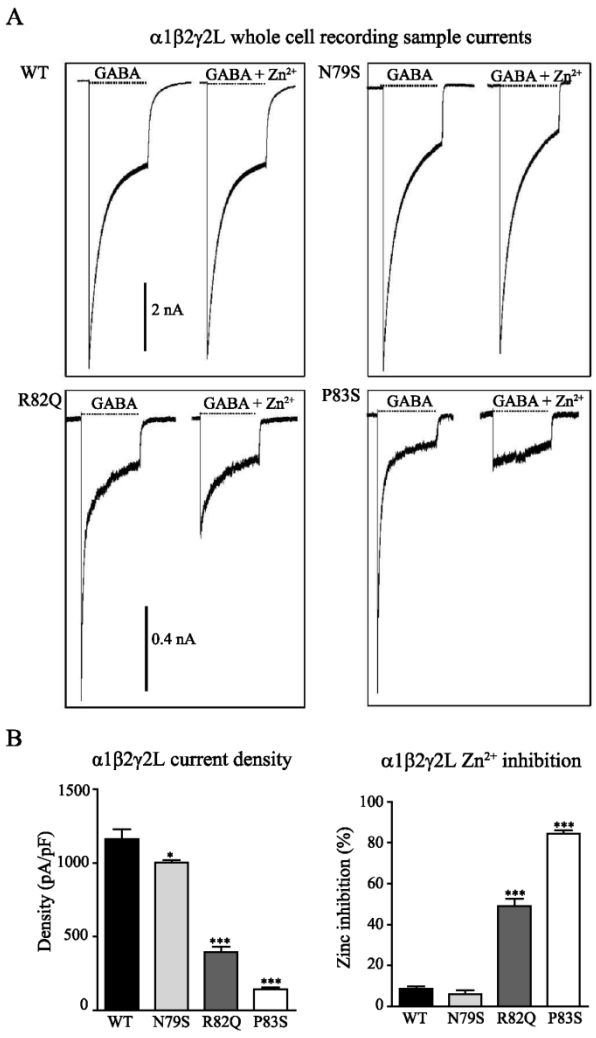

Figure 2-3: Mutant receptors showed decreased whole cell current amplitudes and increased Zn2+sensitivity.

A. Wildtype or mutant γ2L subunits were coexpressed with α1β2 subunits in HEK293T cells. $GABA_A$ receptor currents in response to 4 s applications of 1 mM GABA alone (left traces) or coapplied with 10 μM Zn^{2+} (right traces) to lifted cells containing wildtype and mutant γ2 subunits were shown. Subunit identity and length of GABA application (black line) were indicated above the current traces. Scale bars = 1 nA and 0.4 nA. **B.** Mean current densities (pA/pF, top panel) and Zn^{2+} inhibition (%, bottom panel) from cells coexpressing wildtype or mutant γ2L subunits were calculated. *** indicated $p < 0.001$, * indicated $p < 0.05$, compared with wildtype.

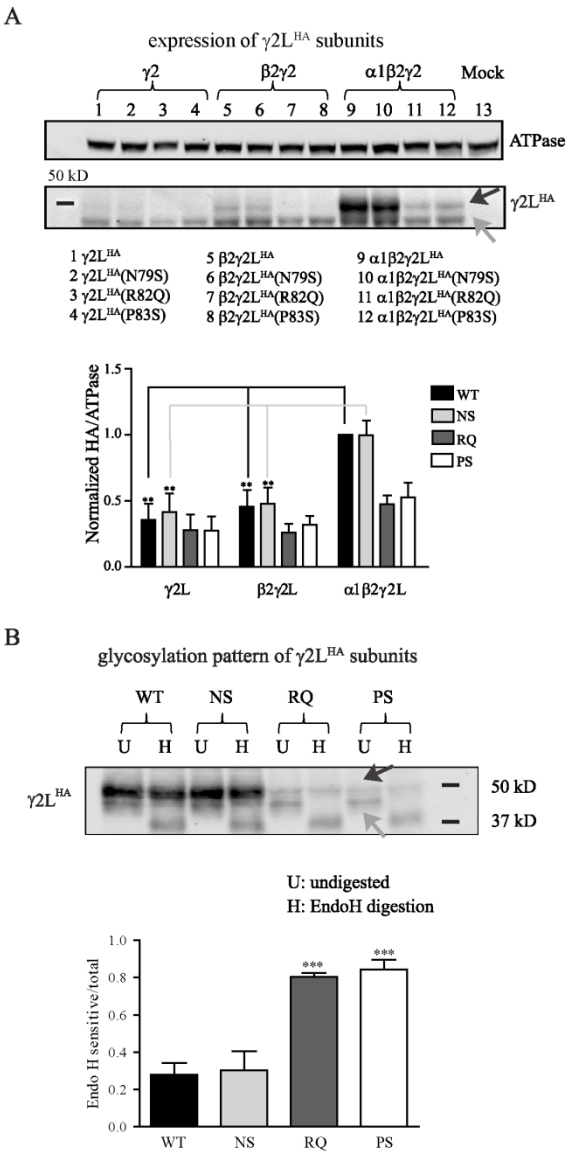

Figure 2-4: Mutant γ2(R82Q) and γ2(P83S) subunits showed immature glycosylation patterns and decreased stability

A. Wildtype or mutant $\gamma2L^{HA}$ subunits were expressed alone, coexpressed with β2 subunits only, or coexpressed with both α1 and β2 subunits in HEK293T cells. The total lysates of transfected cells were collected and blotted by anti-HA and anti-ATPase antibodies. When $\gamma2L^{HA}$ subunits were coexpressed with both α1 and β2 subunits, a top band around 47 kD (black arrow) appeared in addition to the bottom band (grey arrow) around 42 kD. Band intensity of the $\gamma2L^{HA}$ subunit was normalized to the ATPase signal and then normalized to that with cotransfection of wildtype $\alpha1\beta2\gamma2L^{HA}$ subunits (n = 4, mean ± SEM). Results obtained from transfection of wildtype or mutant $\gamma2L^{HA}$ subunits alone or cotransfection of wildtype or mutant $\gamma2L^{HA}$ subunits with β2 subunits were compared to those from cotransfection of corresponding wildtype or mutant $\gamma2L^{HA}$ subunits and α1β2 subunits and were analyzed by one way ANOVA test followed by Dunnett's multiple comparison test. (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$). **B.** Wildtype or mutant $\gamma2L^{HA}$ subunits were coexpressed with α1β2 subunits in HEK293T cells. The total lysates of transfected cells were collected, digested by Endo H, and blotted by anti-HA antibody. The two bands of $\gamma2L^{HA}$ subunits before Endo H digestion were labeled by black and grey arrows. The intensity of the Endo H sensitive

band (bottom band after digestion) was normalized to the total band intensity (top band and bottom band together after digestion) and analyzed by one way ANOVA test followed by Dunnett's multiple comparison test. (*** $p < 0.001$)

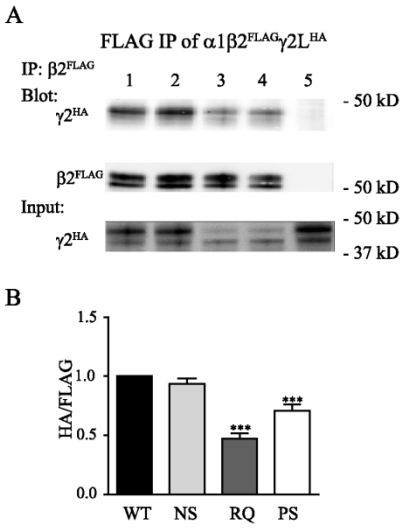

Figure 2-5: Mutant γ2(R82Q) and γ2(P83S) subunits were incorporated inefficiently into receptor pentamers.

A. Wildtype or mutant $\gamma 2L^{HA}$ subunits were coexpressed with α1 and $\beta 2^{FLAG}$ subunits in HEK293T cells. In whole cell lysates, $\beta 2^{FLAG}$ subunits and associated subunits were pulled down by anti-FLAG beads and blotted on Western blots by anti-FLAG and anti-HA antibodies. The total lysates were loaded as input and blotted by anti-HA antibody. **B.** The amount of wildtype or mutant $\gamma 2L^{HA}$ subunits associated with $\beta 2^{FLAG}$ subunits when coexpressed with α1 and $\beta 2^{FLAG}$ subunits were compared (n = 6, mean ± SEM). Differences compared to cotransfection of wildtype $\alpha 1\beta 2\gamma 2L^{HA}$ subunits were analyzed by the one way ANOVA test followed by Dunnett's multiple comparison test. (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$)

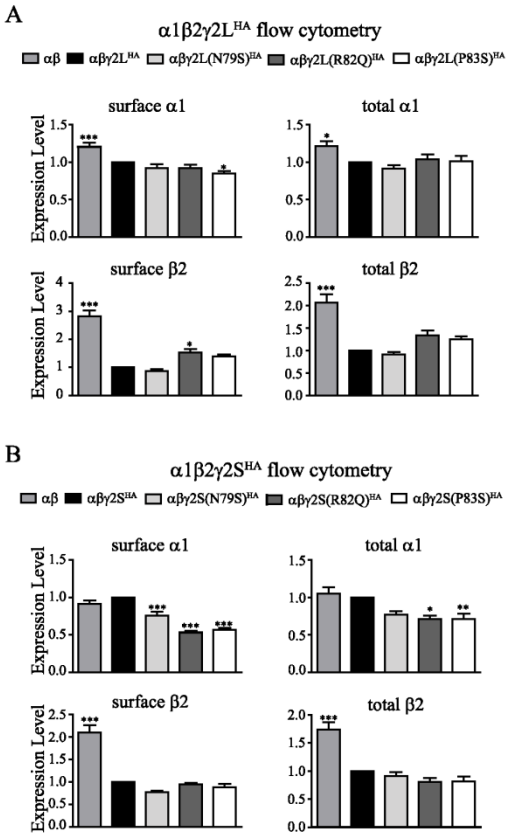

Figure 2-6: Over-expression of mutant γ2 subunits decreased surface levels of partnering subunits

A. B. Wildtype or mutant $\gamma2L^{HA}$ (A) or $\gamma2S^{HA}$ (B) subunits were coexpressed with $\alpha1\beta2$ subunits in HEK293T cells. Surface and total levels of $\alpha1$ or $\beta2$ subunits were evaluated using flow cytometry. The mock-subtracted mean fluorescence value of each subunit under different experimental conditions were normalized to those obtained with cotransfection of wildtype $\alpha1\beta2\gamma2L^{HA}$ (A) ($n \geq 9$, mean ± SEM) or $\alpha1\beta2\gamma2S^{HA}$ (B) ($n \geq 5$, mean ± SEM) subunits. Differences compared to wildtype $\alpha1\beta2\gamma2$ receptor condition were analyzed by the one way ANOVA test followed by Dunnett's multiple comparison test. (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

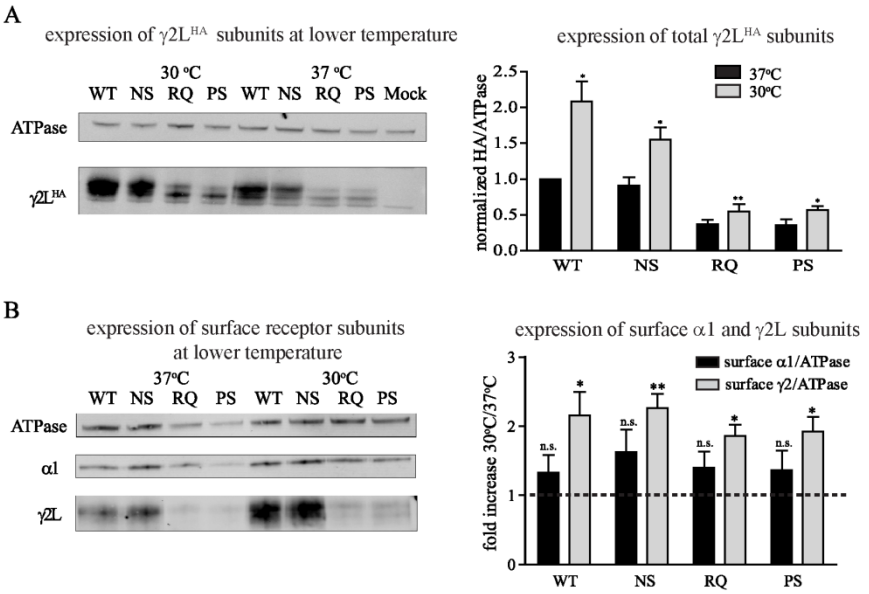

Figure 2-7: Decreased temperature stabilized both wildtype and mutant γ2 subunits

A. Wildtype or mutant $\gamma2L^{HA}$ subunits were coexpressed with α1 and β2 subunits in HEK293T cells and incubated at 37°C or 30°C for 24 hours. The total lysates of transfected cells were collected and blotted by anti-HA and anti-ATPase antibodies. Band intensity of the $\gamma2L^{HA}$ subunit was normalized to the ATPase signal, then normalized to that of wildtype $\alpha1\beta2\gamma2L^{HA}$ subunits (n = 5, mean ± SEM). Differences between 37°C and 30°C incubation were analyzed by t test. (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$). **B.** Wildtype or mutant $\gamma2L^{HA}$ subunits were coexpressed with α1 and β2 subunits in HEK293T cells and incubated at 37°C or 30°C for 24 hours. Surface protein samples were collected through surface biotinylation and blotted by anti-α1, anti-γ2 and anti-ATPase antibody. Band intensities of the α1 and γ2L subunits were normalized to the ATPase signal. The values for the 30°C incubation were further normalized to those obtained at 37°C (n = 4, mean ± SEM) to calculate the fold increase. The significance of fold increase was analyzed by t test. (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

