MOLECULAR PATHOLOGY OF GENETIC EPILEPSIES ASSOCIATED WITH GABA_A RECEPTOR SUBUNIT MUTATIONS

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Mutations in ligand-gated ion channel genes associated with idiopathic generalized epilepsies have been reported in excitatory acetylcholine receptor $\alpha 4$ and $\beta 2$ subunit genes linked to autosomal dominant nocturnal frontal lobe epilepsy and in inhibitory GABA_A receptor $\alpha 1$, $\beta 3$, $\gamma 2$, and δ subunit genes associated with childhood absence epilepsy, juvenile myoclonic epilepsy, pure febrile seizures, generalized epilepsy with febrile seizures plus, and generalized epilepsy with tonic-clonic seizures. Recent studies suggest that these mutations alter receptor function or biogenesis, including impaired receptor subunit messenger RNA stability, receptor subunit protein folding and stability, receptor assembly, and receptor trafficking.

Idiopathic generalized epilepsies (IGEs) presumably are a group of genetic epilepsies that exhibit several clinical phenotypes, including childhood absence epilepsy, juvenile myoclonic epilepsy, generalized epilepsy with febrile seizures plus (GEFS+) and tonic-clonic seizures (1), and are among the most common neurological disorders, affecting about 1% of the population worldwide (2). Genetic advances and functional characterization of the pathophysiological alterations in receptor channel trafficking and function are enhancing the understanding of the cellular pathophysiology of IGEs. It has become clear that transmembrane ion channel mutations are the underlying cause of many IGEs (3). Mutations in voltage-gated ion channels

(sodium, calcium, and potassium) and ligand-gated ion channels (nicotinic cholinergic receptor and GABA_A receptor) have been reported to be associated with IGEs. This article will review the molecular pathology of IGEs caused by mutations in one of the ligand-gated ion channels, the GABA_A receptor, which is the primary mediator of fast inhibitory synaptic transmission in the CNS.

Mutations in GABA_A Receptor Subunit Genes

GABA_A receptors are members of the cys-loop family of ligand-gated ion channels, which also includes nicotinic cholinergic, serotonin 5-HT₃, and glycine receptors. GABA_A receptors are formed by pentameric assemblies of different subunit subtypes ($\alpha 1$ - $\alpha 6$, $\beta 1$ - $\beta 3$, $\gamma 1$ - $\gamma 3$, δ , ϵ , π , θ , and $\rho 1$ - $\rho 3$), and form chloride ion selective channels (4). The majority of GABA_A receptors contain two α subunits, two β subunits, and a γ or δ subunit (5). GABA_A receptors mediate both phasic inhibitory synaptic transmission and tonic perisynaptic inhibition, and the mechanism of action of several antiepileptic drugs is the enhancement of GABA_A receptor currents. GABA_A receptor mutations or variants associated with IGEs have been reported in $\alpha 1$, $\beta 3$, $\gamma 2$, and δ subunits (see Figure 1) (6). Mutations in subunit genes can be divided broadly into four classes: 1) missense, 2) nonsense, and 3) frameshift mutations in coding sequences as well as 4) mutations in noncoding sequences (intronic, 3' downstream, or 5' upstream mutations).

Missense mutations alter the nucleotide sequence in a codon, resulting in the incorporation of a different amino acid in the mature peptide or the signal peptide and altering the triplet genetic code. The altered amino acid can result in a benign polymorphism, a variant that can confer susceptibility to a disease, or a mutation that is associated with a disease. In general, GABA_A receptor subunit missense mutations impair surface expression of receptors and/or impair receptor channel kinetic properties (6). Reduced surface expression of receptors harboring missense mutations is due to multiple mechanisms, such as altered folding, impaired assembly, and endoplasmic reticulum retention, often leading to loss of the mutant proteins by endoplasmic reticulum-associated degradation. GABA_A receptor missense mutations in mature subunits include: GABRA1(A322D), GABRB3(G32R), GABRG2(R43Q), GABRG2(R139G), and GABRG2(K289M); GABA_A receptor subunit missense mutations in signal peptides include: GABRB3(P11S) and GABRB3(S15F); and GABA_A receptor subunit missense mutations producing susceptibility variants include: GABRD(E177A) and GABRD(R220H).

