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Epilepsia. Author manuscript; available in PMC 2013 December 01.

Published in final edited form as:
Epilepsia. 2012 December ; 53(0 9): 59–70. doi:10.1111/epi.12035.

mRNA surveillance and endoplasmic reticulum quality control processes alter biogenesis of mutant GABA$_A$ receptor subunits associated with genetic epilepsies

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Summary

Previous studies from our and other groups have demonstrated that the majority of γ-aminobutyric acid (GABA)$_A$ receptor subunit mutations produce mutant subunits with impaired biogenesis and trafficking. These GABA$_A$ receptor mutations include missense, nonsense, deletion, or insertion mutations that result in a frameshift with premature translation-termination codons (PTCs) and splice-site mutations. Frameshift or splice-site mutations produce mutant proteins with PTCs, thus generating nonfunctional truncated proteins. All of these mutant GABA$_A$ receptor subunits are subject to cellular quality control at the messenger RNA (mRNA) or protein level. These quality-control checkpoints shape the cell's response to the presence of the mutant subunits and attempt to reduce the impact of the mutant subunit on GABA$_A$ receptor expression and function. The check points prevent nonfunctioning or malfunctioning GABA$_A$ receptor subunits from trafficking to the cell surface or to synapses, and help to ensure that the receptor channels trafficked to the membrane and synapses are indeed functional. However, if and how these quality control or check points impact the posttranslational modifications of functional GABA$_A$ receptor channels such as receptor phosphorylation and ubiquitination and their involvement in mediating GABAergic inhibitory synaptic strength needs to be investigated in the near future.

Keywords

GABA$_A$ receptors; Nonsense mediated decay; Premature translation-termination codons; Endoplasmic reticulum associated degradation; Endoplasmic reticulum retention; Unfolded protein response; Mutation; Idiopathic generalized epilepsies

Genetic Generalized Epilepsies—Channelopathies

Genetic generalized epilepsies (GGEs) (formerly called idiopathic generalized epilepsies) affect about 3% of the general population and account for approximately 30% of all epilepsies (Hauser, 1994). GGEs include multiple epilepsy syndromes that vary in clinical

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Disclosures
The authors have no conflicts of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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severity from the relatively benign childhood absence epilepsy (CAE) to the more severe generalized epilepsy with febrile seizures plus (GEFS+). A subpopulation of patients with GEFS+ have the severe Dravet syndrome that is associated with severe recurrent seizures and cognitive decline (Berkovic et al., 2006b). It has become increasingly clear that mutations of transmembrane ligand- and voltage-gated ion channel genes cause the majority of GGEs, leading them to being referred to as "channelopathies." These channelopathies have many forms of inheritance that include rare (approximately 2% of GGEs) fully penetrant monogenetic alleles found in small pedigrees with autosomal dominant inheritance, common but less penetrant alleles of large effect, polygenetic alleles of small effect in large pedigrees with complex inheritance, and sporadic cases with de novo mutations (Berkovic et al., 2006a; Carranza et al., 2011; Klassen et al., 2011).

Monogenic GGEs with autosomal dominant inheritance have been reported with missense and nonsense mutations in immature and mature ion channel subunit proteins and mutations in noncoding regions including the promoter region and intron spice donor sites. Polygenic GGEs with complex inheritance have been more difficult to investigate. Recently, exome sequencing has demonstrated a common occurrence of nonsynonymous single-nucleotide polymorphisms (nsSNPs) in coding regions of human epilepsy (*hEP*) and non-*hEP* ion channel genes and SNPs in noncoding regions, but SNP frequency or burden was equally high in GGE cases and in family member controls (Klassen et al., 2011). It is likely that the effects on ion channel biogenesis and function of the SNPs found in individuals with GGEs are responsible for their isolated or complex form of GGE, but there are currently no valid approaches to correlate the function of multiple SNPs in all ion channel genes with the epilepsy phenotype. In addition, the genetic background and/or modifying genes (Hawkins et al., 2011) may also contribute to the final clinical presentation, thus further complicating the effects of the SNPs on the epilepsy phenotype. The most direct way to correlate epilepsy mutations with ion channel function has been to study the rare monogenic epilepsy mutations in vitro and in vivo. In vitro studies permit direct characterization of the effects of the mutation itself on ion channel biogenesis and function without the impact of complex neuronal development and network activities. These studies provide data that will guide studies of polygenic mutations of lesser effect, and the results likely are relevant to effects of the mutations in vivo, since the protein synthetic/secretory pathway is highly conserved. In vivo studies in knock-in mice permit evaluation of the function of the mutant ion channels on neuronal and neuronal network function as well as of disease phenotype presentation and progression.

Transcription of mutant genes produces mutant messenger RNAs (mRNAs), and translation of mutant mRNAs produces mutant subunit proteins that alter ion channel biogenesis and/or function. In response to the presence of a mutation, most mutant mRNAs and proteins activate quality control mechanisms to reduce their impact. In this review we discuss these molecular quality control mechanisms and review their involvement in shaping the cellular response to GGE-associated γ-aminobutyric acid $(GABA)_A$ receptor channel subunit gene nonsense and missense mutations. Although we focus on the effects of quality control regulation of $GABA_A$ receptor subunit gene mutations, the general features of quality control regulation of mutant gene expression are applicable to all ion channel gene mutations.

