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$GABA_A$ Receptor Variants in Epilepsy

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Abstract: Epilepsy is one of the most common episodic neurological disorders, affecting 1% population worldwide. The genetic variations of γ-aminobutyric acid type A ($GABA_A$) receptor, including missense, nonsense, splice site and frameshift variants in *GABRA1-6*, *GABRB1-3*, *GABRG1-3*, and *GABRD*, have been identified as some of the primary genetic causes of epilepsy. However, the lack of a complete understanding of the association between epilepsy syndromes and $GABA_A$ receptor variants makes it challenging to develop effective therapeutics. Here, we summarize a comprehensive list of over 150 epilepsy-associated variants in the major α1, β2, β3, and γ2 subunits of $GABA_A$ receptors and their functional defects. In addition, their spatial distribution is visualized in the cryo-EM structures of $GABA_A$ receptors. Many of the variants lead to reduced receptor surface expression and thus loss of function due to protein conformational defects and impaired trafficking. This knowledge aids the development of precision medicine-based therapeutic strategies to treat epilepsy.

Keywords: epilepsy syndromes; epilepsy-associated variants; $GABA_A$ receptors in epilepsy; precision medicine; trafficking

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INTRODUCTION

Epilepsy affects 1–2% of population worldwide. According to the Centers for Disease Control and Prevention, in the United States, about 3 million adults and 470,000 children were diagnosed with epilepsy in 2015 (1). Epilepsy is not a single neurological disorder, but a highly heterogeneous group of conditions starting from the brain. It affects individuals of any age, sex, and ethnicity (2). The majority of the epileptic syndromes have an early onset, occurring during the first year of life (3). Thus, it presents as an enormous burden to the affected families and public health. Recurrence of episodic seizures constitutes the primary symptom of epilepsy. It not only affects health but also encumber qualify of life. In 2012, the Global Burden of Disease study investigated 291 diseases and ranked epilepsy as the second-most-burdensome neurological disease (4). Although it has been recognized for many years and numerous diagnostic methods are available, epilepsy is still poorly understood by the public due to its complicated forms and multiple causes.

Seizures, the landmark of epilepsy, are classified as focal or generalized. Focal seizures are theoretically limited to part of one cerebral hemisphere, accounting for almost 60% of all epilepsies (5). Generalized seizures originate in localized brain regions and are then bilaterally distributed, causing widespread brain pathology. Generalized seizures are divided into specific subtypes, such as, absence, generalized tonic-clonic (GTC), myoclonic, and atonic (6). The classification of seizures assists the diagnosis of epilepsy types, which is vital for determining the antiepileptic treatment at the first line (7). In 2010, the International League Against Epilepsy (ILAE) revised the classification guideline to expand epilepsy syndromes to genetic, structural-metabolic, and unknown representations (8).

During the last two decades, much effort has been deployed in unraveling the genetic factors of epilepsy. Genes harboring pathogenic variants have been identified, a majority of which are located at neuronal ion channels or genes involved in ion channel function, resulting in neuronal hyperexcitability or inhibition of excitability (9). Among the causative genes, the γ-aminobutyric acid type A receptor ($GABA_AR$) has been recognized as one of the major genetic causal agents in epilepsy. Many studies have linked the variants in genes encoding $GABA_ARs$ with a broad phenotypic range of epileptic encephalopathies (EEs), in which the variants impaired the whole channel gating or receptor trafficking (10). Additionally, $GABA_AR$ has been a prime target of anti-seizure treatment for epilepsy caused by various etiologies.

STRUCTURE OF $GABA_A$RECEPTORS

The $GABA_AR$ is the primary inhibitory neurotransmitter receptor in the central nervous system (CNS), belonging to the Cys-loop ligand-gated ion channel superfamily, including serotonin 5-HT_3, nicotinic acetylcholine, and glycine receptors (11, 12). Upon GABA or other agonist bindings, the receptor allows the influx of negatively charged chloride ions and mediates neurotransmission to reduce neuronal excitability and firing (13). A fully assembled $GABA_AR$ has a pentameric

structure that consists of five subunits arranged around a central hydrophilic pore (Figure 1A) (14, 15). It is composed of 19 different subunits (α1-6, β1-3, γ1-3, δ, ε, π, θ, ρ1-3), leading to a complex structural assembly. Although the large numbers of subunits could potentially form a huge variety of receptors, only a limited number of possible combinations have been found *in vivo*. General forms of $GABA_AR$ are the combination of alternating two essential α subunits and two essential β subunits, with one γ subunit or one δ subunit on the fifth position. $GABA_ARs$ with different subunit compositions exhibit different intrinsic properties including agonist binding affinity, kinetics, conductance, expression and accumulation during development, and distributions through the brain (16). For instance, receptors containing α1-3, β1-3, and γ2 subunits are typically localized in the postsynaptic sites, where a high concentration of GABA can open the receptor leading to the increase of anion conductance in a short time period. On the other hand, receptors containing α4-6, β2/3, and δ subunits are localized in the extrasynaptic sites, and they have high GABA affinity, but low desensitization time compared with postsynaptic $GABA_ARs$ (17). The most prominent population of $GABA_AR$ subtypes in mammalian brains are the combination of α1, β2/3, and γ2 subunits. The majority of epilepsy-causing variants of these subunits and their associated receptors provide direct evidence linking the receptor functional deficiency with neuronal hyperexcitation (18). Currently, more than 150 *de novo* and