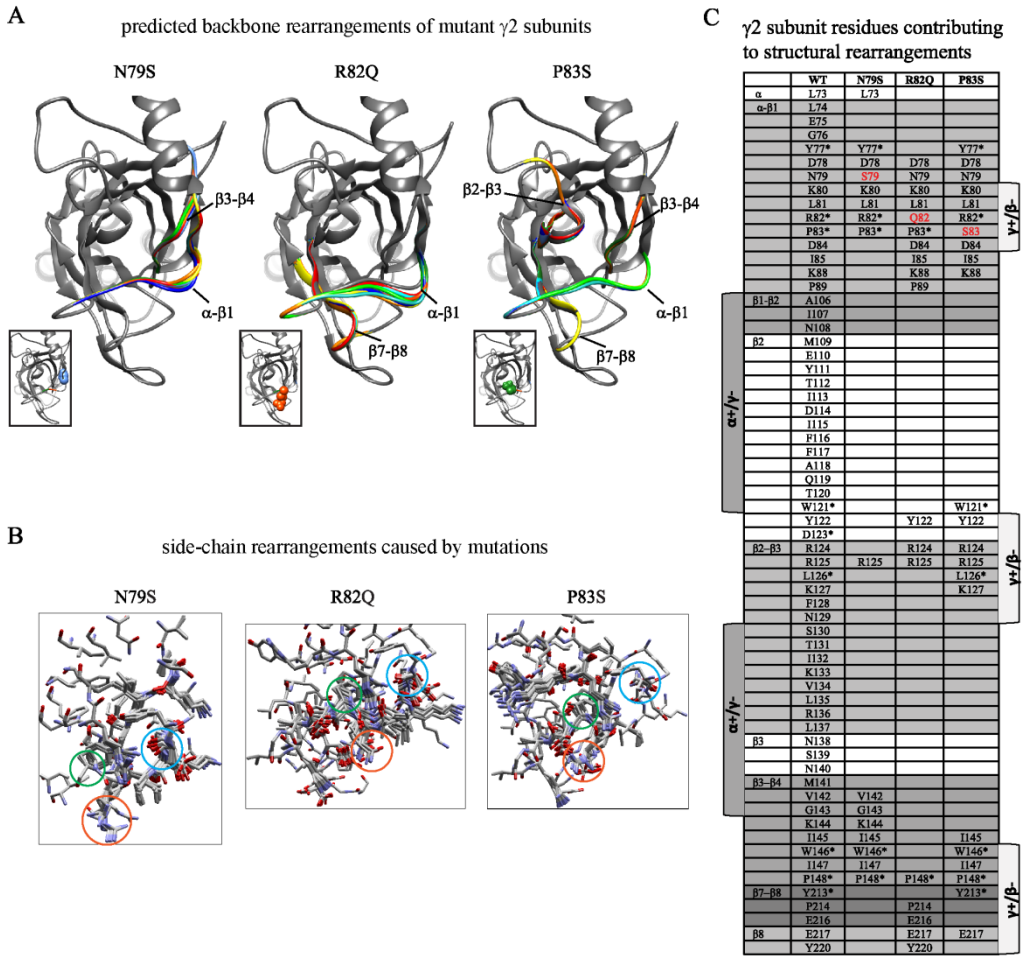

Figure 2-8: Structural simulation predicted mutation-induced changes in subunit structure

A. Superpositions of structural models for up to 10 of the best-scoring low-energy generated backbones of wildtype and mutated N79S, R82Q and P83S γ2 subunits were made. In ribbon representation, the native secondary structure was shown in gray, and the mutated secondary structures were represented by other colors. Structural domains as shown in panel C were also represented. Sites of missense mutations in γ2 subunits were shown in space-filling representation in the inserts: N79 in blue, R82 in orange, and P83 in green. **B.** Local side-chain rearrangements observed for mutated N79S, R82Q and P83S γ2 subunit residues were displayed. Neighboring residues within an 11 Å radius were shown in stick representation and color by element (CPK representation). Sites of wildtype or mutated N79S, R82Q or P83S residues were labeled in blue, orange or green circles. **C.** A table of predicted amino acids contributing to side-chain rearrangements for mutated residues at positions 79, 82 and 83 was categorized by structural domains. The mutated residues were shown in red, and identical residues among the Cys-loop family were labeled with an asterisk.

Chapter 3 : The *GABRG2* Nonsense Mutation, Q40X, Associated with Dravet Syndrome Activated NMD and Generated a Truncated Subunit That was Partially Rescued by aminoglycoside-Induced Stop Codon Read-through

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* Both authors contribute equally to this work

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1. Abstract

The *GABRG2* nonsense mutation, Q40X, is associated with the severe epilepsy syndrome, Dravet syndrome, and is predicted to generate a PTC in the $GABA_A$ receptor γ2 subunit mRNA in a position that codes for the first amino acid of the mutant subunit. 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 by NMD. Undegraded mutant mRNA was translated to a truncated peptide, likely the signal peptide, which was cleaved further. We also found that mutant γ2(Q40X) subunits did not assemble into functional receptors, thus decreasing 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. As a first approach, we investigated use of the 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, Dravet syndrome, generalized epilepsy, γ2 subunit, *GABRG2(Q40X)* mutation, loss of function, gentamicin

2. Introduction

Epilepsy is a common neurological disorder that affects about 1% of the world's population (*223*). Epilepsy syndromes are usually either symptomatic and due to a known brain injury or idiopathic and not due to brain injury. Genetic generalized epilepsy syndromes (GGEs) comprise ~30% of all cases and can vary in severity from the mild childhood absence epilepsy syndrome to the severe Dravet syndrome (*224, 225*). While many GGES 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. About one half of Dravet syndrome-associated mutations are nonsense mutations in genes such as voltage-gated sodium channels that create PTCs, and thus, truncated subunit proteins (*252*). Although rare, nonsense mutations in $GABA_A$ receptor subunit genes have been identified also in Dravet syndrome patients (*179*). *GABRG2(Q40X)* is a nonsense mutation located in $GABA_A$ receptor γ2 subunits that has been associated with Dravet syndrome (*253*).

$GABA_A$ 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 have been shown to decrease channel function by altering receptor biogenesis or channel function (*114*). The *GABRG2(Q40X)* mutation was shown to impair $GABA_A$ receptor channel function and to form granules in neurons (*253*). 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 (*254, 255*). 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 gene *CFTR*, further confirming the clinical feasibility of this strategy (*256, 257*). 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 γ2(Q40X) 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 truncated 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 subunit truncation caused by the Q40X mutation. To evaluate the plausibility of 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 of full-length γ2 subunits, and that the rescued γ2 subunits assembled with α1β2 subunits to form functional α1β2γ2 $GABA_A$ receptors.

3. 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 (*258*). 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 4^{th} and 5^{th} residue of the mature peptide of both wildtype and mutant γ2S subunit) to facilitate our experiments (*137*). To detect the truncated protein generated by the mutation, we also inserted an HA epitope at the N terminus of the unprocessed subunit, while an FLAG epitope was inserted between the 4^{th} and 5^{th} residue of the mature peptide, 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 (*259*). The oligonucleotide sequences for BAC recombineering are available upon request. A reporter gene containing an SV40 early promoter-driven eGFP was integrated to BACs using Cre (NEB) recombination (*260*). 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 Kit (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* and *GAPDH* mRNA, 18S rRNA, or *eGFP* mRNA (part number 4331348 [Custom Taqman Gene Expression Assay Service]) were used to quantify the amount of transcribed cDNA. Samples were obtained in triplicate for each experiment, and the average threshold cycle (Ct) 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* mRNA, 18S rRNA or *eGFP* mRNA levels 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 glycans, cell lysates were incubated with the enzyme PNGase F (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 (*228*). 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 (*229*). 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 lifted HEK293T cells 24-72 hrs after transfection with $GABA_A$ receptor subunits as described previously (*230*). 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 activated 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. Diazepam sensitivity was determined by co-application of 1 μM diazepam with 2 μM GABA for 4 s. Peak currents before and after diazepam co-application were compared to determine the % enhancement by diazepam. All currents were low-pass filtered at 2 kHz, digitized at 5-10 kHz, and analyzed using the pCLAMP 9 software suite.

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.

4. Results

1) The γ2S subunit mutation, Q40X, decreased γ2S subunit transcripts.

The nonsense mutation, Q40X, generated a PTC in the second exon of the nine exon *GABRG2* (Figure 3-1A). Because nonsense mutations located at least 50-55 nt upstream of an exon-exon junction activate NMD to degrade susceptible transcripts (*171*), the mutant γ2S(Q40X) subunit mRNA level should be lower. NMD efficiency is an inherent property of cells and varies among cell types (*177*). In HEK 293T cells, the mRNA level of an NMD-competent construct was degraded by about 60% (*261*). 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 (Figure 3-1B). 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 the *GABRG2* 5' exon 4 and 3' exon 5 border and normalized to GFP or GAPDH 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 (1.03 ± 0.08 fold, n = 6) after UPF1 knock down (Figure 3-1C). The mutant γ2S(Q40X) subunit mRNA level in cells transfected with mutant *GABRG2(Q40X)* BACs, however, was increased by UPF1 siRNA

(1.94 ± 0.23 fold, n = 6, $p < 0.05$) (Figure 3-1C). Thus, blocking NMD rescued the mutant γ2S(Q40X) subunit mRNA, 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).

2) <u>The γ2S subunit mutation, Q40X, generated a truncated peptide.</u>

Because not all mutant mRNA was degraded by NMD, we studied the protein generated by the mutant γ2S(Q40X) subunit cDNA. The Q40X nonsense mutation is located in the 40th residue of the immature γ2S subunit, which is the first residue of the predicted mature subunit (*262*). Thus, this mutation is predicted to generate a truncated protein encoding the 39 amino acid 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 4th and 5th residue of the mature γ2S subunit cDNA, generating a double tagged SP^{HA}-γ2S^{FLAG} subunit (Figure 3-2A). Signal peptides are composed typically of a positively charged 'N domain', a hydrophobic 'H domain' and a slightly polar 'C domain' (*263, 264*). The additional HA tag at the N terminus of the immature γ2S subunit did not significantly affect the hydrophobicity pattern of the signal peptide calculated *in silico* using the ProtScale software (*265*) (Figure 3-2B). Insertion of an epitope in the N domain should not change signal peptide topology or function (*266, 267*).

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 3-2C). In cells transfected with γ2S^{HA} subunits, a large band was detected by anti-HA antibody at about 44 kDa, and as expected, no signal was detected by anti-FLAG antibody (Figure 3-2C, lane 2). In cells transfected with SP^{HA}-γ2S^{FLAG} subunits, a large band around 44 kDa was detected by anti-FLAG antibody and a small band around 7.5 kDa was detected by anti-HA antibody (Figure 3-2C, lane 4). The size of the higher molecular mass FLAG-band was consistent with mature, glycosylated γ2S subunits (*180*), 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 3-2C, lane 3), indicating that synthesis of full length γ2S subunits was abolished by the *GABRG2(Q40X)* mutation. Interestingly, two different small peptides around/below 7.5 kDa were detected by anti-HA antibody in the mutant SP^{HA}-γ2S(Q40X)^{FLAG} subunit transfected cells (Figure 3-2C, lane 3), which may have been caused by a further cleavage of the signal peptide by signal peptide peptidase (*268*).

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 (Figure 3-2C, lane 4). 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 3-2D). 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.

3) The γ2S subunit mutation, Q40X, disrupted the membrane insertion of γ2S subunits and changed the composition of GABAA 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 4th and 5th 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 3-3A). The fluorescence indices of each subunit under different experimental conditions were normalized to those obtained with cotransfection of α1β2γ2S^{HA} subunits. Cotransfection of either α1β2 or α1β2γ2 subunits can produce functional $GABA_A$ receptors on the cell surface (*137, 269*). 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 (*46, 237*). Our flow cytometry analysis revealed a significant increase of surface β2 subunit levels with cotransfection of α1β2 subunits compared to cotransfection of α1β2γ2S^{HA} subunits (α1β2: 2.14 ± 0.23; α1β2γ2S^{HA}: 1.00; n = 7) with no change in the relative amount of surface α1 subunits (α1β2: 0.92 ± 0.05; α1β2γ2S^{HA}: 1.00; n = 7) (Figure 3-3A). In the presence of the Q40X mutation, no surface γ2S(Q40X) ^{HA} signal was detected by anti-HA antibody (Figure 3-3A), consistent with finding that synthesis of the full-length γ2S(Q40X) subunits was disrupted by the mutation (Figure 3-2C). With cotransfection of α1β2γ2S(Q40X)^{HA} subunits, surface α1 subunit levels were similar to those obtained with cotransfection of α1β2 and α1β2γ2S^{HA} subunits (α1β2: 0.92 ± 0.05; α1β2γ2S^{HA}: 1.00; α1β2γ2S(Q40X)^{HA}: 0.91 ± 0.03; n = 7). However, with cotransfection of α1β2γ2S(Q40X)^{HA} subunits, surface β2 levels were increased significantly compared to those obtained with cotransfection of α1β2γ2S^{HA} subunits, reaching the levels of α1β2 receptors (α1β2γ2S(Q40X)^{HA}: 1.99 ± 0.20; n = 7; $p < 0.05$) (Figure 3-3A). We also evaluated the total cell expression of the receptor subunits (Figure 3-3B). The total levels of α1 and β2 subunits with cotransfection of α1β2γ2S(Q40X)^{HA}

subunits were also similar to those obtained with cotransfection of α1β2 subunits. These data indicated that mutant γ2S(Q40X) subunits did not incorporate into surface receptors, and thus $GABA_A$ receptors assembled in the presence of mutant γ2S(Q40X) subunits were binary αβ receptors.

To determine how mutant γ2S(Q40X) subunits affected $GABA_A$ receptor function, 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 3-3C). Peak current amplitude recorded from cells coexpressing α1β2 subunits was 1351 ± 158 pA (n = 9), approximately 33% of currents recorded from cells coexpressing α1β2γ2S^{HA} subunits (4106 ± 156 pA, n = 15, $p < 0.001$) (Figure 3-3C, left traces), a difference consistent with previously reported data (*269-271*). Peak current amplitude from cells coexpressing α1β2γ2S(Q40X)^{HA} subunits was also decreased significantly (1778 ± 232 pA, n = 18) to about 43% of 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+} (Figure 3-3C, right traces). The fractional Zn^{2+} inhibition of currents evoked from cells coexpressing α1β2γ2S(Q40X)^{HA} subunits was significantly higher than inhibition of currents from cells coexpressing α1β2γ2S^{HA} subunits (93 ± 1%, n = 18; 9 ± 2%, n = 15, respectively, $p < 0.001$) but similar to inhibition of currents evoked from cells containing α1β2 subunits (94 ± 1%, n = 17, $p > 0.05$). 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 not incorporated into ternary α1β2γ2S(Q40X) receptors, thus leading to expression primarily of binary α1β2 receptors on the cell surface.

4) 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 functional, 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 (*220, 272*). 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 (*273*). To maximize read-through efficiency, we replaced the original TAG stop codon with the TGA stop codon (Figure 3-4A) and then transfected γ2S(Q40X,TGA)^{HA} subunit cDNA into HEK cells. Eighteen hours after transfection, varying concentrations of gentamicin were added to the culture media. Forty-eight hours later, the transfected cells 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 3-4B) .

In the absence of gentamicin treatment, mature, full-length, HA-tagged γ2S subunits were detected from wildtype transfected cells (Figure 3-4B, lane 9), but mature, full-length, HA-tagged γ2S(Q40X,TGA)^{HA} subunits were not detected from mutant transfected cells (Figure 3-4B, 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 3-4B, lanes 2-6). No HA signal was detected from mock transfected cells in the presence or absence of gentamicin (Figure 3-4B, 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 3-4D, 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 (*273, 274*). 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 3-4C) in a gentamicin concentration-dependent fashion (Figure 3-4D, filled squares).

5) 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 α1β2γ2S(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 γ2S^{HA} subunits (Figure 3-5A, 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 3-5A, 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 blotted 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.

6) Gentamicin-rescued γ2S subunits were functional.

