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CANDIDATE REVIEWS

Epilepsy associated mutations in the γ2 subunit of $GABA_A$ receptor

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Abstract

Idiopathic generalized epilepsies are a group of neurological disorders with genetic origins. Genes coding for ion channels are involved in the disease development. Mutations identified from epilepsy patients have been located to type A γ-aminobutyric acid (GABA) receptor ($GABA_AR$). $GABA_ARs$ are GABA gated pentameric chloride channels mediating fast inhibitory neurotransmission in the brain. Nineteen homologous subunits have been cloned and γ2 containing receptors are the major native compositions. In this review, we discuss several well studied epilepsy associated mutations in γ2 subunit: the missense mutations *R43Q* and *K289M*, the premature translation termination codon (PTC)-generating mutations *Q351X* and *IVS6+2T→G*. Different etiology of these mutations will be addressed.

Keywords: *epilepsy, $GABA_AR$, missense mutation, PTC mutation*

Introduction

Epilepsy is a neurological condition affecting 1% of the population and is characterized by recurrent and usually unpredictable seizures, . A hyperexcitable or hypersynchronized state of the neuronal network underlie syndromes observed in epilepsy patients. Although acquired factors such as trauma and infection could cause epilepsy, idiopathic generalized epilepsies (IGE) are of familial origin[1-3]. Given neuronal excitability is controlled by various ion channels, impairments on channel activities could lead to disease development. Mutations in ion channels have been reported to associate with epilepsy, such as voltage gated sodium channel[4], voltage gated potassium channel[5], nicotinic acetylcholine receptor[6] as well as type A γ-aminobutyric acid (GABA) receptor ($GABA_AR$)[7-8].

GABA is the main inhibitory neurotransmitter in the central nervous system. One of the major postsynaptic targets activated by GABA is $GABA_AR$, through which chloride ions enter to hyperpolarize the cell membrane, maintaining inhibitory tone. Widely expressed across the brain, $GABA_ARs$ are targets of several pharmaceutical drugs such as benzodiazepine (BZD) and barbiturates; they are also modulated by neurosteroids[9-10]. Besides, $GABA_ARs$ are associated with multiple disorders, including depression[11], schizophrenia[12], alcoholism[13] and epilepsy. Pentylenetetrazol, a $GABA_AR$ antagonist, has been widely used to induce generalized clonic seizures in rodent model[14-15]. The oscillatory synchrony recorded in mice brain was dramatically intensified when the $GABA_AR$ mediated inhibition was abolished in the reticular nucleus[16]. Moreover, multiple mutations in $GABA_AR$ have also been revealed in genetic study on epilepsy patients[7-8,17], implying $GABA_ARs$ play a role in the pathophysiology of epilepsy.

Here we address how a variety of epilepsy associated mutations identified in γ2 subunit of $GABA_AR$ could impair the receptor function, and how these impairments might increase the epilepsy susceptibility. First, a discussion on the subunit composition of $GABA_AR$; next, several well studied mutations will be discussed.

$GABA_AR$: Subunit Composition

$GABA_ARs$ belong to the family of pentameric cys-loop ligand gated ion channels. In the mammals, this subfamily also includes nicotinic acetylcholine receptors (nAChR), serotonin type 3 receptors, and glycine receptors. Five homologous subunits are arranged pseudo-symmetrically to form a central ion passing pore. Similar to other subunits from cys-loop family, $GABA_AR$ subunits contain a large N terminal extracellular domain, followed by four transmembrane segments (M1~M4) and a small C terminal tail. The GABA and BZD binding sites are located in a different extracellular subunit interface, while the M2 segments line the inner pore[18-19].