Ion Channel Biogenesis

As with all membrane proteins, once translated, voltage- and ligand-gated ion channel subunit proteins enter the secretory pathway in the endoplasmic reticulum (ER), resulting in processing of individual immature subunits to immature multimeric ion channels that are trafficked through the Golgi apparatus to become mature multimeric ion channels on the

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surface membrane of neurons (Fig. 1). In the first step of this process, multiexon ion channel subunit genes are transcribed in the nucleus to produce premature mRNAs that then interact with the mRNA-splicing machinery to remove introns and to produce mature mRNAs that are translocated to the ER where ion channel protein synthesis occurs. Following translation by ribosomes and translocation into the ER lumen, nascent unfolded subunits are glycosylated and interact with folding catalysts to form disulfide bonds and associate with ER-resident chaperones of the heat shock protein family such as the immunoglobulin binding protein or glucose regulated protein GRP78/BiP(BiP) and lectin chaperones such as calnexin and calreticulin (Kim & Arvan, 1995). The immature subunits also interact with ER quality control enzymes that segregate them to prevent them from aggregating, thus providing time for proper folding (Fink, 1999). During ER retention, an ER-timing mechanism monitors subunit folding and promotes ER-associated degradation (ERAD) of terminally misfolded or unfolded subunits (Mori et al., 1996). For multimeric ion channels, the subunits must oligomerize with appropriate channel subunit partners. When successfully folded and assembled, the immature ion channels are trafficked to the Golgi apparatus where ER core glycans are trimmed and mature glycans are attached, whereas the subunits inside ER remain core-glycosylated or unglycosylated (Kang et al., 2009a,b; Lo et al., 2010). Fully mature ion channels then become cargo in endocytic vesicles and are trafficked to the surface membrane. There are many check points for mRNA and protein quality control during the process of ion channel biogenesis that ensure that $GABA_A$ receptor channels on the cell surface and at synapses are mature and functional. These quality control check points shape the neuronal response to the presence of mutant ion channel subunit proteins and include nonsense-mediated mRNA decay (NMD), ER retention, and ERAD.

Mutations that Produce Premature Translation-Termination Codons May Activate mRNA Quality Control Mechanisms

About one third of human genetic diseases are caused by nonsense, insertion, or deletion/frameshift and splice donor site mutations that generate premature translation-termination codons (PTCs) (Frischmeyer & Dietz, 1999). Translation of mRNAs containing PTCs results in production of truncated subunit proteins that are often, but not always, associated with more severe genetic diseases than are associated with missense mutations (Kang et al., 2009a). Cellular mRNA surveillance mechanisms often degrade mutant PTC-containing mRNAs by NMD, which is a posttranscriptional, but translation dependent, mRNA quality control mechanism (Maquat, 2005). During the pioneering round of translation, the NMD quality control machinery recognizes and initiates degradation of mRNAs that contain a PTC that is 50–55 nucleotides upstream from an exon–exon junction (Isken & Maquat, 2007) or mRNAs with an aberrantly configured 3′ untranslated region (UTR) (Amrani et al., 2004). NMD is intron splicing-dependent and during splicing requires deposition of an exon junctional complex at an exon–exon junction located at least 50–55 nucleotides 3′ of the PTC. Exon junctional complexes contain mRNA decay factors including UPF-1, which is an essential factor for activation of NMD. The majority of transcripts containing PTCs are degraded by NMD, thus reducing intracellular levels of potentially deleterious truncated proteins (Kuzmiak & Maquat, 2006). However, NMD efficiency varies among cell types, and thus, NMD is often not complete, and the levels of intact, undegraded mRNAs vary among different cell types. Most nonsense transcripts are reduced by cellular mRNA surveillance processes, including NMD, to approximately 5–25% of wild-type levels (Kuzmiak & Maquat, 2006).

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Truncated, Misfolded, and Unassembled Ion Channel Subunits Are Subject to ER Quality Control

PTCs in the last exon of multiexon genes, in the penultimate exon of multiexon genes <50–55 nucleotides from the last exon–exon junction, and in single exon genes, do not activate NMD, and thus their mRNAs are not degraded and generate mutant truncated proteins. Because NMD is rarely complete, mutant transcripts that escape NMD will be translated into mutant truncated proteins that are usually misfolded and misrouted, and thus are trafficking deficient (Fig. 1; Kang et al., 2009a). In addition, missense mutations that significantly alter protein structure often result in misfolding of ion channel subunits, which impairs, but may not abolish, subunit–subunit oligomerization and thus impairs assembly of multimeric ion channels. As a result, most of these mutant ion channel subunits also will be trafficking deficient (Kang & Macdonald, 2004; Tan et al., 2007). The cellular fate of misfolded and trafficking-deficient missense mutant subunits is similar to that of mutant truncated $GABA_A$ receptor subunit proteins (Kang & Macdonald, 2004, 2009; Gallagher et al., 2007). Similar to mRNA surveillance at the mRNA level, at the protein level, trafficking-deficient mutant subunits are subject to ER protein quality control, leading to ER retention and ERAD after translation (Stephenson & Maquat, 1996). The mechanisms by which ERAD targets misfolded proteins include the ubiquitin–proteasomal system (Turnbull et al., 2007) and the autophagy–lysosome pathway (Cuervo, 2004; Fig. 1). However, the degradation rate of different subunits harboring different mutations may differ. The relative stability of mutant subunits may vary with the stability of different subunit subtypes and with the nature and location of the mutation. In addition, because NMD efficiency is variable, there may be different amounts of reduction of mutant mRNA by NMD or of reduction of mutant protein by ERAD in neurons in different regions of the brain, during different developmental stages or among different individuals.