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GABA_A receptor subunit gene mutations associated with epilepsy

FIGURE 1. Schematic representation of the GABA_A receptor subunit topology, showing the location of autosomal dominant epilepsy mutations associated with different idiopathic generalized epilepsies. SMEI, myoclonic epilepsy of infancy; CAE, childhood absence epilepsy; GEFS+, generalized epilepsy with febrile seizures plus; JME, juvenile myoclonic epilepsy; FS, febrile seizures.

Nonsense mutations produce alteration of the nucleotide sequence in codons that result in the introduction of a stop codon or premature translation–termination codon. Premature translation–termination codon-generating mutations in the last exon of a multi-exon gene or less than 50–55 nucleotides upstream of the last exon–exon junction produce a truncated protein, while premature translation–termination codon-generating mutations that are not in the last exon of a multi-exon gene or are more than 50–55 nucleotides upstream of the last exon–exon junction produce mRNA degradation through activation of nonsense-mediated decay (NMD)—a cellular mRNA quality-control system that eliminates production of the mutant protein (7). GABA_A receptor nonsense mutations include: GABRG2(Q1X), GABRG2(Q351X), and GABRG2(W390X).

Frameshift mutations occur because the deletion or insertion of one or two nucleotides causes a change in downstream codons, with or without a change in the frameshifted codon. In addition to altering the downstream amino acid sequence, these frameshifts often result in production of a premature translation–termination codon. One GABA_A receptor subunit frameshift mutation, GABRA1(975delC, S326fs328X), has been reported. Mutations can also occur in noncoding sequences, such as in introns, or either upstream or downstream of the GABA_A receptor open reading frame. A GABA_A recep-

tor subunit mutation in a noncoding intron splice donor site GABRG2(IVS6 + 2T→G) has been described.

GABA_A Receptor Subunit Missense Mutations

γ2 subunit missense mutation, R43Q

The GABA_A receptor $\gamma 2$ subunit mutation, R43Q, is located in the distal N-terminus of the $\gamma 2$ subunit and is associated with childhood absence epilepsy and febrile seizures (see Figure 1) (8). The functional and cellular consequences of this mutation have been shown in vitro and in vivo due to reduction of surface $\gamma 2$ subunit protein with heterozygous expression (9). Investigators have demonstrated that expression in HEK 293-T cells of heterozygous $\alpha 1\beta 2\gamma 2/\gamma 2(R43Q)$ or homozygous $\alpha 1\beta 2\gamma 2(R43Q)$ receptors resulted in reduced peak current and surface receptors (9,10). In vivo, in mice engineered with the mutation $\gamma 2(R43Q)$, a small but significant reduction of GABA_A receptors in cortical pyramidal neurons also was observed (11). Using fusion proteins, in which DNA-encoded enhanced yellow fluorescent protein was inserted between amino acids four and five of the mature $\gamma 2$ subunit and fluorescence microscopy, Tan et al. demonstrated that the reduced surface expression of the mutant protein resulted from endoplasmic retention of the mutant protein. It has been shown that the mutation impairs subunit oligomerization (12). Thus, the reduced

surface expression of heterozygous $\alpha 1\beta 2\gamma 2S(R43Q)$ receptors was a consequence of receptor endoplasmic retention, secondary to impaired subunit folding or assembly and/or to impaired receptor trafficking.

$\gamma 2$ subunit missense mutation, K289M

The GABA_A receptor $\gamma 2$ subunit mutation, K289M, is located in the short extracellular loop between transmembrane domains M2 and M3 (M2–M3 loop) (see Figure 1), a region implicated in the gating of ligand-gated ion channels and associated with an autosomal dominant generalized epilepsy, a condition similar to GEFS+ (13). The effects of this mutation were investigated using a rapid application concentration jump technique (open tip application rise time of 1 msec), applying GABA for brief (2–5 msec) durations, and the excised outside-out patch clamp recording technique (14); $\alpha 1\beta 3\gamma 2L(K289M)$ receptors had more rapid current deactivation than wild-type receptors. Single channel currents from homozygous $\alpha 1\beta 3\gamma 2(K289M)$ receptors had mean open times that were one-fourth as long as wild-type $\alpha 1\beta 3\gamma 2L$ receptor currents, consistent with its more rapid whole cell current deactivation time (10). Brief, rapid GABA applications to excised macropatches evoked currents that were similar to IPSCs. Therefore, reduction of the duration of rapid GABA-evoked current by the $\gamma 2L(K289M)$ subunit mutation suggests that it would result in reduced IPSC duration, thus producing disinhibition that may lead to epilepsy.