Nineteen different $GABA_AR$ subunits, α1-6, β1-3, γ1-3, δ, θ, ε, π, and ρ1-3, have been cloned, and the heterogeneity is increased by alternative splicing[20], RNA editing[21], as well as posttranslational modification[22]. However, only certain subunit combinations could form functional receptors. Study on the unary, binary, and ternary combination of α1, β2 and γ2L subunits showed that although several distinct oligomers existed in endoplasmic reticulum (ER), only αβ and αβγ combinations could form functional channel on the surface. Immature oligomers were retained in ER and degraded quickly[23-24]. The major native receptor composition found in the brain is αβγ, but extrasynaptic αβ receptor was also reported[25]. Comparison of these two receptor types in mammalian cell lines revealed that αβ receptor displayed a lower single channel conductance, smaller macroscopic amplitude and higher zinc sensitivity[26-28] (Figure 1). However, the generation of αβ receptor seems to be relatively inefficient, as the αβγ composition is preferred in the presence of γ subunit. A 2α:2β:1γ receptor stoichiometry was suggested for αβγ composition[29-30], in a counterclockwise arrangement of γ-β-α-β-α when viewed from the extracellular space[31] (Figure 1 inset).

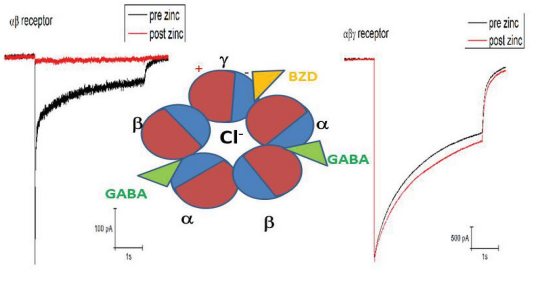

Figure 1: Whole cell recording showing GABA mediated chloride current through αβ and αβγ receptors. Black line: 1mM GABA only; red line: 1mM GABA + 10uM zinc2+ after 10s zinc2+ inhibition. The vertical scale bar is 100pA and 500pA respectively. The inset shows the schematic view of a pentameric αβγ receptor viewed from the synaptic cleft. Yellow triangle: BZD binding site; Green triangle: GABA binding site.

Epilepsy associated mutations in γ2 subunit

As a subunit of the major $GABA_AR$ isoform, γ2 subunit is distributed throughout the brain.

Immunohistochemistry staining revealed strong signals in most regions including cerebral cortex, basal ganglia, hippocampus, and relatively weak signal in thalamus[32]. Postsynaptic clusters of $GABA_AR$ and gephyrin was greatly reduced when γ2 subunit was deleted[33]. The majority of homozygous γ2 knockout mice die within a few days after birth, and the surviving mice showed retarded growth, sensorimotor dysfunction and reduced life-span[34]; the heterozygous knock out displayed a higher level of anxiety[35]. Several epilepsy associated mutations have been identified in γ2 subunit (Figure 2): some are missense mutations where a specific residue is replaced by another; others generate a premature translation termination codon (PTC), stop codon caused by nonsense mutation, or frameshift mutation.

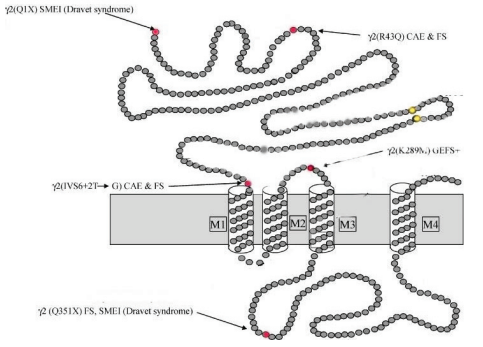

Figure 2: The topology of γ2 subunit of $GABA_AR$. The subunit is composed of a big extracellular N terminal region, four transmembrane domains (M1~M4) and a small C terminus. The red circles represent mutations identified in epilepsy patients. The yellow circles represent the two conserved cysteine residues.