Misfolded and Truncated Ion Channel Subunits Retained Inside ER May Activate the Unfolded Protein Response (UPR)

We previously demonstrated that several epilepsy mutant $GABA_A$ receptor subunits were trafficking incompetent and retained in the ER (Kang & Macdonald, 2004). The ER-retained mutant subunits were subject to ERAD and removed from the cells. However, the presence of an overwhelming load of misfolded proteins may activate the UPR, which is an ER cellular stress response. Normally, the ER is capable of recognizing misfolded or unfolded proteins without causing disruption to the functioning of the ER. In conditions of prolonged ER stress, an overwhelming load of misfolded proteins activates a signaling network and activates the UPR. The UPR increases the biosynthetic capacity of the secretory pathway through upregulation of ER chaperone and foldase expression. In addition, the UPR decreases the biosynthetic burden of the secretory pathway by down-regulating expression of genes encoding secreted proteins (Schroder & Kaufman, 2005). For example, accumulation of misfolded or unfolded proteins requires more of the available ER chaperone BiP/Grp78 to bind to their exposed hydrophobic regions. The aim of these responses is to reduce the accumulated protein load while preventing any further addition to the stress, so that normal function of the ER can be restored as soon as possible. The goal of the UPR changes from being one that promotes cellular survival to one that commits the cell to a pathway of apoptosis.

GGEs and $GABA_A$ Receptor Mutations

$GABA_A$ receptors are the primary mediators of fast inhibitory synaptic transmission in the central nervous system, and reduction of $GABA_A$ receptor–mediated inhibition plays an

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important role in many animal models of seizures (Evans et al., 1994; Kapur & Macdonald, 1997; Karle et al., 1998; Poulter et al., 1999; Kohling et al., 2000; Feng et al., 2001; Cohen et al., 2003). These inhibitory receptors are pentamers formed by assembly of multiple subunit subtypes (α1–α6, β1–β3, γ1–γ3, δ, ε, π, θ, and ρ1–ρ3), that are each encoded by a different gene; form chloride ion channels; and most commonly contain two α subunits, two β subunits, and a, γ, or δ subunit. $GABA_A$ receptors mediate both phasic, inhibitory synaptic transmission and tonic, perisynaptic inhibition, and several antiepileptic drugs including benzodiazepines, tiagabine, and barbiturates act by enhancing $GABA_A$ receptor currents (Feng et al., 2004; Jones-Davis et al., 2005).

Mutations in $GABA_A$ receptor subunit hEP genes, *GABRA1*, *GABRB3,* and *GABRG2,* and variants in *GAB-RD* have been associated with multiple GGE syndromes (Fig. 1). Most of these mutations have autosomal dominant inheritance or are sporadic and have been associated with epilepsy syndromes with pure febrile seizures (FS) (Audenaert et al., 2006), CAE (Tanaka et al., 2008), mixed afebrile and febrile seizures (CAE and FS and GEFS+ including Dravet syndrome), or afebrile seizures (Baulac et al., 2001; Wallace et al., 2001; Harkin et al., 2002; Kananura et al., 2002; Dibbens et al., 2004; Sun et al., 2008). The epilepsy mutations include missense, nonsense, and insertion or deletion/frameshift mutations in coding regions as well as promoter and splice donor site mutations in noncoding regions.

GABR Nonsense, Insertion/Deletion-Frameshift, and Splice Donor Site Mutations that Produce PTCs

Six mutations have been reported that generate PTCs among the >20 mutations/variants in *GABRs* that have been associated with genetic epilepsies (Fig. 2; Table 1). The PTCs are in the hEP genes, *GABRA1* and *GABRG2,* and are produced by nonsense mutations (*GABRG2(Q40X)* (Hirose, 2006), *GABRG2(Q390X)* (Harkin et al., 2002))and *GABRG2(W429X)* (Sun et al., 2008), insertion/deletion-frameshift mutations (*GABRA1(S326fs328X)* (Maljevic et al., 2006; Kang et al., 2009b) and *GABRA1(K353de-lins18X*) (Lachance-Touchette et al., 2010), and an intron 6 splice donor site mutation (*GABRG2(IVS6 + 2T → G),* Kananura et al., 2002; Tian & Macdonald, 2012).

GGE-Associated GABR Nonsense Mutations that Activate NMD and ERAD

Four mutations in the $GABA_A$ receptor subunit hEP genes that have been reported to be associated with GGEs have been shown to activate NMD and ERAD (Table 1). These mutations are in *GABRG2* (Q40X, Hirose, 2006; IVS6 + 2T → G, Kananura et al., 2002) and in *GABRA1* (K353delins18X, Lachance-Touchette et al., 2010), 975delC, S326fs328X (Maljevic et al., 2006; Fig. 1). All of the mutations produce mRNAs that are partially degraded by NMD, and truncated proteins that are degraded by ERAD (Table 1; Kang & Macdonald, 2009; Lachance-Touchette et al., 2011; Huang et al., 2012; Tian & Macdonald, 2012).

GABRG2(Q40X)

The *GABRG2* nonsense mutation, Q40X, is located in exon 2 of the 9 exon gene and was identified in heterozygous dizygotic twin sisters with Dravet syndrome (Hirose, 2006). The mutation is in the first amino acid of the predicted mature γ2 subunit (Fig. 2). We found that mutant γ2S subunit mRNA levels were increased significantly after knockdown of the essential NMD factor UPF1, demonstrating that the mutant mRNA was degraded by NMD (Huang et al., 2012). Because Q40 is the first amino acid of the predicted mature γ2 subunit, production of a truncated protein composed only of the signal peptide would be predicted. We found that synthesis of full-length γ2-subunit protein was abolished by Q40X and only

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signal peptide was translated (Huang et al., 2012). Surprisingly, we found that the signal peptide was further cleaved, probably by a signal peptide peptidase (Martoglio, 2003; Xia & Wolfe, 2003) It is possible that the cleavage site for the signal peptide peptidase was better exposed in truncated γ2(Q40X) subunits, thus leading to further cleavage. Although likely, it has not been shown that the truncated γ2(Q40X) subunit is processed by ERAD. Therefore, the *GABRG2(Q40X)* mutation likely causes haploinsufficiency, primarily due to its extreme N terminal location that results in translation of no mature subunits and to activation of NMD that reduces truncated signal peptide production. It has been reported that in addition to membrane targeting, signal peptide fragments may interact with signaling molecules (Martoglio et al., 1997) or be processed as antigenic epitopes (El Hage et al., 2008). It is not known if the novel cleavage pattern of the γ2(Q40X) subunit signal peptide also contributes to epileptogenesis.