$\gamma 2$ subunit missense mutation, R139G

The GABA_A receptor $\gamma 2$ subunit mutation, R139G, is in the N-terminus of the $\gamma 2$ subunit and is associated only with febrile seizures (15). The R139 residue is conserved among $\gamma 2$ subunits across species, and basic residues are conserved among other γ subunits. Within the cys-loop family, polar and charged amino acid residues occur at this position. Residue R139 is located at a highly conserved and likely aqueous accessible part of the amino terminus in a hairpin loop between β sheets 5 and 6. It has been demonstrated that mutant $\alpha 1\beta 3\gamma 2L(R139G)$ receptors had subtly altered current kinetics and reduced benzodiazepine sensitivity. Future study of the mutant protein maturation and receptor trafficking will further elucidate the molecular defect of this mutation in epilepsy.

$\alpha 1$ subunit missense mutation, A322D

The GABA_A receptor $\alpha 1$ subunit mutation (A322D) introduces a negatively charged aspartate into the middle of the M3 transmembrane helix of the $\alpha 1$ subunit at residue A322 and is associated with autosomal dominant juvenile myoclonic epilepsy (16). Cossette and colleagues found that when co-expressed with wild-type $\beta 2$ and $\gamma 2$ subunits, the mutant $\alpha 1(A322D)$ subunit reduced both total and surface $\alpha 1$ subunit levels and had an intermediate effect on heterozygous subunit expression ($\alpha 1:\alpha 1(A322D) = 1:1$ cDNA ratio) (17). Peak

GABA-evoked currents were significantly reduced in both heterozygous and homozygous conditions. Gallagher et al. demonstrated that this nonconserved mutation in a transmembrane domain destabilized M3 α helix formation and impaired $\alpha 1$ subunit folding and pentamer assembly (18). The loss of the misfolded mutant protein was due to an endoplasmic retention quality-control process, endoplasmic retention-associated degradation (19), and lysosomal degradation (20).

$\beta 3$ subunit missense mutations, P11S, S15F, and G32R

Two mutations (P11S, S15F) in the GABA_A receptor $\beta 3$ subunit signal peptide in exon 1a and a mutation (G32R) in the mature $\beta 3$ subunit of exon 2 have been associated with childhood absence epilepsy (21). The underlying molecular defect might be abnormal protein expression and/or impaired receptor trafficking, but the mechanism is still under study. Heterozygous and homozygous null mutant *GABRB3* mice show absence-like features, including the EEG characteristics and pharmacological responses to antiepileptic drugs that are typical for absence seizures. *GABRB3* deletion also has been associated with Angelman syndrome in which absence seizures are present. In addition, a *GABRB3* promoter haplotype impairing transcriptional activity has been associated with childhood absence epilepsy. Thus, mutation of the *GABRB3* subunit likely contributes to generation of childhood absence epilepsy, but this involvement needs to be clarified further.

δ Subunit Missense Variants, E177A, R220C, and R220H

Monogenic mutations only account for the pathogenesis of a small portion of IGE syndromes. Most of the idiopathic epilepsies are polygenic, requiring additive actions of a set of susceptibility genes. Dibbens et al. reported the first GABA_A receptor susceptibility gene, *GABRD*, for IGEs (22). Two putative missense mutations in *GABRD* were identified: $\delta(E177A)$ was detected in a small GEFS+ family and $\delta(R220C)$ was detected in a second small GEFS+ family. Both mutations were heterozygously associated with epilepsy in these pedigrees. In addition, a polymorphic allele, $\delta(R220H)$, has been associated with juvenile myoclonic epilepsy patients but also is found in the general population.