We then evaluated assembly of α1β2γ2S(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 α1β2γ2S(Q40X,TGA)^{HA} subunits were substantially sensitive to Zn^{2+} inhibition (Figure 3-3B), consistent with assembly of only α1β2 receptors. In contrast, after 24 h gentamicin treatment, the fractional Zn^{2+} inhibition of currents recorded from treated cells containing α1β2γ2S(Q40X,TGA)^{HA} subunits was significantly smaller than those recorded from untreated cells (Figure 3-5B,C) (79 ± 1%, n = 19, treated, 93 ± 1%, n = 18, untreated; $p < 0.001$). This appearance of Zn^{2+} insensitive currents indicates the existence of αβγ receptors on the cell surface. We also determined the diazepam sensitivity of α1β2γ2S(Q40X) receptors since γ subunits are required for potentiation of $GABA_A$ receptor currents by diazepam (*270, 275*). In the absence of gentamicin, currents recorded from cells containing α1β2γ2S(Q40X,TGA)^{HA} subunits were not potentiated by diazepam application (1.9 ± 1.9%, n = 5) (Figure 3-5B,C), consistent with the insensitivity to diazepam potentiation of αβ receptors (*270*). In contrast, after gentamicin treatment, the peak current amplitudes recorded from cells containing α1β2γ2S(Q40X,TGA)^{HA} subunits was significantly enhanced (Figure 3-5B,C) (302 ± 55%, n = 11, treated; 2 ± 2%, n = 5, untreated; $p < 0.05$). Taken together, these results suggested that gentamicin caused 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 α1β2γ2S receptors on the cell surface.

5. Discussion

1) The GABRG2 mutation, Q40X, may induce epilepsy through haploinsufficiency.

The *GABRG2(Q40X)* mutation was identified from heterozygous dizygotic twin sisters with Dravet Syndrome (*253*). 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 *GABRG2* 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 (*274*). 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 (*176*). 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 (*177*). Thus, we also characterized the mutant protein generated by this mutation. Q40 is the first 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 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}-γ2S$(Q40X)^{FLAG}$ subunits was further cleaved (Figure 3-2B), probably through signal peptide peptidase (*268, 273*). 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 (*276*) or be processed as antigenic epitopes (*277*). 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 (*54*), and homozygous γ2 knockout mice died within a few days after birth (*275*). 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 (*150*). Several epilepsy-associated *GABRG2* mutations have been identified in families with (GEFS+ (*114*). Hence, loss of one functional *GABRG2* allele in patients carrying the *GABRG2(Q40X)* mutation combined with other unidentified modifier genes is likely responsible for development of the Dravet syndrome phenotype.

2) <u>The expression and function of mutant γ2(Q40X) subunits were partially rescued by gentamicin in vitro.</u>

Out of the seven epilepsy-associated mutations identified in *GABRG2*, four generated PTCs (*114*), and out of mutations identified from Dravet Syndrome patients, 50% were nonsense mutations (*252*). Aminoglycosides, including G418 and gentamicin, promote read-through of PTCs by disturbing stop codon recognition during translation. *In vitro*, in animals *in vivo* and in preclinical studies in humans, successful rescue of the mutant phenotype has been reported for several different disease models (*220, 278, 279*). 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 nonsense 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 genetic disorders where protein expression is almost null. However, this therapeutic approach may also work in autosomal dominant disorders (*280*), including epilepsy. It is possible that a small amount of rescued γ2 subunits during a critical time period could benefit patients substantially. GABA acts as a trophic factor during neural development (*281-283*) and disrupting postsynaptic γ2 subunit clusters decreased presynaptic GABAergic innervation (*284*). Study of heterozygous $\gamma2^{R82Q/+}$ mice revealed that $GABA_A$ receptor dysfunction during development increased seizure threshold in adulthood (*285*). 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 of 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 subunit 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 (*180*). 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 (*286*). 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 (*180*). 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 interesting to evaluate read-through of *GABRG2(Q390X)* subunit mRNA. Besides, as mutations in neuronal sodium channel SCN1A account for approximately 70% of all Dravet patients, it will be worthwhile to study how the chemical read-through approach could rescue SCN1A-associated nonsense mutations.

Long term use of aminoglycosides could cause nephrotoxicity and ototoxicity (*287*). 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 with cystic fibrosis (*288*) and Duchenne muscular dystrophy (*278*) carrying PTCs, it is necessary to explore other less toxic drugs. PTC124 (Ataluren®) is a nonaminoglycoside compound with superior read-through efficacy and lower toxicity (*257, 289*). A phase II prospective trial showed that PTC124 administration reduced abnormalities in cystic fibrosis patients (*290*). Another strategy is to use suppressor tRNA (*291, 292*). However, both transfection and read-through efficiency of suppressor tRNA is not high, and high level of suppressor tRNA was shown to be toxic to cells (*292, 293*). Recently, pseudouridylation has been suggested to target a specific nonsense codon into sense codon, but the rescue efficiency of this method is similar to that of aminoglycosides (*294*). Compounds with better efficacy and therapeutic window could be identified in future and our work shows a possible direction for epilepsy therapy.

6. Acknowledgements

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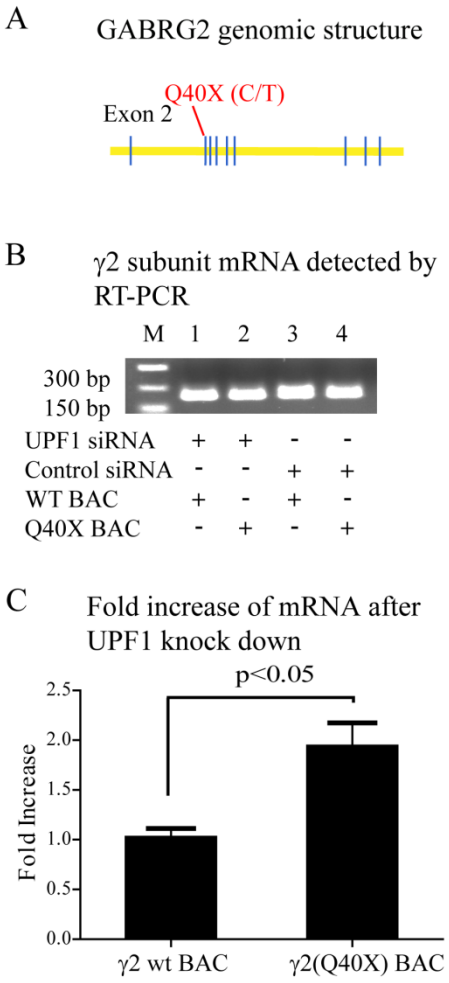

Figure 3-1: Mutant mRNA was degraded by NMD.

A. A schematic representation of the genomic structure of *GABRG2*. Vertical blue lines represent the exons composing the 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 (n = 6, mean ± SEM).

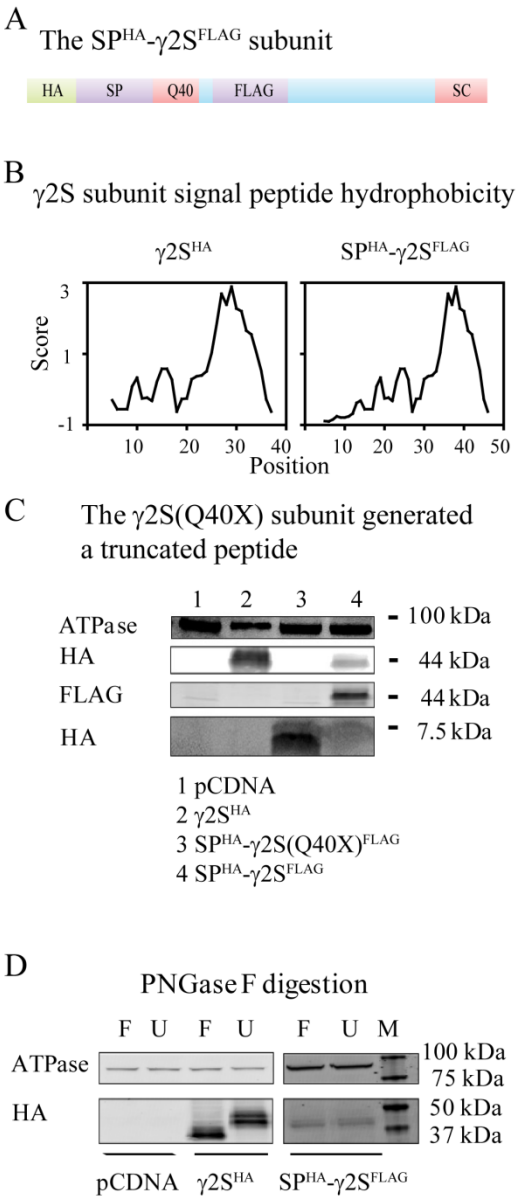

Figure 3-2: The GABRG2(Q40X) mutation generated a truncated peptide.

A. To identify the protein generated by the *GABRG2(Q40X)* mutation, an HA tag was inserted into the N terminal signal peptide and a FLAG tag was inserted between the 4th and 5th residue of the mature γ2S subunit protein to produce wildtype SP^{HA}-$\gamma2S^{FLAG}$ or mutant SP^{HA}-$\gamma2S(Q40X)^{FLAG}$ subunits. SP: signal peptide. SC: stop codon. **B.** The hydrophobicity patterns of γ2S and SP^{HA}-$\gamma2S^{FLAG}$ 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 $\gamma2S^{HA}$, wildtype SP^{HA}-$\gamma2S^{FLAG}$ or mutant SP^{HA}-$\gamma2S(Q40X)^{FLAG}$ subunits. Cell lysates (10 µg) from wildtype $\gamma2S^{HA}$ subunit transfected cells and cell lysates (50 µg) from wildtype SP^{HA}-$\gamma2S^{FLAG}$ or mutant SP^{HA}-$\gamma2S(Q40X)^{FLAG}$ subunit transfected cells were subjected to Western blot by anti-FLAG and anti-HA antibodies. ATPase levels were used as loading controls.

D. Samples from cells transfected with $\gamma 2S^{HA}$ or SP^{HA}-$\gamma 2S^{FLAG}$ 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.

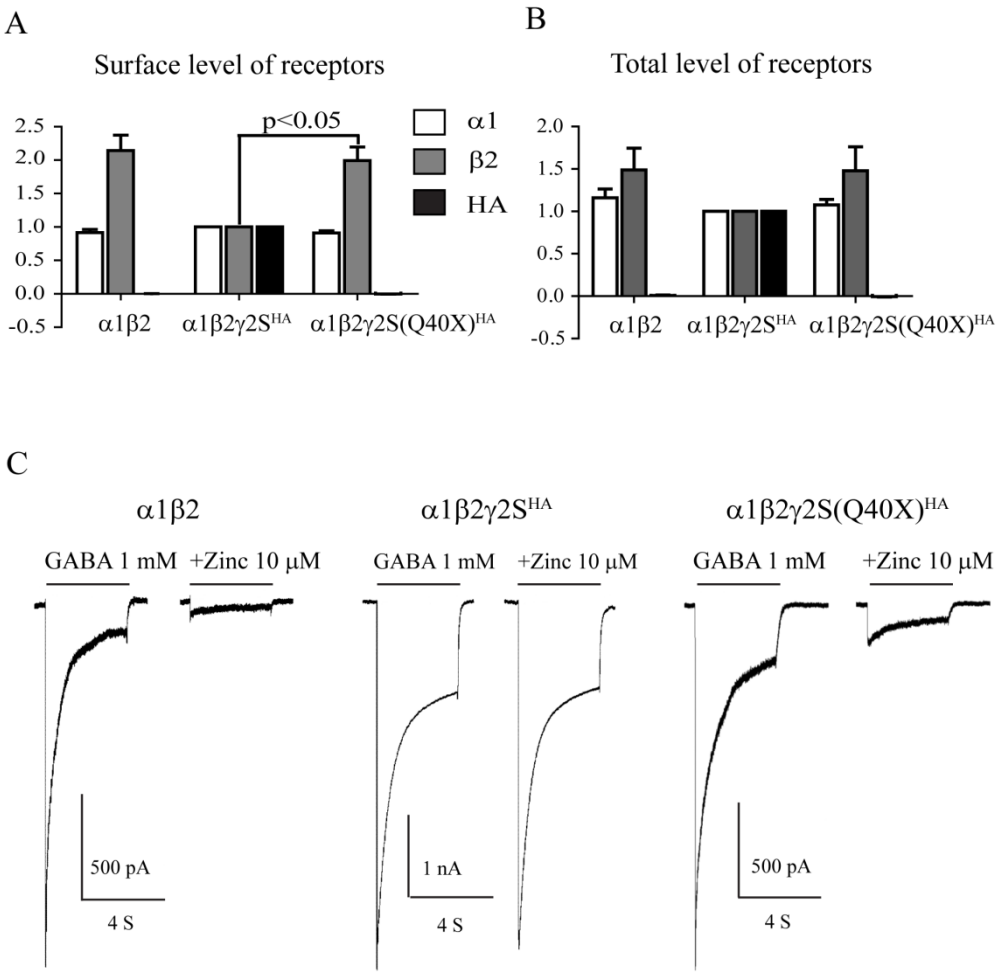

Figure 3-3: The mutant γ2S(Q40X) subunit was not expressed on the cell surface.

A. and B. Wildtype α1β2 or $\alpha1\beta2\gamma2S^{HA}$ or mutant $\alpha1\beta2\gamma2S(Q40X)^{HA}$ 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 $\alpha1\beta2\gamma2S^{HA}$ subunits (n = 7, mean ± SEM). Group differences were analyzed by the one way ANOVA test. **C.** Sample traces of whole cell recordings of currents evoked by 1 mM GABA from cells expressing α1β2, $\alpha1\beta2\gamma2S^{HA}$ or $\alpha1\beta2\gamma2S(Q40X)^{HA}$ subunits were obtained. After a 4.0 sec wash, the currents were recorded again with coapplication of 1 mM GABA and 10 μM Zn^{2+} (n > 9).

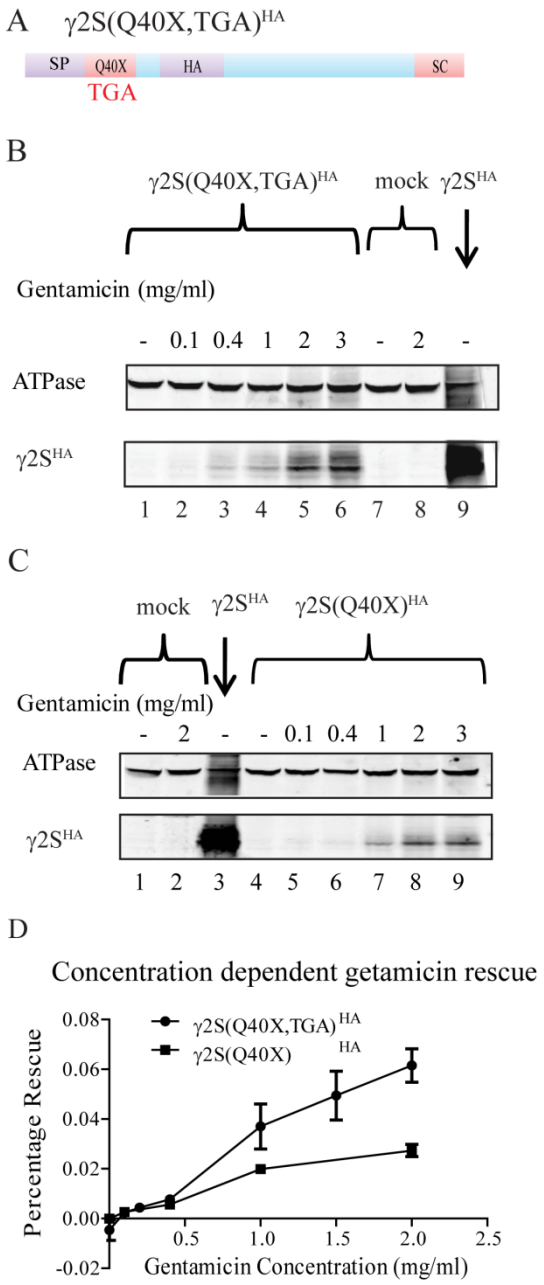

Figure 3-4: Gentamicin partially restored expression of full length γ2S subunits by read-through of γ2S(Q40X) subunit mRNA.