GABRG2(IVS6 + 2T → G)

The *GABRG2* splice donor site mutation, IVS6 + 2T → G, is located in intron 6 and was identified in a family with FS and CAE (Kananura et al., 2002). The mutation was predicted to impair splicing of intron 6. This was confirmed by introducing the mutation into intron 6 of *GAB-RG2* cloned into a bacterial artificial chromosome and expressed in vitro in HEK293T cells and in a transgenic mouse (Tian & Macdonald, 2012). In both cases, a cryptic splice donor site in intron 6 was activated, resulting in retention of a portion of intron 6 and a frameshift that resulted in production of a string of 29 alternate amino acids terminated with a PTC in exon 7 (Fig. 2). The exon 7 PTC had an exon–exon junction downstream and thus activated NMD, which partially degraded the *GABRG2(IVS6 + 2T → G)* mRNA. The mRNAs that escaped NMD were translated to a stable, truncated subunit (the γ2-PTC subunit) containing the first six GABRG2 exons and a novel frame-shifted 29 amino acid C-terminal tail. The γ2-PTC subunit was homologous to the mollusk acetylcholine binding protein (AChBP), but in contrast to the AChBP was not secreted from cells. Similar to all of the trafficking-deficient missense or truncated $GABA_A$ receptor subunits, the novel mutant γ2-PTC subunit was retained in the ER and not expressed on the surface membrane. It did, however, oligomerize with α1 and β2 subunits, and imposed a mild dominant-negative effect on $\alpha\beta\gamma$2 receptor assembly. The mutant γ2-PTC subunit protein was retained in the ER and increased ER stress, as evidenced by increased expression of the ER stress marker BiP.

GABRA1(975delC, S326fs328X)

The *GABRA1* deletion mutation, 975delC, S326fs328X, is located in exon 10 of the 11 exon gene and is associated with CAE (Maljevic et al., 2006). The mutation causes a frameshift in *GABRA1,* producing a PTC in exon 10 that is 84 base pairs upstream of intron 10 and causing a subunit that is truncated in transmembrane 3 (Fig. 2). Using an intron 10–containing minigene, we demonstrated that this PTC activated NMD and reduced mutant α1 subunit mRNA (Kang et al., 2009a). The NMD was incomplete leading to production of some truncated subunit protein. However, the truncated mutant α1 subunit protein was unstable, with a half-life that was only about one third that of the wild-type α1 subunit. Consequently, mutant α1(975delC, S326fs328X) subunits were rapidly degraded by ERAD, leading to minimal mutant subunit protein expression. When α1 subunits were immunoprecipitated, however, the mutant α1(975delC, S326fs328X) subunits had increased conjugation with BiP compared to wild-type α1 subunits. These results suggested that the *GABRA1* deletion/frameshift mutation, S326fs328X, resulted in haploinsufficiency by reduction of both mutant mRNA by NMD and mutant subunit protein by ERAD. Due to their instability, the mutant α1(975delC, S326fs328X) subunits had no dominant negative effect on wild-type subunits (Kang et al., 2009a).

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GABRA1(K353delins18X)

The *GABRA1* insertion mutation, K353delins18X, is located in intron 10 and is associated with late-onset, afebrile, generalized tonic–clonic seizures (GTCS) (Lachance-Touchette et al., 2011). The mutation is a 25-bp insertion in intron 10 that prevents cleavage at the 3′ end of the intron, which results in the retention of the entire intron 10 (1,242 bp). The transcript rearrangement results in transcription of 54 bp coding for 18 novel amino acids terminated by a PTC caused by a frameshift at the exon 10–intron 10 junction and produces a subunit that is truncated in the M3/M4 intracellular loop (Fig. 2). Although it has not been demonstrated, the mutant subunit should be subject to quality control via two molecular pathways like α1(975delC, S326fs328X) subunits (Kang et al., 2009a). The mutation should activate NMD, and undegraded mRNA should produce a truncated subunit with deletion of the fourth transmembrane domain that should be degraded by ERAD. With use of complementary DNAs (cDNAs) carrying the predicted truncated subunit sequence, coexpression of the mutant α1 subunit cDNA with γ2 and β2 subunits resulted in α1 subunit ER retention and no recorded current, suggesting that the truncated subunit was trafficking-incompetent (Lachance-Touchette et al., 2011). However, since the identified patients were heterozygous for the insertion, the effect of the mutant protein on partnering subunits is unknown. Further functional study will help elucidate the detailed molecular pathology of this mutation.

GGE-Associated GABR Nonsense Mutations that Do Not Activate NMD but Do Cause ER Retention of Truncated Subunits

Two nonsense mutations in the last exon of *GABRG2* (Q390X, Harkin et al., 2002 and Q429X, Sun et al., 2008) have been reported to be associated with GGEs (Fig. 2). Both of the mutations produce mRNAs that are stable and undegraded by NMD, and truncated proteins that are degraded incompletely by ERAD (Kang et al., 2009a; Table 1).