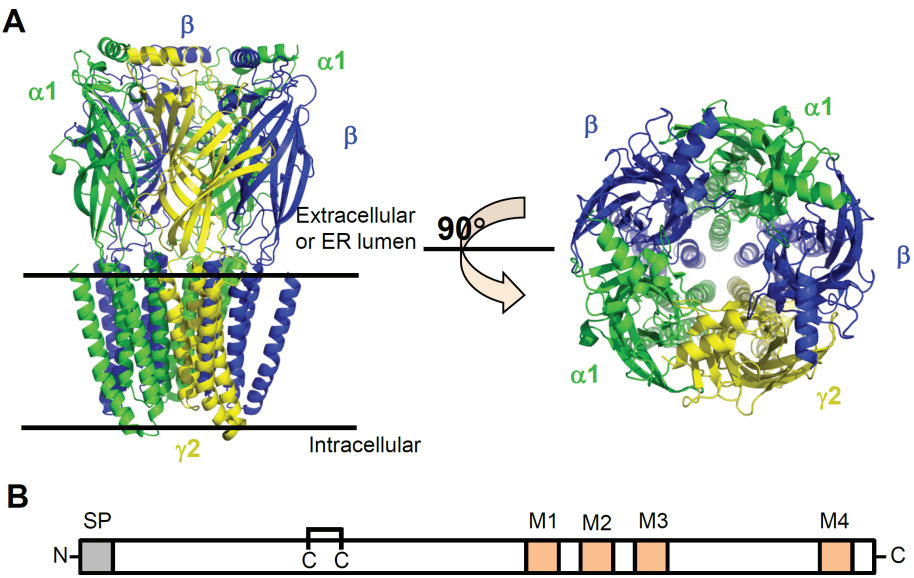

Figure 1. The architecture of GABA$_A$ receptors. A, The most common type in the mammalian central nervous system contains two α1 subunits, two β subunits, and one γ2 subunit. The pentameric receptors, constructed from 6X3S.pdb, are viewed from the side or the extracellular space (also the lumen of the endoplasmic reticulum [ER]). The extended intracellular cytoplasmic domain (ICD) between TM3 and TM4 is absent in the structure. **B,** A Cartoon representation of the primary sequence of $GABA_A$ receptor subunits. Each subunit has a signal peptide (SP) in its N-terminus, a signature disulfide bond in the N-terminal domain (NTD), four transmembrane helixes (TM1 to TM4), and a short C-terminus.

familial variants in *GABRA1*, *GABRB2*, *GABRB3*, and *GABRG2* have been identified in patients with broad phenotypic epileptic syndromes, such as childhood absence epilepsy (CAE), febrile seizures (FSs), generalized epilepsy with FS plus (GEFS+), juvenile myoclonic epilepsy (JME), West syndrome (WS, or infantile spasms [IS]), Lennox-Gastaut syndrome (LGS), and Dravet syndromes (DS) (10, 19, 20).

Epilepsy-associated variants are spread throughout the peptide chain along the primary sequence of $GABA_AR$ subunits (21-122). A select list of variants are presented in Table 1 and Table 2. Each subunit shares common structural motifs with a relatively long extracellular N-terminal domain (NTD), four transmembrane helices domain (TM1-4), an extended intracellular cytoplasmic domain (ICD) between TM3 and TM4, and a short extracellular C-terminus (Figure 1B). The long NTD plays an important role in binding agonists, antagonists, and benzodiazepines (BZDs) (17). Similar to other Cys-loop receptors, the canonical agonist binding site for GABA is an "aromatic pocket" formed at the NTD between α and β subunits. The two GABA binding sites are not identical in structures, but the same in chemical specificity for ligand (21). Structurally, three peptide loops (Loop A-C) in principle β subunit form "positive" side of the binding pocket, whereas three β-sheets (Loop D-E) contributed by adjacent complementary α subunits form the "negative" side (22). The allosteric BZD binding site is located at the interface between the principal α1 subunit and the complementary γ2 subunit (23). Many studies have reported the importance of NTD in the function of $GABA_ARs$. F45 at NTD of α1 subunit is involved in agonist binding and late channel gating transitions (24). N104 and N173 of β2 subunits, which are located around Cys-loop, exhibit the essential role in N-glycosylation (25). Thus, variants located at or near the binding site could directly alter the channel gating functions and cause epilepsy. Two *de novo* variants (D120N and E180G) at the β+/α– interface of *GABRB3* were found in multiple cases diagnosed with LGS, one of the severe forms of epilepsy in the infants and early childhood (26). Both NTD and its adjacent TM1 play an important role in the fast phase of receptor desensitization (27). The TM1 helix interacts with the lipid environment participating in channel gating. Therefore, variants in the β1 subunit TM1 segment (e.g., P228A) perturb the linkage of GABA binding with channel gating (28).