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 $\gamma2S^{HA}$ and $\gamma2S(Q40X,TGA)^{HA}$ (B) or $\gamma2S^{HA}$ and $\gamma2S(Q40X)^{HA}$ (C) subunits and treated with different concentrations of gentamicin for 48 hours. Cell lysates (10 µg) from wildtype $\gamma2S^{HA}$ subunit transfected cells were loaded, while cell lysates (50 µg) from mutant $\gamma2S(Q40X,TGA)^{HA}$ or $\gamma2S(Q40X)^{HA}$ subunits transfected cells were loaded. **D.** Band intensity of the $\gamma2S^{HA}$ subunit was normalized to the ATPase signal and plotted against gentamicin concentration (n = 7 and 5 respectively, mean ± SEM).

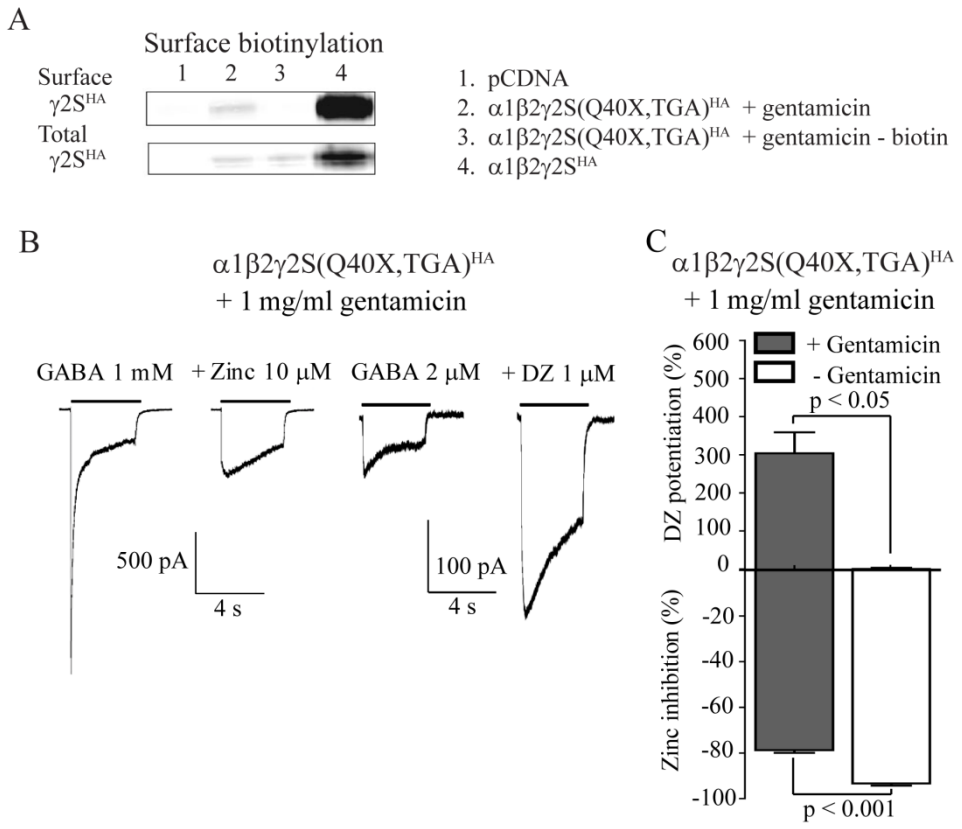

Figure 3-5: Gentamicin increased surface expression of mutant γ2(Q40X) subunits and decreased Zn2+ sensitivity of mutant receptor currents.

A. HEK 293T cells were cotransfected with $\alpha1\beta2\gamma2S^{HA}$ or $\alpha1\beta2\gamma2S(Q40X,TGA)^{HA}$ subunits. Cells were then treated with 1 mg/ml of gentamicin for 48 hours. 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 $\gamma2S^{HA}$ or mutant $\gamma2S(Q40X,TGA)^{HA}$ subunits were used to collect the surface fraction. Cell lysates (10 μg and 50 μg) from wildtype $\gamma2S^{HA}$ or 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 or 2 μM GABA then were recorded. The current amplitudes recorded in the presence of 10 μM Zn^{2+} or 1 μM diazepam were normalized to those recorded in the absence of Zn^{2+} or diazepam. The percentage of current amplitudes inhibited by Zn^{2+} (n = 19, mean ± SEM) or enhanced by diazepam (n = 11, mean ± SEM)) was compared to that obtained from cells untreated with gentamicin (n = 18 and 5, respectively).

Chapter 4 : Overexpressing wildtype γ2 subunits reversed seizure phenotype in a mouse model of genetic epilepsy

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1. Abstract

The $GABA_A$ receptor mutation, *GABRG2(Q390X)*, is an epilepsy-associated mutation identified in a family with GEFS+ (Harkin et al., 2002). It generates a premature stop codon that disrupts assembly of γ2 subunits into $GABA_A$ receptors and affects trafficking of partnering α and β subunits. Heterozygous *Gabrg2*$^{Q390X/+}$ knock-in mice showed reduced cortical inhibition, exhibited a lower seizure threshold and developed epileptic EEGs. In this study, we tried to theoretically rescue the knock-in mice from these deficits by using a gene therapy. To accomplish this we introduced an extra *GABRG2* allele by crossing *Gabrg2*$^{Q390X/+}$ knock-in mice with BAC transgenic mice overexpressing HA tagged human γ2^{HA} subunits. Compared to adult knock-in mice, adult mice carrying both the mutant allele and the transgene expressed γ2^{HA} subunits and had increased expression of wildtype γ2 subunits in the brain, increased miniature IPSC amplitudes in cortical neurons, and reduced intense thalamocortical oscillations. We measured seizure threshold by injecting mice with the chemoconvulsant PTZ and found that adult mice carrying both the mutant allele and the transgene exhibited a higher PTZ seizure threshold compared to the knock-in mice. Our results suggested that the epilepsy phenotype caused by a human epilepsy *GABRG2* mutation with dominant negative effects could be potentially rescued by increasing expression of wildtype γ2 subunits.

Key words: $GABA_A$ receptors, generalized genetic epilepsy, *GABRG2(Q390X)* mutation, gene therapy

2. Introduction

Epilepsy is a common neurological disorder affecting more than 65 million patients worldwide (*4*), in which altered excitatory/inhibitory balance in brain cortex is the likely underlying mechanism. Many patients have no discernable cause for epilepsy, suggesting a genetic origin (*117*). These genetic seizures exhibit varied epileptic and epileptogenesis mechanism and around 20% of them are refractory to treatments (*6, 10, 11, 150, 153, 295*). Meanwhile, many AEDs have undesirable side effects and current therapies provide symptomatic treatment and do not prevent the epileptogenesis process (*14*). Thus, alternative strategies are under development, such as interneuron transplantation (*296*) and neuroprotective factor-based gene therapy (*196, 197*). Gene-replacement therapy targeting the underlying genetic causes of epilepsy could provide another potential strategy.

Epilepsy-associated mutations have been identified in individuals and families with epilepsy, and many of them alter or even disrupt functioning of ion channels such as type-A γ-aminobutyric acid receptors ($GABA_A$Rs) (*119, 123*). $GABA_A$Rs are the major receptor mediating fast inhibitory synaptic transmission and controlling network excitability in the CNS. $GABA_A$Rs play an important role in establishing synapses during development and maintaining inhibitory tone in adulthood and are the molecular targets for multiple anticonvulsant and anxiolytic drugs (*109*). Nineteen different $GABA_A$R subunits have been cloned, but the majority of postsynaptic $GABA_A$R are composed of 2 α, 2 β and 1 γ2 subunits. Among currently known epilepsy-associated mutations identified in $GABA_A$R subunits, half of them are found in *GABRG2*, which encodes the γ2 subunits of the receptor (*114*).

Epilepsy-associated *GABRG2* mutations exhibit a wide range of effects, from subtle kinetic alteration, loss of function, to dominant negative effects (*297*). For example, R82Q is one of the best characterized epilepsy-associated *GABRG2* mutations (*123, 149*) and produces a loss of function and slight dominant

negative effects (*141, 143, 145, 146, 222*); heterozygous knock-in mice carrying this mutation had reduced cortical inhibition and displayed spontaneous spike-wave discharges and thermal-induced seizures (*150, 153*). The *GABRG2(Q390X)* mutation is a GEFS+ associated mutation identified in a patient with the severe Dravet syndrome (*179*). *In vitro* the Q390X mutation not only completely disrupted the trafficking and function of mutant subunits but also affected the trafficking of partnering α and β subunits (*180*). Mutant γ2(Q390X) subunits were slowly degraded and formed aggregates (*181*). Recent *in vivo* data in our lab also showed that heterozygous *Gabrg2*$^{+/Q390X}$ knock-in mice carrying this mutation showed decreased expression of γ2 subunits, reduced cortical inhibition, elevated anxiety, as well as behavioral and electrographic seizures, which is much more severe compared to heterozygous *Gabrg2*$^{+/-}$ knock-out mice (Kang et al., under review).

Here we explored whether a gene-replacement therapy could rescue the seizure phenotype of *Gabrg2*$^{+/Q390X}$ knock-in mice. It has been shown before that mice overexpressing γ2 subunits were indistinguishable from wildtype mice except for decreased alcohol tolerance (*298*). To compensate for the reduced inhibition caused by mutant γ2(Q390X) subunits, we introduced HA-tagged wildtype γ2 subunits in knock-in *Gabrg2*$^{+/Q390X}$ mice using a BAC transgene. We found that the transgene not only restored the total amount of wildtype γ2 subunits, but also reverse the decreased miniature(m) IPSCs, seizure threshold, and mitigated the intensified thalamocortical oscillation in adult heterozygous KI mice.

3. Materials and Methods

Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University. The Tg(*hGABRG2*^{HA}) transgenic mice were generated in C57BL/6J mice by pronucleus injection. *Gabrg2*$^{+/Q390X}$ knock-in mice were generated in a C57BL/6J ;129svJ mixed background and were backcrossed into the C57BL/6J background for more than 8 generations.

Tg(*hGABRG2*^{HA}) transgenic mice were bred with *Gabrg2*$^{+/Q390X}$ knock-in mice, generating offspring with four different genotypes, as described in Figure 4-1A.

Mouse tail samples collected at P14 – P21 were extracted using red Extract-N-AMP tissue PCR kit (Sigma-Aldrich) according to the manufacturer's manual. Mice were genotyped with the following primer sets: Tg(*hGABRG2*^{HA}): forward primer (5'-TACCCCTACGACGTGCCCGACTACGCC-3') and reverse primer (5' -CACCTCTCCCACTCATAGGCCTGAATG-3') (324 bp); *Gabrg2*: forward primer (5'-ATGGCGATGGAAGTTGACA-3') and reverse primer (5'-TGATGTTGCTCATGCCTCTC-3') (323 bp for wildtype allele and 405 bp for mutant allele).

Immunohistochemistry

Adult mice were anesthetized using isoflurane, followed by transcardial perfusion of 20 ml ice-cold 4% paraformaldehyde. Brains were removed and postfixed at 4 °C overnight, then cryoprotected in 30% sucrose at 4 °C for 48 hours. Brain sections of 50 µm thickness were collected in microtome and stored at -20 °C until immunostaining. Slices were incubated in rabbit monoclonal anti-HA antibody (1:500; Cell Signaling) in PBS with 0.3% Triton X and 4% horse serum at 4 °C for two nights, followed by 2 h incubation in IRDye800-conjugated donkey anti-rabbit IgG secondary antibody (1:1000; Li-COR). Immunolabeled slices were mounted by propyl gallate and scanned using Odyssey imaging system (LI-COR) with a resolution of 42 µm.

Western blot

Brains were removed from CO2-euthanized adult mice, then cortices were dissected. Cortical protein was collected in RIPA buffer with 1% protease inhibitor (Sigma), and extracted by sonication. Collected samples were subjected to gel electrophoresis using NuPAGE® (Invitrogen) precast gel and then transferred to PVDF-FL membranes (Millipore). Monoclonal anti-HA antibody (Covance or Cell signaling) and polyclonal anti-γ2 antibodies (Millipore) were used to detect HA tag and $GABA_A$ receptor γ2 subunits respectively. Anti-sodium potassium ATPase antibody (Abcam) was used as a loading control. After incubation with primary antibodies, IRDye® (LI-COR Biosciences) conjugated secondary

antibody was used, and the signals were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences). The integrated intensity value of each specific band was calculated using the Odyssey 3.0 software (LI-COR Biosciences).

PTZ-induced seizure threshold

Male adult mice (2 – 4 month old) were administered with 55 mg/kg pentylenetetrazol by intraperitoneal injection. The mice were then video-monitored for 20 min. The latency to GTCS and death were recorded.

Whole cell slice recording

Brain slice were prepared as the method in.(*299*) and (*300*). Briefly, adult mice (2-6 month old, either gender) were anesthetized with isoflurane and transcardially perfused with ice-cooled dissection solution (4°C) (mM: 2.5 KCl, 0.5 $CaCl_2$, 10 $MgSO_4$, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 11 Glucose, 214 Sucrose). Then mice were decapitated and the brains were removed. Coronal section slices (300 μm) were prepared using a LEICA VT-1200S vibrotome (Leica Inc) with oxygenated (bubbling with 95%O_2/5%CO_2) dissection solution. The slices were transferred to an incubation chamber containing oxygenated ACSF (mM: 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$, 10 Glucose, pH 7.4). After 40 min incubation at 35-36°C, the slices were recovered at room temperature for at least 1 hour before experiments.

Slice recordings were carried out with an upright NIKON Eclipse FN-1 IR-DIC microscope (Nikon) and one MultiClamp 700B amplifier and Digidata 1440A (Molecular devices Inc.). Since the thalamocortical circuitry is involved in epileptogenesis (*299*, *301*), Layer VI pyramidal neurons in the somatosensory cortex were chosen for recording, based on their apical dendrites and location right above the white matter. mIPSCs were isolated by including 10-20 μM NBQX and 1 μM tetrodotoxin (TTX) in the ACSF (flow rate: 1-1.5 ml per min). The internal solution for recordings contained (as (*299*), mM): 135 CsCl, 10 HEPES, 10 EGTA, 5 QX-314, 5 ATP-Mg (290-295 mOsm, pH = 7.3) and filled glass electrodes had 3-5 MΩ resistance. Access resistances during recording were continuously monitored and less than 20-25 MΩ. The access resistances were compensated by 70% and cell capacitance. Unstable

recordings with access resistance variation (>20%) or larger than 25 MΩ were discarded. Junction potentials were compensated when electrodes were in ACSF. The reversal potential of chloride anion was close to 0 mV and cells were clamped at -60 mV. Data were collected by using the Clampex program 10.2 (Molecular devices Inc.) and synaptic currents were filtered at 2000 Hz and digitized at 10 KHz. All recordings were continuously made for 20-30 min after rupture of membrane. All recordings were performed at room temperature (24°C).