GABRG2(Q390X)

The *GABRG2* nonsense mutation, Q390X, is located in the intracellular loop of the γ2 subunit between transmembrane domains M3 and M4 and was identified in a family with GEFS+ and Dravet syndrome (Harkin et al., 2002). The PTC is located in the last exon (exon 9) of *GABRG2*, and therefore, would not be expected to activate NMD and should produce a subunit that is truncated in the M3/M4 loop (Fig. 2). We expressed an intron 8–containing γ2 subunit minigene in HEK 293T cells and demonstrated that intron 8 was spliced out and that mutant mRNA was transcribed that was stable and not degraded (Kang et al., 2009a). When cDNAs containing the truncated mutant subunit were cotransfected with α1 and β2 subunits into HEK293T cells, translation resulted in production of a truncated protein that lacked its C-terminal 78 amino acids and was retained in the ER. Because the γ2-subunit mutation Q390X had a dominant negative effect on wild-type partnering α1 and β2 subunits, no GABA-evoked currents were recorded from cells transfected with α1, β2, and γ2(Q390X) subunits (Harkin et al., 2002; Kang et al., 2009a). Currents recorded following heterozygous expression of $\alpha1\beta2\gamma2/\gamma2$(Q390X) subunits were reduced relative to hemizygous control ($2\alpha1{:}2\beta2{:}1\gamma2$) subunit expression. In addition, with heterozygous expression of $\alpha1\beta2\gamma2/\gamma2$(Q390X) subunits, partnering α1 and β2 subunit and wild-type γ2S subunit levels were all reduced more than with hemizygous expression, suggesting that the mutation produced a loss of function of the mutant allele as well as a dominant negative effect on wild-type subunits (Kang et al., 2009a), further reducing wild-type $GABA_A$ receptor channel function. In addition, when expressed alone or with their wild-type partnering subunits, γ2(Q390X) subunits formed high molecular mass protein complexes (Kang et al., 2010) that were very stable and had a half-life more than twice that of wild-type γ2 subunits. With ^{35}S radio labeling, the mutant γ2(Q390X) subunits formed

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the high molecular mass protein complexes within 5 min of the start of protein synthesis. We have studied several different truncated γ2 subunits and found that formation of high molecular mass protein aggregates is a common phenomenon among all the different truncated γ2 subunits. However, some truncated γ2 subunits may be more prone to form the high molecular mass protein complex than others (Kang et al., 2010). The pathologic effect of these high molecular mass protein complexes needs further investigation.

GABRG2(W429X)

The *GABRG2* nonsense mutation, W429X, is located in the intracellular loop of the γ2 subunit between transmembrane domains M3 and M4, and it was identified in a family with GEFS+ (Sun et al., 2008). The PTC is located in the last exon of *GABRG2* and downstream of the *GABRG2(Q390X)* mutation (Fig. 2), and therefore, also would not be expected to activate NMD. Similar to the Q390X mutation, the W429X mutation would be expected to produce truncated γ2 subunits, but for this mutation with the loss of the C-terminal 39 amino acids. The truncated γ2(W429X) subunit would be expected to be trafficking-deficient and subject to degradation by ERAD. However, the function, trafficking, and protein metabolism of mutant *GABRG2(W429X)* subunits, and if the mutant subunit protein has any dominant negative effect on the wild-type subunits are unknown.

GGE-Associated GABR Missense Mutations that Cause ER Retention and Activate ERAD and the UPR

Nine missense mutations in the $GABA_A$ receptor subunit hEP genes and two variants in *GABRD* have been reported to be associated with GGEs (Fig. 3). These mutations are in *GABRA1* (*A322D*, Cossette et al., 2002; *D219N*, Lachance-Touchette et al., 2011), *GABRB3* (P11S, S15F, and G32R, Tanaka et al., 2008), and *GABRG2* (K328M, Baulac et al., 2001; R82Q, Wallace et al., 2001; R177G, Audenaert et al., 2006; *P83S*, Lachance-Touchette et al., 2011; Fig. 3). At least five of the mutations, *GABRG2(R82Q), GABRG2(P83S), GAB-RG2 (R177G), GABRA1(D219N)* and *GABRA1(A322D)* disrupt $GABA_A$ receptor biogenesis (Bianchi et al., 2002; Kang & Macdonald, 2004; Sancar & Czajkowski, 2004; Hales et al., 2005; Feng et al., 2006; Eugene et al., 2007; Tan et al., 2007; Kang et al., 2009a,b; Lachance-Touchette et al., 2010), and the mutant subunits to varying extent were reported to have impaired folding and impaired oligomerization with other subunits and to be subject to ER retention and subsequent degradation by ERAD (Table 1).

GABRG2(R82Q)

The *GABRG2* missense mutation, R82Q, is located in the distal N terminus of the γ2 subunit (Fig. 3) and is associated with FS (Wallace et al., 2001). An autosomal dominant form of CAE was also present in the family pedigree, and it was demonstrated that an interaction of *GABRG2* with another gene or genes is required for the CAE phenotype in this family (Marini et al., 2003). *GABRG2(R82Q)* knock-in mice are the first knock-in mice to harbor a human epilepsy mutation in a *GABR* subunit gene, and heterozygous mice displayed the CAE phenotype in both C57/BL and DBA/2J backgrounds, arguing against the requirement of other genetic factor for CAE phenotype manifestation in this mutation, at least in mice (Tan et al., 2007). Alignment of γ2 subunit and AChBP sequences revealed that R82 is positioned at the γ2/β2 subunit–subunit interface, and it was demonstrated that the mutation impaired γ2 and β2 subunit oligomerization (Hales et al., 2005). This impairment was likely the basis for this mutation's reduction of surface $\alpha1\beta2\gamma2$ receptors (Bianchi et al., 2002; Kang & Macdonald, 2004; Sancar & Czajkowski, 2004; Eugene et al., 2007; Frugier et al., 2007), ER retention of unassembled γ2(R82Q) subunits (Frugier et al., 2007; Kang & Macdonald, 2004), and reduction of $GABA_A$ receptor currents (Bianchi et al.,

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2002; Kang & Macdonald, 2004). In γ2(R82Q) knock-in mice, total levels of mutant γ2S(R82Q) subunits were reduced compared to wild-type mice (Tan et al., 2007), suggesting that mutant γ2(R82Q) subunits were also subject to ERAD (Table 1). However, the impact of the mutant γ2(R82Q) subunits on the wild-type $GABA_A$ receptor subunit expression profile and on neuronal development are unknown.