Missense Mutations: *R43Q*

The *R43Q* mutation is one of the first epilepsy associated mutations identified in $GABA_AR$, and also the most extensively studied one. It was originally found in a large Australian family with epilepsy[8,36], segregating with patients with childhood absence epilepsy, febrile seizure or febrile seizure plus. A single nucleotide substitution was found in the *GABRG2* gene of these patients, causing a highly conserved arginine residue in γ2 peptide to be replaced by glutamine. This 43rd arginine in the mature peptide is located in the extracellular N terminus.

Several mechanisms have been suggested for *R43Q* mutation, including altered channel kinetics[37], interruption of BZD binding[8,37], decreased amplitude of GABA induced current[38-40], and decreased surface expression of γ2 subunit[39-43]. These discrepancies might be explained by the different heterologous expression systems and techniques. Initially, electrophysiological recordings in oocytes suggested this mutation disrupted BZD potentiation, decreasing the efficacy of inhibitory neurotransmission and generating a hyperexcitable state in the thalamocortical network[8]. This was supported by observations showing that arginine43 is inside one of the two important BZD binding domains[44]. Positron emission tomography revealed BZD binding was reduced in patients harboring *R43Q* mutation, especially in the anterior region of the brain[45]. However, as gamma subunit is necessary for BZD binding[33-34,46], a loss of γ2 subunit would also impair the BZD effects.

Biochemical evidences from surface biotinylation, radioactive ligand binding and immunofluorescence imaging agreed the *R43Q* mutation impaired the surface expression of γ2 subunit of $GABA_AR$, reducing the postsynaptic inhibitory response. According to the homology model derived from the crystal structure of acetylcholine binding protein, the R43 residue of the γ2 subunit was located in the face contributing to the β/γ interface rather than the α/γ interface where BZD binds. A salt bridge may connect the R43 and E178 residues in γ2 subunit with the R117 residue in β2 subunit. In addition, a pull down assay identified the 15-residue segment surrounding R43 capable to mediate subunit binding. This interaction was abolished when the arginine residue was changed to glutamine. Similarly, mutagenesis on neighboring Asp39 and Pro44 disrupted the membrane insertion of γ2 subunit. It is likely the conserved region around R43 plays a role in the receptor assembly and surface insertion of γ2 subunit[40-41,43], thus the mutant γ2 subunit was trapped in ER[39]. However, it is still controversial whether the retained mutant γ2 subunit would affect the surface insertion of its coupling partners. Overexpressing γ2(*R43Q*) subunit in hippocampal neurons, Eugene and colleagues found the mutant did not affect the synaptic IPSP, which is largely contributed by $GABA_ARs$ containing α1 subunit. However, the mutant reduced the extrasynaptic tonic currents by preventing the surface expression of α5 subunit[42]. Using an overexpressed amount of γ2 subunit in COS-7 cells, Frugier and colleagues suggested the retained γ2(*R43Q*) subunit did not affect the surface targeting of α3β3 complexes. A decreased number of α3β3γ2 receptors was accompanied with an increase of α3β3 receptors[43]. Flow cytometry data from our lab suggested that while a small amount of mutant αβγ receptor was expressed on the cell surface, a large amount of surface receptors switched to αβ receptor composition (unpublished data). As αβ receptors are extrasynaptic and exhibit smaller single channel conductance, this switch might affect the balance between tonic and phasic inhibition or decrease the efficiency of inhibitory neurotransmission.