Thalamocortical oscillation recording

Horizontal slices (350-400 µm) containing the thalamocortical circuitry were prepared (*302*)and put on top side of a self-made nylon mesh interface (only one side of a slice contacted ACSF solution). The network oscillation was recorded by using one tungsten electrode (MultiClamp 700B, current-clamp mode) in the Ventrabasal area (VB) and one concentric bipolar stimulating electrode placed in the internal capsule was used for stimulation. The multiunit recordings were band-filtered (between 100 Hz and 3 KHz) (*303*). Stimuli were 0.1 or 0.3 ms in duration. These experiments were performed at 31-32ºC.

Data analysis

The mIPSC data were analyzed with Clampfit (Molecular Devices Inc.), using threshold detection (at least 2.5X baseline RMS with no clear synaptic events) (*300, 303*). Histogram and accumulative graphs were constructed. The network oscillation data were analyzed with both Clampfit (for spike histogram and autocorrelation function) and Matlab to obtain autocorrelograms. As (*302*) and (*304*), oscillation indices were calculated to compare among different groups (both littermates and cogenic mice were used). Numerical data were reported as mean ± S.E and statistical differences were determined by the pair wise Student's t-test/One-Way ANOVA or Mantel-Cox method.

4. Results

1) The total amount of wildtype γ2 subunits was restored by introducing exogenous human GABRG2 allele to Gabrg2$^{+/Q390X}$ mice

Previously we generated BAC transgenic mice Tg(*hGABRG2*^{HA}) expressing HA-tagged human γ2 subunits under the control of the endogenous *GABARG2* promoter (*187*). The human γ2^{HA} subunits were expressed in a pattern that was similar to that of endogenous γ2 subunits in mouse brain, and the transgenic mice were indistinguishable from their wildtype littermates. We crossed the transgenic mice with the heterozygous *Gabrg2*$^{+/Q390X}$, knock-in mice that carried a GEFS+-associated mutation and developed spontaneous GTCS (Kang et al., under review). Offspring with four different genotypes, WT;0 (*Gabrg2*$^{+/+}$), Het;0 (*Gabrg2*$^{+/Q390X}$), WT;Tg (*Gabrg2*$^{+/+}$; Tg(*hGABRG2*^{HA})), and Het;Tg (*Gabrg2*$^{+/Q390X}$; Tg(*hGABRG2*^{HA})), were generated in an equal ratio, consistent with Mendelian inheritance (Figure 4-1A, B). This suggested that overexpressing wildtype γ2 subunits did not affect the birth rate of any of the mouse genotypes and could be a potential method for gene therapy.

We collected brain sections (50 μm) from WT;0 and Het;Tg littermates and stained the transgenic human γ2^{HA} subunits using an anti-HA antibody. As reported in the transgenic mice, the exogenous γ2^{HA} subunits were expressed across the whole brain of Het;Tg mice, including cortex, hippocampus, thalamus, and cerebellum (Figure 4-2A). Although the transgene was well expressed, the total amount of γ2 subunit protein may not be changed. To study whether the total amount of wildtype γ2 subunits was up-regulated by the transgene, we prepared cortical lysates from adult mice and performed western blotting for wildtype γ2 subunits using an antibody recognizing the M3-M4 loop of both human and mouse γ2 subunits but not the truncated mutant γ2(Q390X) subunits (Figure 4-2B1). While the total amount of wildtype γ2 subunits was significantly reduced in the cortex of Het;0 mice (0.68 ± 0.04, n = 7, p = 0.0001, two-tailed t test), it was significantly restored in Het;Tg mice (1.27 ± 0.18, n = 7, p = 0.01, two-tailed t test) (Figure 4-2B2). A similar restoration was also observed in thalamic lysates (Het;0: 0.68 ± 0.04,

Het;Tg: 0.96 ± 0.06, n = 4) (WT;0 vs. Het;0, p = 0.0019; Het;0 vs. Het;Tg, p = 0.0262). Thus, the total amount of wildtype γ2 subunits, which was reduced in the Het;0 mice, was restored to the Wt;0 level with the exogenous transgene.

2) <u>The PTZ-induced seizure threshold was reversed by overexpressing wildtype γ2 subunits in *Gabrg2*$^{+/Q390X}$ mice</u>

To determine whether the overexpression of the wild type γ2 subunit could rescue the seizure phenotype in *Gabrg2*$^{+/Q390X}$,mice, we compared the pentylenetetrazol(PTZ)-induced seizure threshold among the four genotypes, WT;0 (*Gabrg2*$^{+/+}$), Het;0 (*Gabrg2*$^{+/Q390X}$), WT;Tg (*Gabrg2*$^{+/+}$; Tg(*hGABRG2*^{HA})), and Het;Tg (*Gabrg2*$^{+/Q390X}$; Tg(*hGABRG2*^{HA}). PTZ induces GTCS in rodents (*305*), mimicking one of the seizure phenotypes observed in *Gabrg2*$^{+/Q390X}$ mice. We i.p. injected 55 mg/kg PTZ to 2-4 month-old mice and video monitored the mice after injection to assess development of GTCSs. Het;0 mice developed GTCSs quickly after injection, showing limb extension, tail-jerks, rearing or jumping, and many of the mice also died during seizures. We plotted the survival curve for PTZ-induced GTCS (Figure 4-3A) and death (Figure 4-3B) among four different genotypes and found that the Het;0 mice showed a lower seizure threshold compared to mice with the other three genotypes. The Het;0 mice developed GTCSs and died much faster compared to WT;0 littermates, while the seizure threshold of Het;Tg mice was not significantly different from littermate WT;0 mice (GTCS: Wt;0 (n = 5) vs. Het;0 (n = 7): p = 0.0009; Het;0 (n = 7) vs. Het;Tg (n = 9): p = 0.0001; Wt;0 (n = 5) vs. Het;Tg (n=9): p = 0.7713. death: Wt;0 (n = 5) vs. Het;0 (n = 7): p = 0.0034; Het;0 (n=7) vs. Het;Tg (n=9): p = 0.0004; Wt;0 (n = 5) vs. Het;Tg (n=9): p = 0.5050), indicating the restored expression and function of wildtype γ2 subunits by transgene was enough to rescue the seizure threshold of *Gabrg2*$^{+/Q390X}$ mice.

3) Reduced GABAergic synaptic transmission in Gabrg2$^{+/Q390X}$ mice was rescued by overexpression of wildtype γ2 subunits.

Spontaneous generalized seizures including GTCS and myoclonic jerks evolved in heterozygous *Gabrg2*$^{+/Q390X}$ mice, as detected in daily manipulation and video-EEG recording (Kang et al., under review). Here we characterized inhibitory synaptic transmission in thalamocortical circuitry, which was involved in generalized epilepsy and epileptogenesis (*306, 307*) Pyramidal neurons in cortical layer VI were visually identified by their upright apical dendrites. After blocking the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (AMPARs) and kainic acid (KA) receptors (KARs) with NBQX and with the chloride anion reversal potential set at -0.3 mV, inward $GABA_A$ receptor-mediated mIPSC were recorded while cells were voltage-clamped at -60 mV. All mIPSCs from slices from wildtype littermates exhibited fast rising and slow decaying phases, which are similar to previous reports(*308*). Consistent with western blot results for total γ2 subunit expression, mIPSCs in WT;0 mice were significantly larger in amplitude (39.65 ± 2.69 pA, n = 11 cells), compared with those in littermate Het;0 mice (31.02 ± 1.51 pA, n = 7 cells, t-test p = 0.018). This is also indicated by the right shift of the cumulative probability curve generated from recordings from WT;0 mice (Figure 4-4). Meanwhile, the mIPSCs in WT;0 mice occurred more frequently (4.96 ± 0.58 Hz, n = 11 cells) than those in Het;0 mice (2.45 ± 0.34 Hz, n=7 cells, t-test; p = 0.003) (Figure 4-4). This indicated dysfunction of γ2 subunits not only reduced the amount of postsynaptic receptors, but also decreased the amount of innervated presynaptic terminals, consistent with the notion that $GABA_A$ receptors are important for synaptogenesis during early development (*29-31*). With exogenous wildtype γ2 subunits in Het;Tg mice, layer VI pyramidal neurons exhibited similar mIPSC amplitudes (41.23 ± 3.70 pA, n = 11 cells, t-test p = 0.739) to those in littermate WT;0 mice, and significantly larger than those in littermate Het;0 mice (t-test, p = 0.024). However, mIPSC frequency recorded from Het;Tg mice only showed a trend for recovery (3.59 ± 0.64 Hz, n = 11 cells; t-test with het, p = 0.315), compared with those in Het;0 mice. All together these

data suggested that overexpressing wildtype γ2 subunits in heterozygous *Gabrg2*$^{+/Q390X}$ mice could rescue the reduced mIPSCs in neurons.

4) <u>The intensity of thalamocortical network oscillation was reduced by overexpression of wildtype γ2 subunits in Gabrg2$^{+/Q390X}$ mice.</u>

Network oscillation in the thalamocortical circuitry plays a significant role on epileptogenesis and generalized epilepsy (*89, 299, 302*). To examine whether the network oscillation or neuronal synchrony in *Gabrg2*$^{+/Q390X}$ mice was enhanced, we made extracellular multiunit recordings in VB nucleus of thalamus. Both spontaneous (Figure 4-5A1) and evoked (Figure 4-5A2) neuronal firing were observed and rhythms was self-repeated for a period in horizontal slices from Het:0 mice. These firings exhibited different spike amplitudes (based on spike-sorting) as well as oscillatory characteristics of network activity (shown by oscillatory pattern with autocorrelation of neuron firing) indicating the involvement of different neurons (Figure 4-5B, Het;0 mice for both spontaneous and evoked firing autocorrelation). However neuronal synchrony/oscillation was less frequently observed in WT;0 mice. Sometimes only burst activity was observed in wt;0 mice. Compared with that in WT;0 mice (oscillatory index: 0.49 ± 0.10, n=8 slices, Figure 4-5C), neuronal oscillation index in Het;0 mice was larger (oscillatory index; 0.70 ± 0.07, n=8 slices, t-test p=0.03). Moreover oscillation duration in het;0 mice had a long episode (2.46 ± 0.98 s, n=10 slices), much longer than that in WT;0 mice (0.72 ± 0.32 s, n=8 slices; t-test p=0.04) (Figure 4-5). With wildtype γ2 subunits introduced into heterozygous knock-in mice (Het;Tg), spontaneous and evoked oscillation were significantly reduced (oscillation index 0.52 ± 0.018, n=10 slices) compared with Het;0 mice and was very similar to those oscillation in WT;0 mice (Figure 4-5C1). Meanwhile, the oscillation duration was shorter (0.94 ± 0.26 s, n=8 slices) than het;0 mice (Figure 4-5C2), suggesting that introducing wildtype γ2 subunits into the Het;0 mice could also rescue altered neuronal synchrony and network oscillation.

5. Discussion

1) The seizure phenotype was rescued in a mouse model of genetic epilepsy

In humans the *GABRG2(Q390X)* mutation has been found to be associated with GEFS+ and Dravet syndrome (*179*). Here we report the rescue of the seizure phenotype in the *Gabrg2*$^{+/Q390X}$ mouse bearing one mutant allele with the *GABRG2(Q390X)* mutation. These mice had a reduced threshold for chemically- and thermally-induced seizures, and displayed spontaneous myoclonic jerks and GTCS (Kang et al., under review). Compared to heterozygous *Gabrg2*$^{+/-}$ knock-out mice, deficits caused by *GABRG2(Q390X)* mutation were much more severe, including a larger reduction of wildtype γ2 subunits, a larger decrease of mIPSC amplitude/frequency and more frequent and severe seizures. These are all consistent with *in vitro* characterization of the pathophysiological effects of the mutation that demonstrated that mutant γ2(Q390X) subunits were not only trapped in the endoplasmic reticulum but also prevented the trafficking of partnering subunits. Thus, the *GABRG2(Q390X)* mutation is a severe epilepsy-associated mutation with loss-of-function and dominant-negative-effects *in vitro* (*180*).

To determine if the loss of function and dominant negative effects of mutant γ2((Q390X) subunits could be reversed by overexpression of wildtype γ2 subunits, we introduced exogenous wildtype γ2 subunits using a BAC transgenic mouse, the Tg(*hGABRG2*^{HA}) mouse, that expressed HA-tagged human γ2 subunits under the control of the endogenous *hGABRG2* promoter (*187*). Similar to other transgenic mice expressing β-actin driven γ2 subunits (*298*), Tg(*hGABRG2*^{HA}) mice exhibited normal behavior and life span and had a PTZ-induced seizure threshold that was similar to that of wildtype mice. To overexpress wildtype γ2 subunits in the mutant mice, we crossed *Gabrg2*$^{+/Q390X}$ and Tg(*hGABRG2*^{HA}) mice determined whether overexpressing wildtype γ2 subunits could rescue deficits caused by *GABRG2(Q390X)* mutation. We found a complete recovery of total γ2 subunit protein level and mIPSC amplitude, and the mIPSC frequency was partially reversed. The incomplete recovery of mIPSC frequency indicated that presynaptic deficits were not fully rescued, which could be caused by the

dominant negative effects of mutant γ2(Q390X) subunits, especially during early development. However, the intensity of thalamocortical oscillation, which is a hallmark of generalized epilepsy, was still greatly reduced by the transgene. The PTZ-induced seizure threshold was also restored to control level. These results are consistent with a substantial restoration of GABAergic neurotransmission by overexpression of wildtype γ2 subunits in *Gabrg2*$^{+/Q390X}$ mice greatly improving the seizure phenotype.

2) <u>Implication for future therapy</u>

Target-specific gene therapy has been explored in a variety of disease models including the genetic neurological disorder Rett syndrome (*55*, *309-312*). Although different genetic information, including those encoding Kv1.1 potassium channel (*205*) and $GABA_A$ receptor α1 subunits (*313*) have been delivered into brain to inhibit the hyperexcitability and seizure development in several epilepsy models, none has been reported for genetic epilepsies. Here we found that target-specific gene therapy could become a future direction to treat genetic epilepsy caused by *GABRG2* mutations. As *GABRG2(Q390X)* is the most detrimental epilepsy-associated mutation identified in γ2 subunits, the same strategy is speculated to also rescue other less severe *GABRG2* mutations.

However, we only provided a proof-for-principle here. In the future, gene delivery strategies using AAV or lentivirus and small chemical or genetic molecules regulating the gene expression of γ2 subunits may have more promising clinical application. Here we introduced exogenous wildtype γ2 subunits from the beginning of gastrulation, but it would be important to study whether there is an important time window for therapeutic intervention. In addition, in this study we also overexpressed the transgene across the whole brain. Thalamocortical oscillation was suggested to be hijacked in generalized epilepsies (*307*) and and optogenetic inhibition of TC neurons in thalamus has been shown to inhibit thalamocortical seizures induced by stroke (*207*). Thus it would be interesting to investigate whether overexpressing wildtype γ2 subunits in a specific brain region is enough to attenuate seizures.