The R82Q mutation also caused intracellular retention and reduced surface expression of $GABA_A$ receptors in cortical pyramidal neurons (Tan et al., 2007), reduced miniature inhibitory postsynaptic currents in layer II/III cortical neurons, and electrographic and behavioral seizures in R82Q knock-in mice. Endogenous expression of α5 subunits in cultured hippocampal neurons was reduced when coexpressed with γ2(R82Q) subunits, indicating that γ2(R82Q) subunits conferred a dominant negative effect (Eugene et al., 2007). In addition, it is possible that a deficit in γ2 subunits caused a compensatory increase in other subunits, such as δ or β subunits. Because $\alpha\beta\delta$ and $\alpha\beta$ receptors are extrasynaptic or perisynaptic, this compensatory increase may result in a relative increase in tonic currents. Recently, it has been reported that extrasynaptic GABAergic “tonic” inhibition was increased in thalamocortical neurons from both genetic and pharmacologic models of absence epilepsy (Cope et al., 2009), consistent with this conclusion.

GABRG2(P83S)

The *GABRG2* missense mutation, *P83S*, is located in the distal N terminus in a location adjacent to R82 (Fig. 3), and in a large family was associated with FS and GGEs over three generations (Lachance-Touchette et al., 2011). Surface expression of the receptors containing the mutant subunit was not reported, but GABA-evoked currents recorded from cells coexpressing α1 and β2 subunits with wild-type γ2 or mutant γ2(P83S) subunits were similar as, were their benzodiazepine and zinc sensitivities. It is unclear why there was no alteration in current produced by this mutation. Given the adjacency of this mutation with *GABRG2(R82Q)*, it is likely that this mutation may have similar trafficking and functional consequence as *GABRG2(R82Q)* mutation, but this needs to be confirmed.

GABRG2(R177G)

The *GABRG2* missense mutation, R177G, is located in the N-terminus 13 amino acids N terminal to the first cystine in the Cys loop (Fig. 3) and has been associated with FS (Audenaert et al., 2006). The γ2 subunit R177 residue is conserved among γ2 subunits across species. Basic residues are conserved among other γ subunits, and in other Cys-loop receptors; polar and charged amino acid residues occur at this position. Mutant α1β3γ2L(R177G) receptors had altered current kinetics and reduced benzodiazepine sensitivity (Audenaert et al., 2006), but the underlying molecular mechanisms for FS associated with this mutation are unclear. Investigation is needed to determine if the reduced benzodiazepine sensitivity is due to impaired incorporation of γ2 subunits into the pentamers. We have demonstrated that the mutant γ2L(R177G) subunit has impaired oligomerization with α1 and β3 subunits and is retained in the ER (Schwartz E, Gurba K, Botzolakis I, Stanic, Macdonald RL, unpublished data).

GABRA1(D219N)

The *GABRA1* missense mutation, D219N, is located in the N terminus of the α1 subunit 39 amino acids C terminal to the second cystine in the Cys loop and replaces a charged aspartate residue with a small, neutral residue (Fig. 3). The mutation is associated with FS with or without GTCS and absence seizures (Lachance-Touchette et al., 2011). When α1(D219N) β2γ2 subunits were coexpressed in vitro, surface expression of α1(D219N) subunits was present but reduced (Lachance-Touchette et al., 2011). Whole-cell current amplitudes were also reduced, and the kinetic properties of the currents were altered by the

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mutation with slower desensitization and faster current deactivation. Based on these findings, it is likely that mutant α1(D219N) β2γ2 receptors have impaired trafficking with ER retention and possibly impaired channel function; however, further study is needed to identify whether impaired trafficking or channel function are most affected.

GABRA1(A322D)

The *GABRA1* missense mutation, A322D, replaces a small, neutral residue with a larger negatively charged aspartate residue in the M3 transmembrane helix (Fig. 3), and it is associated with an autosomal dominant form of juvenile myoclonic epilepsy (Cossette et al., 2002). This nonconserved mutation was shown to impair α1-subunit folding by destabilizing insertion of the M3 domain into the lipid bilayer (Gallagher et al., 2007). When mutant α1(A322D), β2, and γ2 subunits were coexpressed in HEK293T cells, both total and surface α1 subunit levels were reduced and an intermediate effect was found with heterozygous subunit expression. Loss of the misfolded mutant subunit was due to ERAD (Gallagher et al., 2005) and lysosomal degradation (Bradley et al., 2008). Peak GABA-evoked currents were significantly reduced with both heterozygous and homozygous α1(A322D) subunit expression, consistent with the impaired folding and assembly of the mutant α1(A322D) subunits (Table 1; Fisher, 2004; Gallagher et al., 2004; Maljevic et al., 2006). Recently, we demonstrated that the presence of the undegraded, misfolded α1(A322D) subunit produces a small dominant negative effect that alters the composition and further reduces the expression of wild-type $GABA_A$ receptors (Ding et al., 2010).

Discussion

Although the intracellular processing, posttranslation modifications, and channel function of the mutant $GABA_A$ receptor subunits have been characterized in great detail, there is still lack of understanding of the impact of these mutations in a complex neuronal milieu and in a neuronal context, due to the limited mutation knock-in animals and the unavailability of human patient brains. The number of postsynaptic $GABA_A$ receptors determines inhibitory synaptic strength, and a defect receptor function due to a mutation in a $GABA_A$ receptor subunit would impair overall inhibitory strength. However, there are multiple types of GABAergic interneurons, and the impaired inhibition could be both location- and time-dependent given the differential spatial distribution, synaptic contact, and innervation of neurons (Jadi et al., 2012). Furthermore, the magnitude of changes observed in vitro with a simple cell system may not be easily translated to the change in vivo in a complex brain network. Nevertheless, the basic cell quality control machineries such as mRNA surveillance, ER retention, and ER-associated protein degradation should be conserved from non-neuronal cell system to neurons and from mice to humans.