A heterozygous knock-in mouse model carrying the *R43Q* mutation was generated[47]. The mutant mice displayed spontaneous absence seizures characterized by 5-8 Hz, high amplitude spike-and-wave discharges (SWDs) on electroencephalography, which could be blocked by antiepileptic drug ethosuximide. This is similar to the typical 3-4 Hz SWDs recorded in patients of childhood absence epilepsy[48]. The threshold of seizure induced by pentylenetetrazol was also significantly reduced in *R43Q* heterozygous mice. Interestingly, a small but significant reduced sIPSC was observed in cortical pyramidal neurons, but absent in thalamic reticular nucleus or ventralbasal thalamus neurons. How the mutant mice developed such specific deficit needs further investigation. Another conditional knock-in mouse model was also generated where the temporal expression of the γ2(*R43Q*) allele in the forebrain could be controlled[49]. When one copy of wild-type allele was normally expressed, activation of the *R43Q* allele increased the seizure threshold, and inactivation of the *R43Q* allele during development would decrease the threshold in adulthood. The epileptic phenotype of the heterozygous *R43Q* mice indicates a subtle reduction of cortical inhibition might underlie the pathogenesis of epilepsy. The expression of one *R43Q* allele seems to increase the seizure susceptibility compared to haploinsufficiency, and the changed channel activity during development might play an important role in disease onset in later life.

Generally, the *R43Q* mutation disrupts surface expression of the γ2 subunit. This could be caused by deficit of the subunit oligomerization or membrane insertion. The *R43Q* mice recapitulate some epilepsy syndromes, indicating disruption of $GABA_AR$ could result in the hyperexcitable state observed in epilepsy.

K289M

K289M mutation was found to segregate with various phenotypes of generalized epilepsy with febrile seizure plus in a French family[7]. It was

caused by a single nucleotide change, converting a highly conserved lysine residue in the M2-M3 linker of γ2 subunit to methionine.
This mutation might cause decreased amplitude[7,50] or altered kinetics[38,42] of GABA evoked macroscopic current. The lysine residue in the extracellular M2-M3 linker is conserved among $GABA_AR$ and glycine receptor subunits, participating in channel gating[51]. During gating, the homologous K278 residue in the M2-M3 linker of α1 subunit was proposed to interact electrostatically with negative charged aspartic acid residues in the subunit interface. In the presence of GABA, K278 of the α1 subunit was in proximity to extracellular D149 in loop 7, indicating these two regions move closer during gating process[52]. Single channel recordings showed the mean open time of γ2(K289M) containing receptors was briefer, similar to α1(K278M) containing receptors[38,53]. The channel opening equilibrium constant of γ2(K289M) was also shown to be five fold lower than wild-type[50]. Although reduced macroscopic current amplitude was identified in oocytes and mammalian cells[7,50], no such difference was observed by some other groups[38,42,53]. Instead, both macroscopic currents elicited by rapid agonist application on HEK cells and mIPSCs of hippocampal neurons showed accelerated deactivation rate of γ2(K289M) mutation. The faster deactivation of $GABA_AR$ could also result in reduced inhibitory neurotransmission, as the total volume of chloride ion transferred is reduced. No significant change of protein expression or channel conductance was identified[42,53]. Compared to the R43Q mutation destroying surface targeting, K289M mutation affects the channel activity of type A GABA receptors.

PTC mutations: *Q351X*

Q351X is a nonsense mutation identified in a family with GEFS+[54], and the proband was diagnosed with the severe myoclonic epilepsy in infancy. A single nucleotide substitution in exon 9 of *GABRG2* was identified in three members of this family, suggested to form part of the epilepsy inheritance pattern. This mutation results in a premature stop codon in the glutamine351 residue of mature γ2 subunit.

The *Q351X* mutation displays dominant negative effects. As glutamine351 is located in the big M3-M4 cytoplasmic loop, a truncated peptide lacking the fourth transmembrane domain and the small C-terminal tail was generated by *Q351X* mutation. Expression of mutant γ2(*Q351X*) with α1 and β2 subunit in oocytes and HEK cells showed the GABA induced currents were completely abolished and mutant γ2 subunit was retained in ER complex. The detrimental consequence of this mutation suggested the mutant γ2 subunit might also affect the assembly of α1 and β2 subunits into functional receptor[54]. Kang *et al*. studied the protein expression and channel function of γ2(*Q351X*) in different conditions. They compared the hemizygous condition where half dosage of the wild-type subunit is transfected,with heterozygous condition where half dosage of the wild-type and half dosage of the mutant subunit are co-transfected.They found that the mutant γ2 subunit was trapped in the ER, and also prevent the membrane insertion of wild-type α1, β2 and even γ2 subunits through subunit oligomerization[55].