6. Acknowledgements

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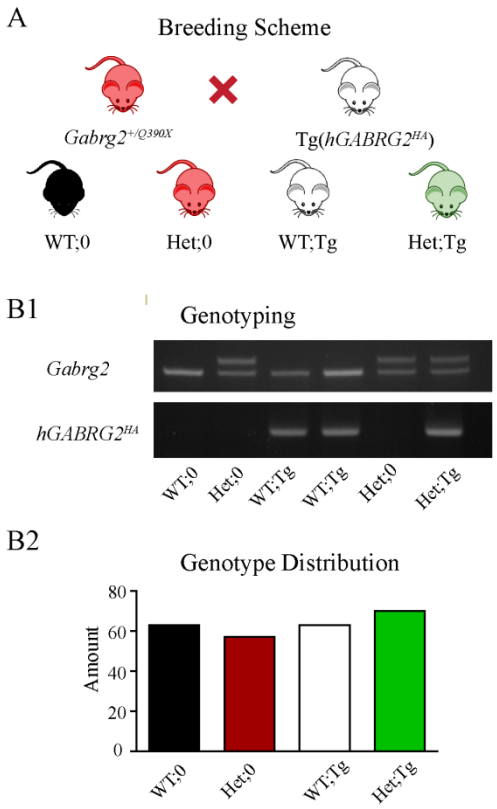

Figure 4-1: Exogenous $\gamma 2^{HA}$ subunits were introduced in Gabrg2$^{+/Q390X}$ mice by crossing them with Tg(hGABRG2HA) mice.

A. The schematic diagram shows the breeding strategy. *Gabrg2*$^{+/Q390X}$ knock-in mice were crossed with Tg(*hGABRG2*^{HA}) transgenic mice, generating offspring with four different genotypes. WT;0 denotes *Gabrg2*$^{+/+}$ mice. Het;0 denotes *Gabrg2*$^{+/Q390X}$ mice. WT;Tg denotes *Gabrg2*$^{+/+}$;Tg(*hGABRG2*^{HA}) mice. Het;Tg denotes *Gabrg2*$^{+/Q390X}$;Tg(*hGABRG2*^{HA}) mice. **B1.** PCR and gel electrophoresis was used for genotyping and the gel presents results from littermates with four different genotypes. Primers amplifying the endogenous *Gabrg2* allele generated one 323 bp band for the wildtype allele and one 405 bp band for the mutant allele. Primers amplifying the transgenic *hGABRG2*^{HA} allele generated one specific band of 324 bp for the transgene. **B2.** Mice with each of the 4 genotypes were born with equal frequency.

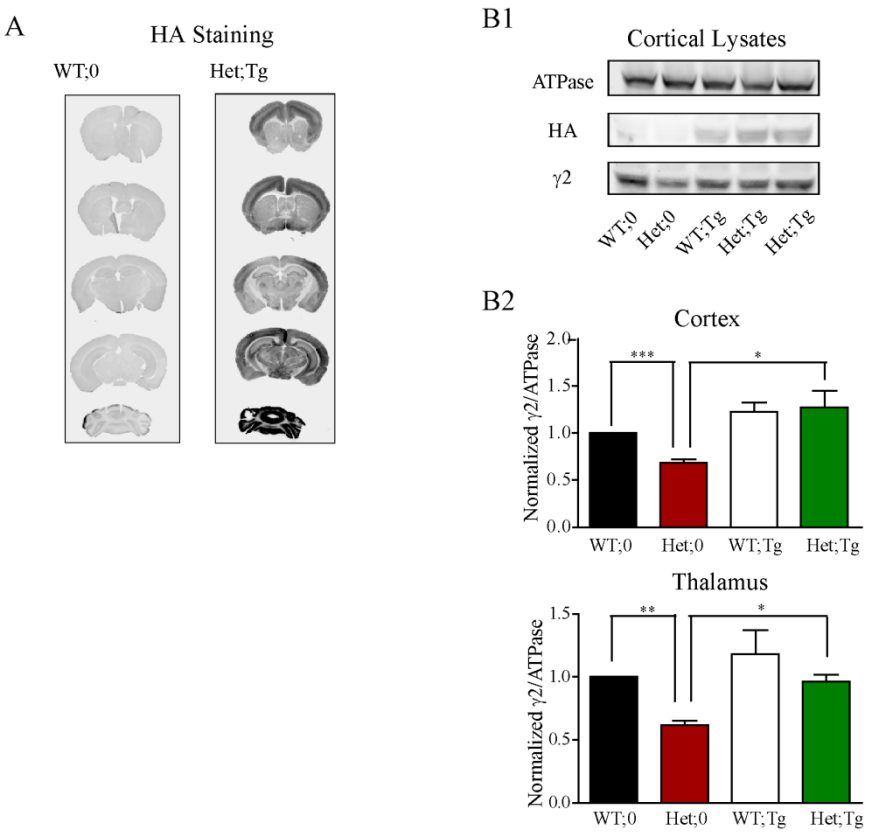

Figure 4-2: Total amount of wildtype γ2 subunits was restored by the transgene

A. Coronal brain sections from adult Wt;0 and Het;Tg mice were stained by an anti-HA antibody. **B1.** Cortical protein was collected from adult Wt;0, Het;0 and Het;Tg mice and blotted by anti-ATPase, anti-HA and anti-γ2 antibodies. The anti-γ2 antibody only recognized the wildtype γ2 and $\gamma2^{HA}$ subunits. **B2.** Expression levels of wildtype γ2 subunits in cortex and thalamus from adult Wt;0, Het;0 and Het;Tg mice were plotted. The band intensity of γ2 subunits was normalized to that of ATPase, and further normalized to that of WT;0 littermate (n = 7, mean ± SEM). Differences between littermates were analyzed by two-tailed paired t test (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

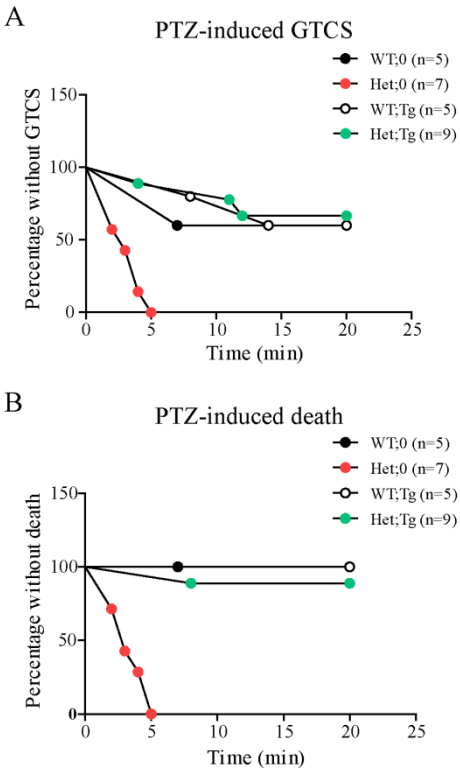

Figure 4-3: PTZ-induced seizure threshold was reversed by the transgene.

Mice were i.p. injected with PTZ (55 mg/kg) to induce seizures. The susceptibility to **A**. PTZ-induced GTCS and **B.** death was assessed by survival curves. Differences between littermates were analyzed by Mantel-Cox method. (**A.** Wt;0 vs. Het;0: p = 0.0009; Het;0 vs. Het;Tg : p = 0.0001; Wt;0 vs. Het;Tg: p = 0.7713. **B.** Wt;0 vs. Het;0: p = 0.0034; Het;0 vs. Het;Tg : p = 0.0004; Wt;0 vs. Het;Tg: p = 0.5050).

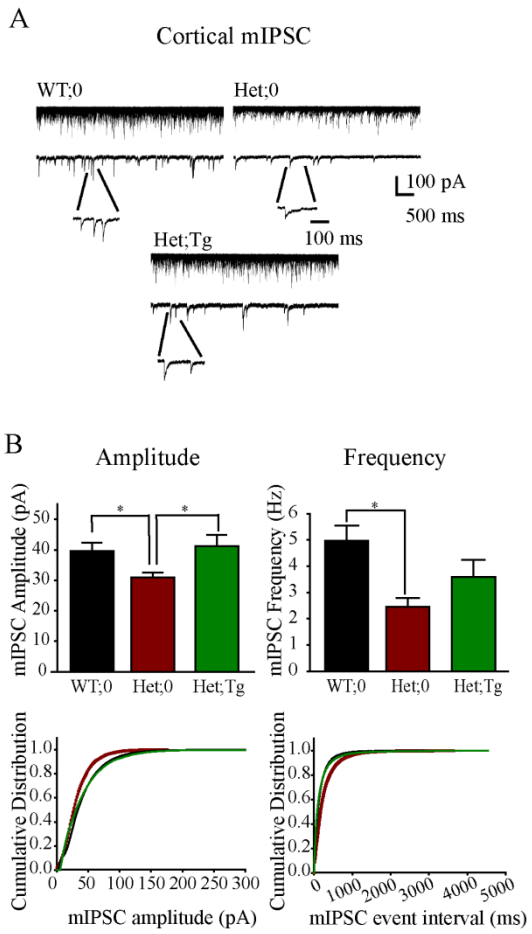

Figure 4-4: Cortical mIPSC was restored by the transgene.

A. Sample traces of mIPSC recorded from layer 6 cortical pyramidal neurons of littermates. **B.** Summary of the averaged mIPSC amplitudes and frequency(top) and normalized cumulative curves of mIPSC amplitude and intervals.

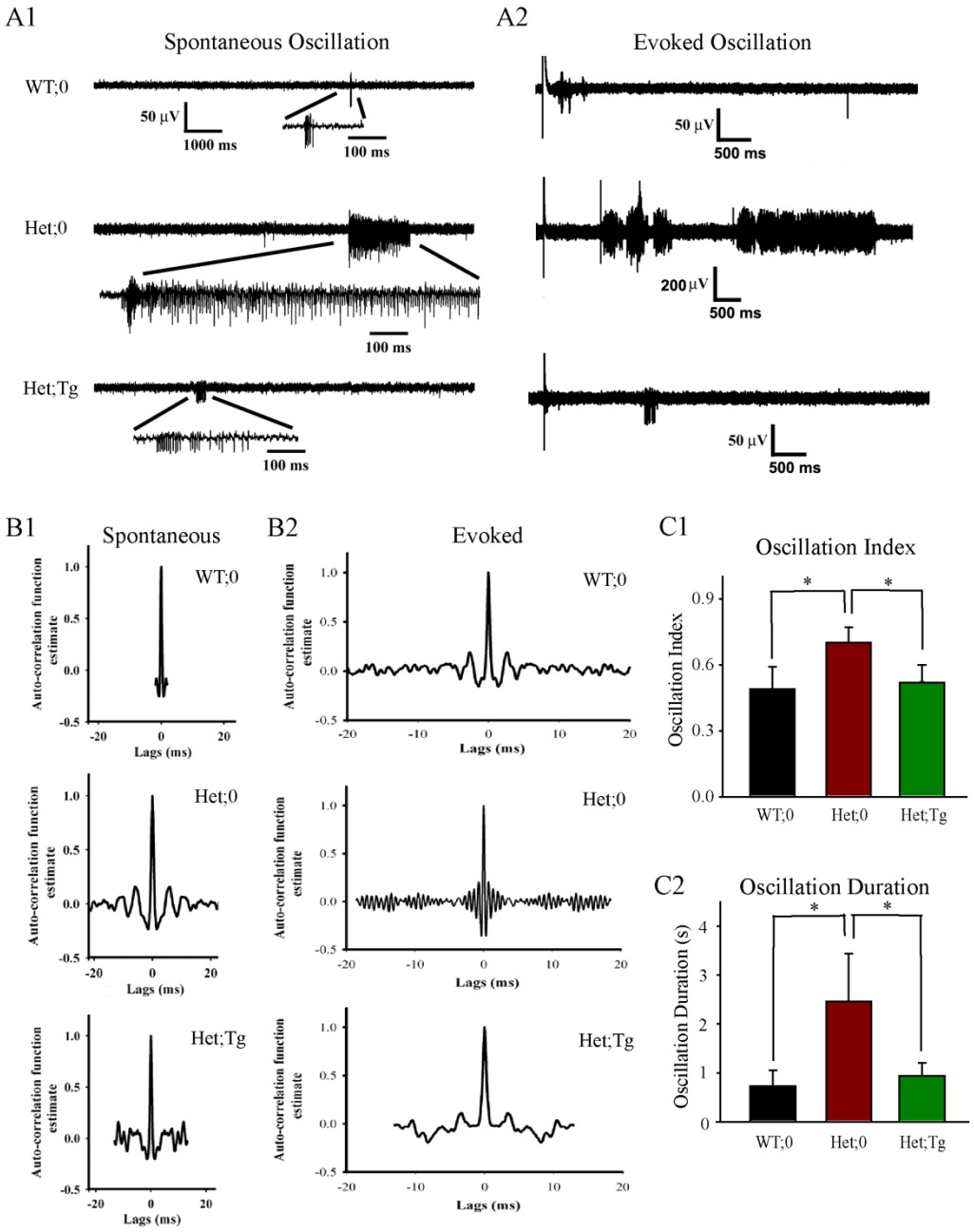

Figure 4-5: Spontaneous thalamocortical oscillation was less intense in Het;Tg mice.

A. Representative extracellular multiple unit recordings (A1, spontaneous and A2, evoked) from VB neurons in horizontal slices. **B**. Corresponding autocorrelograms showing oscillation patterns. **C**. Oscillation index and duration were compared.

Chapter 5 : Discussion and Future Directions

Epilepsy is a neurological disorder affecting almost 1% of the population, and genetic epilepsy is present in 30-60% of the affected individuals. *In vitro* characterization of the pathogenesis of mutations associated with genetic epilepsy and *in vivo* study of mouse models mimicking genetic epilepsy enable us to better understand underlying pathogenic mechanisms that will improve diagnosis and may lead to more effective treatment. In this dissertation study, I have worked on three projects to characterize different epilepsy-associated mutations in *GABRG2* (Table 5) and searched for possible strategies to rescue the mutation-induced deficits.

GABRG2 encodes for the 475 aa human γ2 subunits of $GABA_A$Rs. They are widely distributed in the CNS and contribute to the majority of synaptic $GABA_A$Rs. Although γ2 subunits are not obligatory for forming receptors, they are important for forming functional GABAergic synapses (*314-316*) and maintaining excitation/inhibition balance in the brain. Total loss of γ2 subunits is lethal (*90*), and dysfunction of γ2 subunits is associated with elevated anxiety (*92*), epilepsy (*150*), and abnormal neurogenesis (*93*).

Out of all epilepsy-associated mutations identified in $GABA_A$R subunits, half of them were found in γ2 subunits (Figure 5-1). Rigorous work has been done to study underlying pathogenic molecular mechanisms of mutations present in virtually all structural domains of the subunit. Interestingly, several mutations have been found in similar locations of γ2 and other subunits suggesting that these mutations may disrupt channel function through similar mechanisms. Now, with the help of next generation sequencing techniques, genetic screening is becoming cheaper and more available, yielding a large amount of genetic information for each individual. As a result, many more epilepsy-associated mutations/variants will be identified. In this section, I will speculate on future genomic medicine of epilepsy using *hGABRG2* mutations as examples.

1. Diagnosis: heterogeneous and complex genetic diseases

Genetic factors play important roles in epilepsy pathogenesis. Many epilepsy-associated mutations have been identified in individuals or families with epilepsy, and it is clear now that loss of certain genes could cause epilepsy. Currently, a whole genome can be sequenced in a single day for ~$1000, generating a personal genomic profile for each individual. However, it remains uncertain how to utilize this ever expanding amount of data for epilepsy diagnosis.