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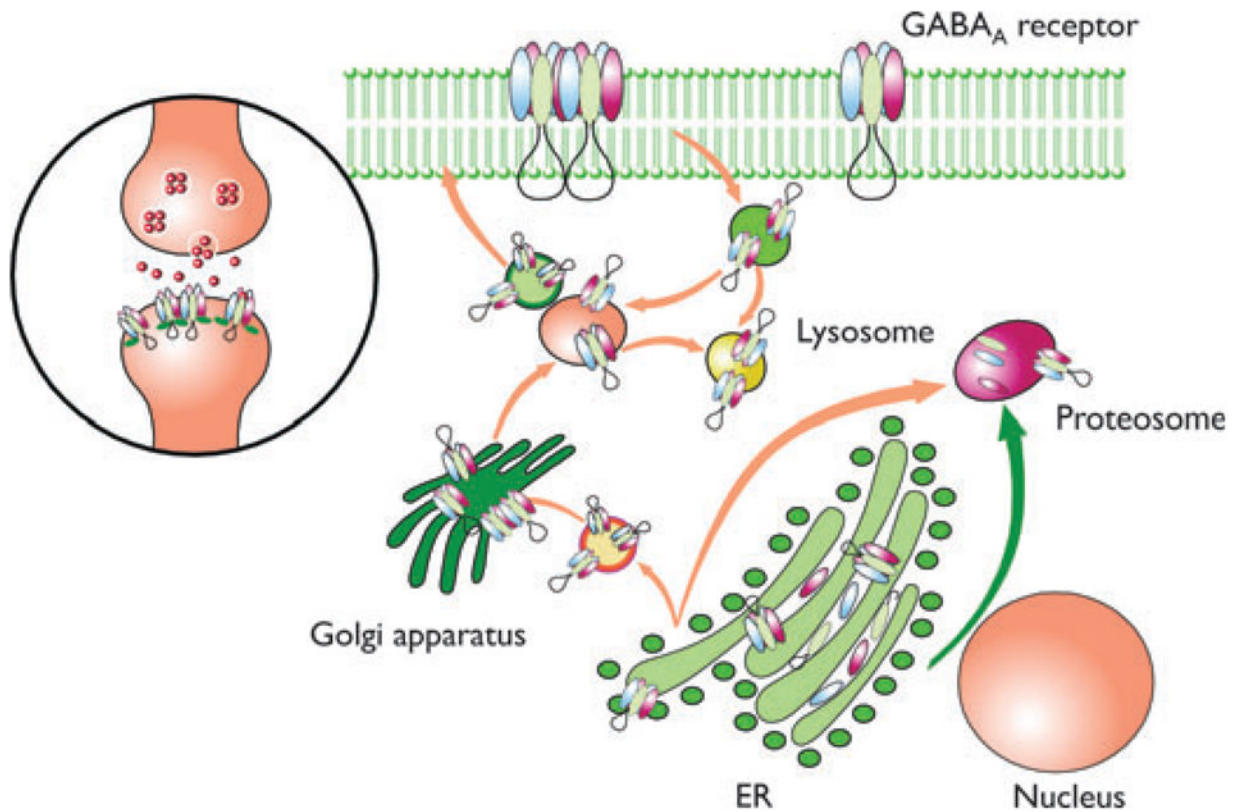

Figure 1.
Schematic representation showing the process of GABA$_A$ receptor subunit biogenesis, assembly, and trafficking. GABA$_A$ receptor subunits are synthesized and directed to the ER by their signal peptide. The signal peptide is then cleaved. The mature subunit peptides oligomerize with their assembly partners into pentamers and are translocated to the Golgi apparatus for further maturation. Finally the mature functional pentameric receptor channels are trafficked to the cell surface and to synapses. The mutant subunits are subject to NMD, ER retention, and ERAD. Therefore, the mutant subunits are unlikely to be present on the cell surface and in synapses as are wild-type receptors. Many PTC-generating mutant mRNAs are subject to NMD. The mRNAs escaping from NMD will be translated and subject to ER retention and ERAD as subunits produced by missense mutations due to the trafficking deficiency of the translated mutant subunits. The arrows designate the targeted subcellular locations of wild-type or mutant subunits. Modified from Kang & Macdonald (2009).
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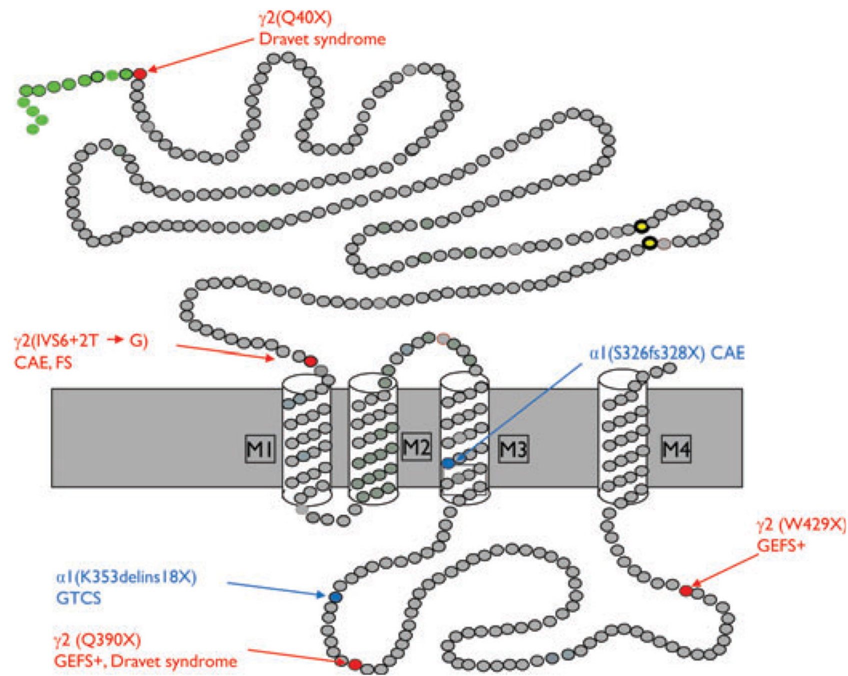