Premature truncation could activate surveillance mechanisms including nonsense-mediated decay (NMD) and ER associated degradation (ERAD)[56]. NMD is a posttranscriptional process to eliminate mRNA that would cause prematurely terminated translation; generally PTCs located more than 50-55 nt upstream of an exon-exon junction could trigger NMD[57]. ERAD is an ER quality control mechanism to eliminate misfolded peptides[58]. Utilizing minigene containing intron 8 sequence, it was shown the mRNA level of the mutant γ2(*Q351X*) was not affected, accordant with the fact the glutamine351 in the last exon is unable to trigger NMD. However, on the protein level, the α1 subunit was degraded more rapidly in the presence of γ2(*Q351X*) subunit, through the proteosome mediated ERAD[55].

To conclude, *Q351X* mutation generated a truncated γ2 subunit with trafficking deficit. The mutant γ2 subunit would retain oligomerization partners in ER and accelerate their degradation. The mutant γ2 subunit also affects the membrane insertion of wild type γ2 subunit, exhibiting a dominant negative effect.

IVS6+2T→G.

γ2(IVS6+2T→G) is an intronic mutation segregated with childhood absence epilepsy and febrile seizures in a small pedigree. It is a single nucleotide change identified in the splice donor site of the intron 6 of *GABRG2*. This mutation could cause exon skipping or alternative splicing using cryptic splice donor sites. Premature truncated protein was predicted for both situation, due to the presence of in-frame stop codons[59].

In contrast to the *Q351X* mutation, the amount of mutant *γ2(IVS6+2T→G)* was affected by NMD. Splicing is a posttranscriptional event to remove introns, which greatly enhances protein diversity. During a typical splicing process, the spliceosome recognizes the 5' terminal splice donor site with dinucleotide GT sequence and the 3' terminal splice acceptor site with AG sequence, followed by intron excision[60]. Intron splicing is affected by some intronic RNA motifs, such as sequences around the donor sites and acceptor sites. Mutations identified in these motifs have been associated with diseases including cancer[61]. Mutations in the splice donor site could cause exon skipping, intron retention or use of cryptic splice sites[62]. Tian *et al*. investigated on the effects of the *γ2(IVS6+2T→G)* mutation using minigene and bacterial artificial chromosome (BAC). A cryptic splicing donor site was activated by the mutation: part of the intron 6 sequence was retained, causing a frame shift in exon 7 and producing a premature stop codon. NMD was shown to be activated by this PTC, and the amount of transcript was reduced[63].

Not all mutant transcripts are cleared by NMD in *IVS6+2T→G* mutants, as a truncated γ2 peptide without transmembrane domain was still produced. Expressed in HEK cells, the mutant γ2 peptide was retained in ER and could not oligomerize with other subunits[63]. In a word, *γ2(IVS6+2T→G)* mutation results in a nonfunctional protein. Different from the dominant negative effects of *Q351X* mutation, the pathogenesis of patients carrying this mutation might be the consequence of haploinsufficiency of γ2 subunit.

Conclusion

As one of the main ion channels mediating inhibitory signal, $GABA_AR$ is involved in the pathogenesis of epilepsy. Here we discuss four well studied mutations identified from small pedigrees, showing impairments on γ2 subunit of $GABA_AR$ could increase the epilepsy susceptibility. In future studies, it will be important to investigate 1) whether these mutations are the main genetic factor in these pedigrees; 2) whether distinct epilepsy subtypes are contributed by different mechanisms of channel dysfunction; 3) how different mutations or polymorphisms work together to affect the individual seizure threshold. A combination of traditional electrophysiology and biochemistry methods and transgenic techniques would improve our understanding of this area.

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