First of all, not each nonsynonymous variant is a disease-associated mutation, even if occurs in disease-associated genes. Many genes, especially those encoding ion channels, are associated with epilepsy, and dysfunction of these human epilepsy genes is assumed to cause epilepsy. However, not every amino acid change in human epilepsy genes is pathogenic. Previous studies have suggested that the presence of an epilepsy and its phenotype cannot be determined simply based on the load of "mutations" in human epilepsy genes (*191*). In one of my projects, we compared three epilepsy-associated mutation/variants in *GABRG2*(N79S, R82Q and P83S) that are clustered in the same region of the γ2 subunit. We found both the R82Q and P83S mutations disrupted the trafficking, and thus the function, of $GABA_A$Rs tremendously while the N79S mutation only produced slight effects. Thus we concluded that *GABRG2(R82Q)* and *GABRG2(P83S)* are epilepsy-associated mutations contributing to the epilepsy phenotype and *GABRG2(N79S)* is likely only a benign rare or susceptibility variant. Software including Polyphen and SIFT provides very useful platforms for prediction of the effects of individual aa substitutions: however, functional characterizations are still needed before we clearly understand the structure-function relationship of subunits and receptors and are able to make accurate predictions how mutation/variant disrupt receptor function.

Secondly, even if the effects of each mutation on protein function are known, it is still difficult to predict the impact of the mutation/variant on individuals that harbor the mutation/variant. Genetic epilepsies are complex, with variable penetrance and a wide spectrum of syndromes, and the majority of genetic epilepsies are polygenetic. Interaction of different genetic factors is the main theme for the

majority of genetic epilepsies. It has been postulated that the effects of multiple variants in several different epilepsy genes can summate to cause epilepsy. Interestingly, it was shown that a combination of two epilepsy-associated mutations with opposing effects could mask the epilepsy phenotype, further increasing the complexity of genetic profiling (*317*). Alternatively, mutations or rare variants in susceptibility genes, producing dysfunction that does not cause epilepsy directly, could also affect the severity of the epilepsy. For example, the potassium channel Kv8.2 was shown to modify the sodium channel *Scn2a* mutation-associated epilepsy phenotype in mice (*318*). Meanwhile, we cannot exclude effects of common SNPs present in the general population, as some of them could increase or decrease disease risk. How to model these complicated interactions is still unclear and may require multiple approaches including genetic, epidemiologic, bioinformatic, and statistical methods. Inducible pluripotent stem cell derived neurons, which are converted from human fibroblasts, can be utilized to compare the neuronal excitability of single cells from different individuals, and also provide a platform for drug screening. Establishment of a highly reproducible standard protocol for iPSC generation and neuron differentiation is required to reduce the variability of this procedure. How to link the neuronal excitability to network excitability still needs further investigation.

Nevertheless, this does not mean that genomic information is not helpful for epilepsy diagnosis. Although we are unable to build a model to accurately predict outcomes, incorporating personal genomic information during diagnosis could assist risk evaluation, guide medical management, and even prevent disease onset in the future. Imagine a child who presents to a physician after his first seizure. Knowing that the child bears a detrimental mutation in an epilepsy gene may change the approach to treatment.

2. Etiology: Does the same group of mutations share a common theme?

A good understanding of epilepsy genetics can improve diagnosis and determination of prognosis, but the ultimate goal is disease prevention and treatment. The traditional treatment strategy for epilepsy, and for most diseases, is based on syndromes, and most treatments are to mitigate symptoms. Anticonvulsant

medication is useful but does not cure the epilepsy. A good understanding of epilepsy etiology would facilitate design of improved target-specific therapies. An interesting initial question is whether or not the same types of mutations share common pathogenic mechanisms. If so, could they be treated similarly?

Here, we tried to group different types of epilepsy-associated *GABRG2* mutations based on their properties and locations. In one of my projects, we studied three missense mutations located in the subunit interface of γ/β subunits in assembled αβγ receptors. We found that, although to different extents, this type of mutation affected subunit interaction during receptor assembly, decreased the efficiency of forming trafficking-competent αβγ pentamers, reduced the trafficking of mutant subunits, altered the stoichiometry of surface receptors, and finally impaired mutant receptor channel function. Mutant subunits that were not incorporated into trafficking-competent pentamers were trapped in the ER and degraded. However, mutant subunits still interacted with partnering subunits during this process, thus slightly preventing their trafficking and exhibiting small dominant negative effects. These observations were partially supported by other studies showing that a β3 mutation located in the γ/β interface (*163*) and a γ2 mutation located in the α/γ interface (*319*) have similar effects, further suggesting that mutations located in subunit interfaces shared the same molecular mechanism. We found that the expression and trafficking of both wildtype and mutant γ2 subunits were stabilized at low temperature. Although we failed to identify molecular chaperones (e.g. heat shock proteins) and pharmacological chaperones (e.g. receptor ligands) that improved the trafficking of mutant subunits, chaperones slowing the biogenesis of receptors may be identified in future through larger scale screening to rescue trafficking-deficient mutant subunits.

Similarly, we also made efforts to group nonsense mutations into pathogenic classes. *GABRG2(Q40X)* and *GABRG2(Q390X)* are two nonsense mutations generating premature translation termination stop codons but exhibiting quite different effects. One of my thesis studies indicated that the *GABRG2(Q40X)* mutation activated the NMD machinery to degrade mutant mRNA and the mutant protein that was

generated was not functional. Thus the *GABRG2(Q40X)* mutation represented a group of nonsense mutations that result in loss-of-function, similar to the mutation *GABRG2(R136X).* The other mutation, *GABRG2(Q390X),* has been extensively characterized previously. As it is located in the last exon of *GABRG2*, it did not elicit NMD. In addition, the mutant protein generated was stable and showed substantial dominant negative effects by trapping partnering subunits in the ER. Mutant subunits also formed aggregates, which may further contribute to their pathogenesis. Both *in vitro* characterization (*180*) and *in vivo* mouse model study (unpublished) indicated that the *GABRG2(Q390X)* mutation represented a group of nonsense mutations that have more detrimental effects than loss-of-function, which includes the *GABRG2(W429X)* mutation. We found the synthesis of functional full-length γ2 subunits could be rescued from the *GABRG2(Q40X)* mutation by using aminoglycoside-induced readthrough, where the translational machinery was not terminated by the nonsense mutation. Although currently available aminoglycosides are toxic and not very efficient, this provided proof of principle of a promising strategy to treat epilepsy caused by nonsense *GABAG2* mutations.

Generally, we have successfully grouped several different mutations based on their location and effects (*222, 251*). Studies from several other projects in our laboratory also suggested that mutations in the same category usually shared the same molecular mechanism, although the severities of these effects vary mutation by mutation and could not be predicted without experimental support (*175, 182, 192, 319*). Treatments targeted to a specific group of mutations may be developed by pharmaceutical companies as individualized medication, a topic that I will elaborate on later.

3. Etiology: Beyond genetics

Study of genetics and associated molecular/cellular mechanisms reveals how a mutation affects channel function and cell excitability. However, how altered cellular excitability cause seizures and how a normal brain progresses into an “epileptic” brain are still unaddressed and will require investigations on *in vivo* systems.

In this dissertation work, our studies involved three typical types of mutations: *GABRG2(Q40X)* represented the group of loss-of-function mutations. Thus, having one allele of the *GABRG2(Q40X)* mutation is similar to the heterozygous knock-out condition. *GABRG2(R82Q)* represents the group of loss-of-function missense mutations with slight dominant negative effects. Different from the heterozygous knock-out condition, the mutant protein is produced, and slightly affects the trafficking of partnering subunits. *GABRG2(Q390X)* is a loss-of-function mutation with severe dominant negative effects, which is located at the other end of the disease spectrum. Mutant *γ2(Q390X)* subunit protein is not only stable, but also toxic.

Consistent with different extents of effects, three mutations are also associated with different epilepsy syndromes. The *GABRG2(Q40X)* mutation was identified in dizygotic twins with severe Dravet syndrome and an apparently healthy father, indicating that the mutation itself will not cause severe epilepsy symptoms and other mutations may be involved in epileptogenesis in these two twins (*174*). The *GABRG2(R82Q)* mutation was identified in a large family with CAE and FS (*123*, *149*). Genetic analysis suggested that the mutation accounted for the FS phenotype and a co-contribution with another unknown factor caused CAE. The *GABRG2(Q390X)* mutation was also identified from members of a family with generalized epilepsy (*179*). While the proband was diagnosed with the severe Dravet syndrome, other carriers in the family were not. It seems that while the mutation is responsible for the epilepsy phenotype, another unknown genetic factor exacerbated the syndrome in the proband. However, it is very difficult to predict the effects of these three types of mutations on brain circuitry and disease development, even though we clearly understand their molecular and cellular mechanisms. Thus it will be very interesting to compare these three types of mutations in representative mouse models, heterozygous *Gabrg2*$^{-/+}$ knock-out, *Gabrg2*$^{R43Q/+}$ knock-in, and *Gabrg2*$^{Q390X/+}$ knock-in mice.

Although controversial, these three mouse models also showed different types of seizures with different severities. While no seizure phenotype was reported when heterozygous *Gabrg2*$^{-/+}$ knock-out mice were first generated, recent studies suggested that these mice showed absence-like spike-wave-

discharges in a seizure-prone background (*94*). Both absence-like spike-wave-discharges and thermal-induced seizures were reported from heterozygous *Gabrg2*$^{R43Q/+}$ knock-in mice, depending on the mouse background and age (*94, 150*). Unpublished data from our laboratory suggested that heterozygous *Gabrg2*$^{Q390X/+}$ knock-in mice exhibited spontaneous myoclonic seizures and GTCS. How does dysfunction of $GABA_A$Rs lead to different epilepsy syndromes and what cause the spontaneous seizures? To connect the known cellular/molecular dysfunction to behavioral/clinic phenotypes, we need to study pathogenic alterations in these mouse models from a higher level, using technologies such as large scale transcriptome analysis, brain imaging and multi-array recording.

At first, we want to know how brain excitability is altered. EEG recording could provide some general information, but its spatial resolution is low and the signal could be affected by brain states and mouse behaviors. Combining EEG recording with extracellular recording including multi-array recording, network excitability in different brain regions and under different brain states could be compared. **Second,** we want to know how brain connections are altered. GABAergic neurotransmission is not only to inhibit neuronal activities. During early development, $GABA_A$Rs are excitatory and important for neuronal growth. $GABA_A$Rs are also pivotal for establishment of GABAergic synapses. If these *GABRG2* mutations affect synaptic development, is there a critical time window turning a normal brain to an "epileptic" brain? Or do they lead to any structural abnormalities? And if these mutations do change brain connection during development, does this contribute to generation of spontaneous seizures? Imaging study including fluorescence microscopy and electron microscopy to compare morphology and connections of neurons and synapses at different developmental stage may reveal such general deficiencies. **Third**, we want to know how synaptic plasticity is altered. Epilepsy is comorbid with autism, which is associated with abnormal information processing. Epileptic brain shows hyper-excitability, and patterns of neuronal firing could change brain plasticity. It is very likely that an "epileptic" brain responds to stimuli differently than a normal brain. Electrophysiological recordings can be used to compare different types of plasticity. **Fourth**, if any of these speculations are true, we want to

know how gene expression in different types of neurons is altered to cause a series of changes. Transcriptome alterations of different mouse models can be compared using RNAseq (Macdonald and Pimenta, unpublished). Is any common pathway altered? Are similar types of gene expression upregulated or downregulated? Are there any specific types of neurons that are vulnerable to these changes? Is there any brain region that is sensitive to these genetic defects? **Fifth**, if these three mutations/mouse models do correspond to three different epilepsy syndromes, what is the circuitry basis for these clinic outcomes? Generalized genetic epilepsies are manifested by different symptoms, including loss of consciousness, jerking, and muscle stiffening. Absence seizures are very different from generalized tonic clonic seizures, but both phenotypes have been identified from patients carrying $GABA_AR$ subunit mutations. A patient diagnosed with CAE could progress into JME. How are different motor/sensory circuits involved and hijacked in different epilepsy syndromes? Is the severity of phenotype only related to the severity of mutation? Do different types of neurons show different vulnerability to a same dysfunction? Pinpointing brain regions involved using small animal fMRI, comparing the altered network oscillation using in three types of epilepsy, or manipulating the spatial/temporal expression pattern of mutant subunits may be worth trying. Answering these questions could help us understand the development of different types of seizures and may provide some clues to identify candidate biomarkers. Answers to these questions can also help us to identify time windows for potential intervention and to identify drug targeting to pathogenic pathways.

4. Future therapy

A better understanding of molecular, cellular and circuitry mechanisms underlying the genetic epilepsies will provide more opportunities to advance the development of future therapies. Individualized genomic medication is under intensive study and even has appeared during clinic assessments for disease including cancer, Duchenne muscular dystrophy (DMD), cystic fibrosis and some retinal disorders (*320*). Specific treatments will be provided to specific populations of patients based on their genetic information

and associated disease etiologies. Compared to previous generations of AEDs that were found based on experience, or designed to suppress neuronal excitability, new generations of therapies will be more target specific, and even individual specific. New generations of therapies may not only suppress seizures but also prevent epileptogenesis. These different strategies could be applied to different types of genetic epilepsy or at different stages of disease progression. Here, I will summarize several categories of future therapy based on our understanding of genetic epilepsies caused by monogenetic mutations in *GABRG2*.

1) <u>Mutation specific chemical therapy:</u>

Although big molecule drugs including antibodies and peptides are under intensive study and development for many diseases, small chemical molecules still occupy the majority of pharmaceutical market due to their convenience and bioavailability. As $GABA_AR$ γ2 subunits are widely distributed in the brain, administration of chemicals to correct mutant γ2 subunit dysfunction all over the brain may prevent the pathological effects caused by the mutations. A similar approach has been adopted in fields studying cystic fibrosis (*216, 217*). Cystic fibrosis is caused by dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Based on the type of CFTR mutation, different drugs have been designed to partially reverse the effects of these mutations and some are already under investigation in clinical trials. For example, a small molecule compound, ataluren, has been tested in a phase 3 clinical trial to read-through a premature stop codon caused by a CFTR nonsense mutation in patients. Another small molecule compound, ivacaftor, which can potentiate the function of CFTRs that contain a missense mutation, showed clinical benefits in patients and has been approved by the FDA. Similarly, ataluren is also in a clinical trial for DMD to test its effects on patients carrying nonsense mutations (*321*).

Several types of *GABRG2* specific drug therapies could be developed. These include receptor potentiators like benzodiazepines to increase function of both wildtype and mutant receptors. However, administration of benzodiazepines produces side effects including drowsiness, amnesia and drug tolerance.