Figure 2.

Schematic representation of $GABA_A$ receptor subunit topology showing the location of mutation-generated PTCs associated with GGEs. Modified from Macdonald et al., 2012. CAE, childhood absence epilepsy; FS, febrile seizures; GEFS+, generalized epilepsy with febrile seizures plus; GTCS, generalized tonic–clonic seizures.

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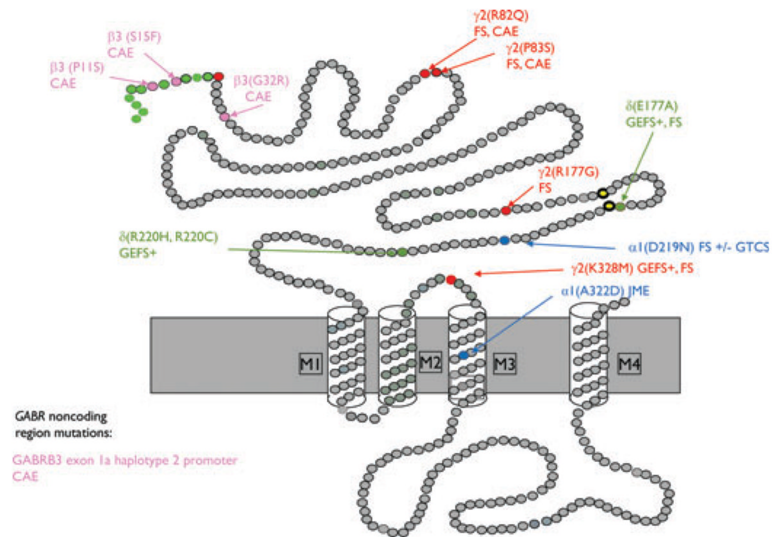

Figure 3.

Schematic representation of $GABA_A$ receptor subunit topology showing the location of missense mutations associated with GGEs. Modified from Macdonald et al. (2012). CAE, childhood absence epilepsy; FS, febrile seizures; GEFS+, generalized epilepsy with febrile seizures plus; JME, juvenile myoclonic epilepsy; GTCS, generalized tonic–clonic seizures. *Epilepsia* © ILAE

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Table 1

$GABA_A$ receptor subunit mutations and variants associated with GGEs and their postulated molecular defects

Gene	Mutations/variants	Locus	Postulated mechanisms	Phenotypes	References
	Nonsense mutations				
GABRG2	Q40X	5q34	NMD, ERAD	DS	Hirose et al. (2005)
GABRG2	IVS6+2T->G	5q34	NMD, ERAD	CAE, FS	Tian & Macdonald (2012)
GABRA1	975delC, S326fs328X	5q34	NMD, ERAD	CAE	Kang et al. (2009a, 2009b)
GABRA1	K353delins18X	5q34	NMD, ERAD?	GTCS	Lachance-Touchette et al. (2011)
GABRG2	Q390X	5q34	ER Retention, dominant negative effect	GEFS+, DS	Harkin et al. (2002)
GABRG2	W429X	5q34	ER Retention, dominant negative effect?	GEFS+	Sun et al. (2008)
	Missense mutations				
GABRG2	R82Q	5q34	Impaired oligomerization, ER retention	FS, CAE	Wallace et al. (2001)
GABRG2	P83S	5q34	Impaired oligomerization, ER retention	FS, CAE	Lachance-Touchette et al. (2011)
GABRG2	R177G	5q34	Impaired oligomerization, ER retention	FS	Audenaert et al. (2006)
GABRA1	D219N	5q34	Impaired oligomerization, ER retention	FS +/– GTCS, CAE	Lachance-Touchette et al. (2011)
GABRA1	A322D	5q34	Impaired folding, ERAD	JME	Cossette et al. (2002)
GABRB3	P11S	15q11-14	Impaired intracellular processing?	CAE	Delahanty et al. (2009)
GABRB3	S15F	15q11-14	Hyperglycosylation?	CAE	Tanaka et al. (2008)
GABRB3	G32R	15q11-14	Gating defect, impaired trafficking?	CAE	Tanaka et al. (2008)
GABRG2	K328M	5q34	Gating defect	FS, GEFS+	Baulac et al., (2001)
	Variants				
GABRD	E177A	1p36	Gating defect	GEFS+	Dibbens et al. (2004)
GABRD	R220H	1p36	Gating defect	JME	Dibbens et al. (2004)
	Other				
GABRB3	-897 T/C polymorphism	15q11-14	Decreased transcription	CAE	Urak et al. (2006)

NMD, nonsense-mediated mRNA decay; ER, endoplasmic reticulum; ERAD, ER-associated degradation; CAE, childhood absence epilepsy; FS, febrile seizures; GEFS+, generalized epilepsy with febrile seizures plus; JME, juvenile myoclonic epilepsy; GTCS, generalized tonic–clonic seizures.