The δ subunit variant, E177A, is adjacent to one of the invariant cysteines that form a disulfide bond—the signature feature of cys-loop receptors. The $\delta(R220)$ residue is localized about in the middle, between the $\delta(E177A)$ variant and the entrance to the first transmembrane domain (M1). The current amplitudes of heterozygous or homozygous receptors harboring either $\delta(E177A)$ or $\delta(R220H)$ subunits were significantly reduced compared to those of wild-type receptors (23). The current amplitudes of heterozygous or homozygous $\alpha 1\beta 2\delta(R220C)$ receptors were not significantly different from those of wild-type receptors, but the finding requires further

investigation. Combining both single channel recording and biochemistry data, the basis for the reduced peak current of these δ variant harboring receptors was determined to be primarily due to the reduced mean channel open time and a small reduction in the surface receptor expression (23).

GABA_A Receptor Subunit Nonsense Mutations

$\gamma 2$ subunit nonsense mutation, Q1X

A GABA_A receptor $\gamma 2$ subunit mutation that introduced a premature translation–termination codon, Q1X, between the signal peptide and mature peptide was identified in a family with severe myoclonic epilepsy of infancy (24). The $\gamma 2$ subunit Q1X mutation likely triggers NMD, although activation of NMD has not been demonstrated. However, if the Q1X mutation resulted in NMD completely and if the mutant $\gamma 2S$ subunit does not interfere with transcription of the wild-type $\gamma 2S$ subunit at the mRNA level, no epilepsy would result (as observed in $\gamma 2$ [+/-] gene deletion heterozygous mice). It is also unclear why, in the pedigree, both the twins carrying the mutation were diagnosed with myoclonic epilepsy of infancy, while the father with the de novo mutation was seizure-free. Future studies focusing on gene expression and the completeness of NMD may elucidate the underlying molecular pathology.

$\gamma 2$ subunit nonsense mutation, Q351X

A GABA_A receptor $\gamma 2$ subunit mutation, Q351X, is associated with a family with GEFS+, including two family members with febrile seizures and a member with severe myoclonic epilepsy of infancy (25). The Q351X mutation is located in the $\gamma 2$ subunit intracellular loop between transmembrane domains M3 and M4 (see Figure 1) and results in a premature translation–termination codon with loss of the downstream 78 amino acids. This mutation was studied by using green fluorescent protein-tagged $\gamma 2$ subunits (25). The receptor containing this mutation was not expressed on the surface, but was retained in the endoplasmic reticulum. Consistent with this finding, no GABA-evoked current was recorded from oocytes expressing the mutant receptors. Genetic mutations producing premature translation–termination codons can result in C-terminally truncated proteins that can produce dominant negative inhibition of full-length proteins, thus potentially harming the cells. It is also intriguing that there is a great intrafamilial phenotype variation with this mutation; yet, the basis for this variation is unknown.

$\gamma 2$ subunit nonsense mutation, W390X

A GABA_A receptor $\gamma 2$ subunit mutation, W390X, is associated with a family with GEFS+, with the majority of family members having febrile seizures plus and one member having febrile seizures (26). The W390X mutation is located in the $\gamma 2$ subunit intracellular loop between transmembrane do-

main M3 and M4 (see Figure 1) and results in a premature translation–termination codon with loss of the downstream 39 amino acids. Identification of this mutation was published recently, and the underlying molecular mechanisms are still unknown.

GABA_A Receptor Subunit Frameshift Mutations

$\alpha 1$ subunit mutation, 975delC, S326fs328X

The GABA_A receptor $\alpha 1$ subunit mutation, 975delC, S326fs328X, is an autosomal dominant mutation associated with childhood absence epilepsy (27), and thus, the patients are heterozygous. The mutation causes frameshift in GABRA1 that results in a premature translation–termination codon in an early exon, exon 8, and is 84 base pairs upstream of intron 8. Based on the 50–55 nucleotides rule, this frameshift mutation is likely to trigger NMD. Using an intron-inclusion minigene (MG) approach, which elicits NMD should it occur, it has been shown that heterozygous expression of $\alpha 1$ MG/ $\alpha 1$ (975delC, S326fs328X)MG $\beta 2\gamma 2S$ subunits were found in HEK 293-T cells and resulted in functional haploinsufficiency. Using real-time polymerase chain reaction, mutant mRNA was demonstrated to be substantially reduced with concomitant loss of the mutant transcripts. Only a minimal amount of mutant $\alpha 1$ (975delC, S326fs328X) subunit protein was detected, and the loss of the mutant protein likely was due primarily to the activation of NMD.