Besides, it has been suggested that these general receptor potentiators may induce absence seizure by increasing inhibition of thalamic neurons (*302, 322*). Due to lack of access, we could not get detailed treatment histories for patients carrying $GABA_AR$ mutations to compare whether benzodiazepines showed better efficacy. Identifying other potentiators that target receptors that contain subunits with specific mutations, like *GABRG2(K328M)*, could be a future direction for drug development. The K328M mutation accelerated channel deactivation of $GABA_ARs$. Chemicals that increase the open duration of receptor channels containing the *GABRG2(K328M)* mutation may correct its dysfunction. Chemical therapies also include molecular or pharmaceutical chaperones to improve receptor biogenesis by stabilizing the biogenesis of wildtype receptors, mutant receptors or both. It was reported that $GABA_A$ receptor ligands could promote receptor trafficking (*250*), and thus small molecules that promote assembly and stabilize mutant receptors may exist. Although we failed to identify such molecules to rescue the function of the missense mutation *GABRG2(R82Q)*, we did show that slowing receptor biogenesis increased subunit stability and trafficking. Chemical therapies also include gentamicin-like aminoglycosides and ataluren to promote read-through of nonsense mutations. We demonstrated in our paper that gentamicin could partially restore the synthesis of full length functional γ2 subunits from subunits containing the *GABRG2(Q40X)* mutation. Although it is not gene specific, this may be useful to prevent the production of toxic mutant protein like γ2(Q390X) subunits. If similar chemicals with higher efficiency and lower toxicity are identified in future, it would be very interesting to test in heterozygous $Gabrg2^{Q390X/+}$ knock-in mice.

2) <u>Mutation specific gene therapy:</u>

Gene therapy is promising because it is very target specific and should work well for monogenic disorders. Lentiviral- and AAV-vector based gene therapy has been taken to clinical trials to treat genetic disorders such as the retina disorder choroideremia (*323*) and Parkinson's disease (*324*). Several anti-sense oligonucleotide drugs, which can induce exon skipping to avoid generating out-of-frame mRNA

caused by deletion mutation, have been taken to clinic trials for DMD (*321*). Gene therapy has emerged as a powerful tool to treat neurological disorders with improvements in vector design and delivery methods (*195*). Following demonstration of substantial effects in preclinical animal models, gene therapy related clinical trials are already undergoing for neurodegenerative disease and brain tumors.

For *GABRG2* mutations, there are two prospective types of gene therapy. The first kind is to overexpress wildtype γ2 subunits using viral vectors or nonviral gene delivery vehicles. The rational is clear for loss-of-function mutation like *GABRG2(Q40X)*, as this method could restore the loss of one allele. This strategy may also work for other mutations like *GABRG2(Q390X)* as extra wildtype subunit protein could compete with mutant subunit protein, thus restoring the function of wildtype subunit protein while reducing dominant negative effects of mutant subunit protein. Here we have demonstrated that supplementation with an extra *GABRG2* allele could rescue reduced GABAergic inhibition and decrease seizure threshold in $Gabrg2^{Q390X/+}$ knock-in mice. It is necessary to test this principle using a more realistic delivery system such as lentivirus or AAV, which will elicit relatively low immune and inflammatory response and have been used in clinical trials. This would also help us examine whether there is a specific time or a specific region that could maximize the effects of gene therapy, how many copies of wildtype allele are optimal, how much wildtype protein is required, and whether epileptogenesis can be prevented if treatment is delivered before the disease onset.

The second type of gene therapy is to correct the mutation in the genomic sequence, regardless of the type of mutation. It has been shown before that antisense oligonucleotide could prevent abnormal splicing events caused by insertion of a retrotransposon (*325*) and a similar strategy might be applied to splicing mutations like *GABRG2(IVS6+2T->G)* to correct the abnormal splicing. The advances of genomic editing techniques, especially the invention of CRISPR (clustered regularly interspaced short palindromic repeats) techniques (*326*), shed lights on mutation correction for future disease treatment. Utilizing the endonuclease Cas9 and guide RNA, the CRISPR/Cas9 system could induce generation of indels (mismatches, insertions, or deletions) or homologous recombination targeting a specific DNA

sequence. CRISPR techniques have been used to generate genetic modifications in rodents (*327*, *328*), pigs (*329*) and even primates (*330*). CRISPR techniques have also been used to correct genetic mutation in rodent models of hereditary tyrosinemia (*331*) and DMD (*332*) by replacing mutant nucleotides. Although only a small proportion of cells were corrected after the delivery of CRISPR-Cas9 system, it was enough to improve certain symptoms. Due to the difficulty of delivery and the non-dividing property of neurons, it is still very difficult to apply this technique in central nervous system. Future improvements of correction efficiency may enable correction of some detrimental mutations related to neurological disorders.

3) <u>Pathology specific therapy:</u>

Mutation targeting therapies will be specific and with few side effects. However, it may be impractical to develop a single reagent for each individual patient, and more importantly, it is difficult to identify the specific mutation for most patients with polygenic epilepsy. Although the genetic factors contributing to epileptogenesis vary among individuals, multiple syndromes are shared among patients. Traditional AEDs are found or designed to suppress syndromes and are effective for two-thirds of patients. However, from the targeted patient population to the targeted syndrome, traditional AEDs are very nonspecific. Furthermore, various side effects are accompanied, and around 20% of patients are unresponsive to AED treatments. For new generation of AEDs, we want to better specify the patient population and target the pathogenic pathways underlying these epilepsy syndromes (Figure 5- 2). We want to know for each type of epilepsy syndrome, is there a common underlying mechanism? Is there a common brain circuit involved? Is there a shared pattern of altered brain connections that leads to an epileptic brain? If so, therapies correcting the common pathology could be applied to a group of patients.

Studies on iPSCs derived from amyotrophic lateral sclerosis (ALS) patient samples have suggested that different ALS-associated mutations caused several common categories of downstream alterations in motor neurons, and some defects could be partially mitigated by the same drug (*333*, *334*). Genetic

epilepsies that share a similar cohort of syndromes may also exhibit the same core defects. As I have mentioned before, we have already established several different models of genetic epilepsies caused by monogenic mutations in *GABAG2*. Several other genetic mouse models have also been created based on findings in other gene mutations. For example, deletion of *Scn1a* caused severe Dravet syndrome in mice (*209*), with reduced excitability of inhibitory interneurons; specific deletion of the *Scn1a* encoding sodium channels in forebrain interneurons caused seizures and premature death in mice (*335*). Deletion of thalamic PLC-β4 led to absence seizures, which could be suppressed by T-type calcium channel blockers (*336*). Do these models share any common downstream characteristics? Are there any specific cell types prone to damage? Comparisons across different models are necessary in the future and may reveal some surprising common "biomarkers" of genetic epilepsy.

5. Conclusions

Study of epilepsy has improved our understanding of human epilepsy genes and associated protein. Exploration of the brain mystery also advances our understanding, diagnosis and treatment of epilepsy. From the discovery of DNA molecules, to human genomics project, to the accelerating upgrade of sequencing techniques, basic research and biotechnology on genetics has led to grand changes in medical research. For the next decade, what will cutting-edge neuroscience techniques and human connectome plan bring us? What kind of surprise about brain and epilepsy will be revealed then? I have tried to speculate a bit, but more details are waiting to be unraveled.

Table 5. *GABRG2* mutations involved in this study

Mutation	Amino Acid Alteration	Location	Associated Epilepsy Phenotype	*in vitro* Pathological Mechanisms
Q40X	Glutamine-> stop codon	N terminus	dizygotic twins with the severe Dravet syndrome, and their healthy father	unstable mRNA, nonfunctional protein, loss of function
N79S	Asparagine-> Serine	N terminus	one patient with generalized tonic-clonic seizures	slightly decreased trafficking, benign or susceptibility variant
R82Q	Arginine-> Glutamine	N terminus	one family with febrile seizures and childhood absence epilepsy	inefficient receptor assembly, decreased trafficking, loss of function with slight dominant negative effects
P83S	Proline-> Serine	N terminus	one family with idiopathic generalized epilepsy	inefficient receptor assembly, decreased trafficking, loss of function with slight dominant negative effects
Q390X	Glutamine-> stop codon	M3-M4 loop	one family with generalized epilepsy with febrile seizures plus, and the proband was diagnosed with the severe Dravet syndrome	stable mRNA, stable protein, decreased trafficking of mutant subunits and partnering subunits, loss of function with severe dominant negative effects

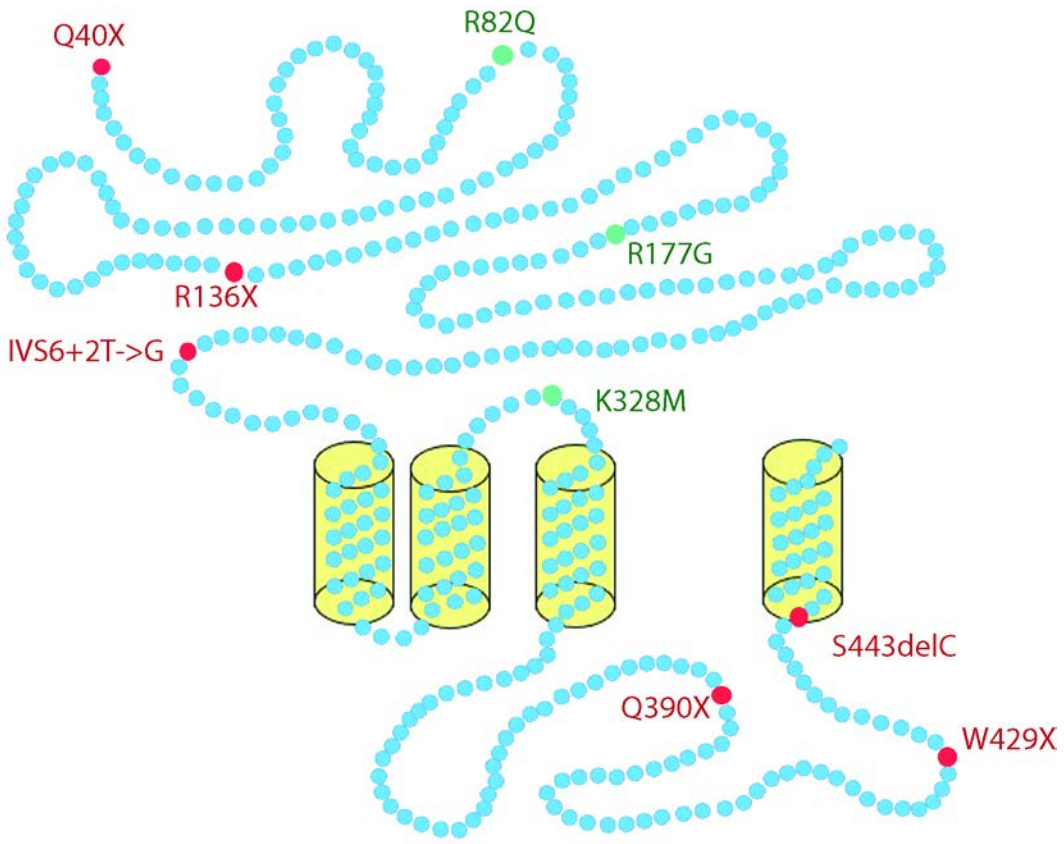

Figure 5-1: Epilepsy-associated mutations identified in GABRG2 gene.

Each blue circle represents one residue of the γ2 subunits. Green circles represent missense mutations and red circles represent mutations generate an alternative stop codon, including nonsense mutations, splice donor site mutations, and frame-shift mutations.

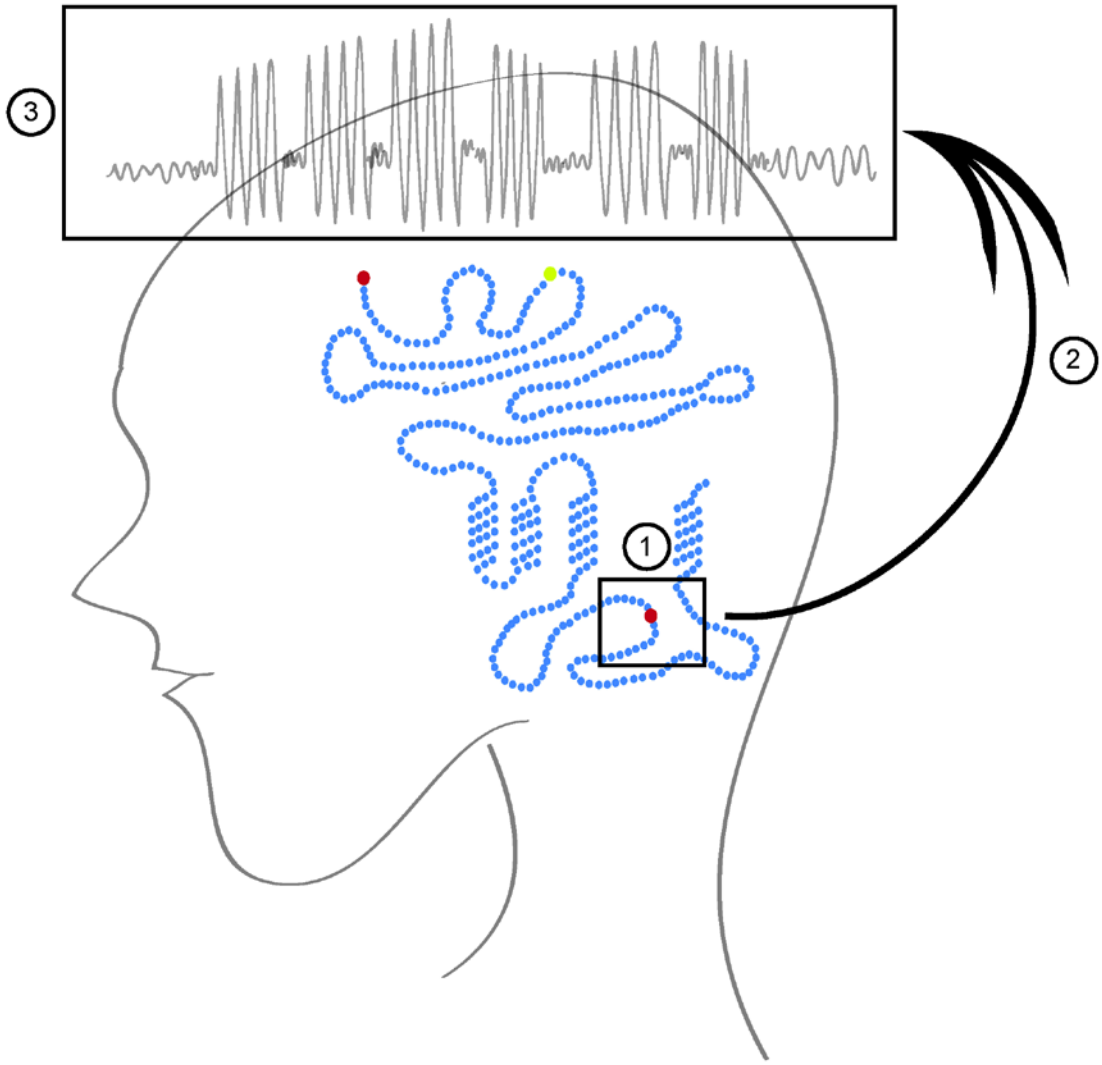

Figure 5-2: Candidate therapeutic targets for treatments of genetic epilepsy.

Mutations ① in epilepsy genes will disrupt the protein function, alter downstream pathway which will change the normal brain to an epileptic brain ②, and finally lead to hyper-excited neuronal firings ③ during seizures. Traditional AEDs are to suppress the hyper-excited neuronal firings while future treatments could be designed to suppress, prevent or erase abnormal hyper-excitability targeting on mutations or pathogenic pathways.

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