GABA_A Receptor Subunit Splice Donor Site Mutations

$\gamma 2$ subunit splice donor site mutation, IVS6 + 2T → G

The GABA_A receptor $\gamma 2$ subunit mutation, IVS6 + 2T → G, is the first mutation identified in a noncoding region of a GABA_A receptor gene. The point mutation is in the splice-donor site in the *GABRG2* intron 6 (IVS6 + 2T → G) and has been identified in a family with childhood absence epilepsy and febrile seizures (see Figure 1) (28). The effect of this mutation on GABA_A receptor function is unknown but was predicted to lead to a nonfunctional protein through exon skipping, which would result in a new premature translation–termination codon at the 5th and 7th exon junction site. Thus, it is very likely that this premature translation–termination codon may also trigger NMD, thus eliminating expression of mutant protein at the mRNA level. Therefore, the underlying mechanism for this splice donor site mutation also may be due to haploinsufficiency. In fact, with the NMD-sensitive minigene approach, it was shown that the mutant $\gamma 2$ (IVS6 + 2T → G) subunit minigene only produced a minimal amount of the mutant protein compared with the wild-type $\gamma 2$ minigene, suggesting that the mutation may activate NMD. Ongoing research at the transcription level will further elucidate the underlying molecular mechanisms.

Conclusions

Despite substantial advances in the understanding of the molecular pathogenesis of various GABA_A receptor epilepsy mutations, there are still many unanswered questions about the pathology of IGEs. It has been difficult to develop an appropriate experimental model that adequately reproduces the functional alterations evidenced by the mutations in these patients. The majority of the studies of the pathophysiology of IGEs have been obtained using heterologous expression systems. Available hemizygous subunit gene deletion mice may reflect the function of some gene mutations, but gene knockout may be different from a loss-of-function mutation. In addition, most of the mutations are not fully penetrant, and there is a great intrafamilial phenotypic variation, suggesting that there are other modifying genes in the background that influence the development and persistence of the epilepsy, but this possibility needs to be clarified. Another unknown variable is the cellular response to the presence of mutant protein. Cells that are overloaded with misfolded proteins are under stress and activate the unfolded protein response, which may alter the biogenesis of wild-type receptors and, thus, produce further compromise of GABAergic inhibition and/or differential response upon cellular stress. The developmental aspects of the expression of a mutant subunit and the compensatory expression of other functionally overlapping subunits have not been studied. Different GABA_A receptor subunit genes are active at different stages of brain development, and the impact of this developmentally regulated expression of subunits on neuronal excitability is likely to be substantial. It has been shown in a number of studies that neurons can compensate for loss of a subunit by regulating partnering subunits either up or down, which may compensate for or exacerbate the loss of inhibition stemming from the loss of a functional subunit. Ultimately, it is essential to determine the specific effect of a mutation on expression of GABA_A receptors in a network context during development. This effect is a critical piece of the puzzle that will allow blending of the molecular pathogenesis of these mutations and the process of epileptogenesis.

Given the complexity of gene transcription and translation, as well as complications involved in receptor trafficking from the endoplasmic reticulum to cell surface and synapses in neurons, it would not be surprising if mutations occurring in other genes of associated proteins (e.g., chaperones, kinases involved in phosphorylation signaling pathways) are also associated with genetic epilepsies. For example, the nonion channel gene mutation in LGI1 has been identified in familial lateral temporal lobe epilepsy, suggesting an additional pathological mechanism for epileptogenesis. Future studies of the effects of GABA_A receptor mutations on gene expression, mRNA stability, posttranslational protein modification, subunit folding and assembly, receptor trafficking, receptor synaptic targeting, and

receptor endocytosis will eventually present a more accurate picture of the molecular pathogenesis of epileptogenesis—and hopefully lead to alternative therapeutic approaches.

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