Since the α1 subunit TM1 domain docks an uncanonical binding site for neurosteroid, variants in this region could potentially affect the positive and negative modulation by neurosteroids (29). The TM2 domain aligns along the central conduction path to form the selective ion channel with a partial contribution from TM1. Besides its important role in the flux of chloride ions, TM2 is linked to zinc inhibition since the zinc binding sites are located at both NTD and the pore. Previous studies suggested that the zinc sensitivity of $GABA_ARs$ changes with the epilepsy onset (30). In 2017, one *de novo* variant P302L at TM2 of γ2 subunit was found in a patient with Dravet syndrome. This variant perturbs the channel conduction pathway and destabilizes the receptor conformation in the open state; however, it does not affect channel chloride permeability but shows zinc insensitive features (31). The short TM2-TM3 loop in the extracellular side plays a critical role in linking the binding-coupling pathway to the pore domain (TMD) and determines the energy transmittance efficiency from ligand binding into channel gating (32). This region is also involved in receptor assembly (33). The R323W variant at the TM2-TM3 loop of *GABRG2* is found in a severe case of EE (34). The TM3 helix, located on the opposite side of TM1 and TM4, is away from the central pore.

TABLE 1 **Select epilepsy-associated variants in the α1, β2, β3, and γ2 subunits of $GABA_A$ receptors**

Domain	α1		β2		β3		γ2
NTD	V74I[a] (47)		M79T[b] (60, 104)		P11S[a,i,iv] (66)	L124F[b] (36)	Q40X[a,i,iv] (98, 105)
	S76R[b,i,ii] (47)		D108Y[b] (63)		S15F[a,i] (66)	K127R[b] (36)	N79S[a,i,iv] (88)
	F104C[a1,i,ii] (47)		A112E[b] (63)		G32R[a,i] (66)	R142L[a] (36)	R82Q[a,i,iv] (79, 82)
	R112Q[b,ii] (29, 50)		D125N[b] (104, 106)		V37G[a2,i] (36)	T157M[a1] (36, 69)	P83S[a2,i,iv] (48, 91)
	N115D[b,ii] (47)		V133M[b] (63)		L52V[b] (107)	M163V[b] (107)	T90M[b]
	L146M[b] (47)		A159S[b] (62)		P54L[u] (108)	L170R[b,i,iv] (109)	T90R[b,iv] (52)
	P181S[b] (110)		M161L[b] (62)		I69T[u] (36)	E180G[b,i,ii] (44)	A106T[b,i,iv] (34)
	Y196C[b] (111)		Y181F[b,v] (52, 62)		S76C[b] (36)	Y182F[b] (69)	I107T[b,i,iv] (34)
	R214C[b,i,iii,iv] (52, 53)		Y183H[b] (62, 106)		E77K[b,v] (112)	Y184H[b,i,ii] (36)	R136X[a,i,iv] (96)
	R214H[b,i] (47, 52)		T184I[b] (63)		V78L[b] (107)	T185I[b] (36)	R177G[a,iii,iv] (85)
	L215P[b,i] (52)		K221R[a1] (113)		M80L[b] (107)	R194X[a1,i,ii] (71)	R177fs[a] (76)
	D219N[a,i,iv] (48)		F224C[b] (63)		N110D[b] (44)	R232X[a2] (36)	G257R[a,iv] (90)
			R240T[b] (63)		R111X[a1,i] (36)	R232Q[b] (114)	
					D120N[b,i,ii] (44, 69)		
TM1	G251D[b,iv] (47)	M263I[b] (29)	Y244H[b] (104)	P252L[b,i] (62)	Y245H[b] (107)	S254F[b] (107, 114)	P282T[b] (91)
	G251S[b,i,ii] (50)	L267I[b,v] (115)	F245S[b] (63)	P252T[b] (62)	Q249K[b] (69)	L256Q[b,iv] (69, 114)	P282S[b,i,iv] (34)
	P260S[b,i,iii,iv] (51)		F245L[b] (62)	L255V[b] (62)	Q249H[b] (107)		
	P260L[b,i] (29)		I246T[b,i] (62)	V262F[b] (62, 116)	P253S[b] (107)		
	M263T[b] (29)		P252A[b] (62, 106)		P253L[b] (114)		

Table continued on following page

TABLE 1 Select epilepsy-associated variants in the α1, β2, β3, and γ2 subunits of $GABA_A$ receptors (Continued)

Domain	α1		β2		β3		γ2
TM2	P280T[b] (117)	T289A[u] (47)	L277S[b] (104, 106)	I288S[b,i] (62)	L278F[b] (107)	T287I[b,v] (118)	P302L[b,i,iv] (31)
	V287I[b,i] (52)	T292I[b] (44)	V282A[b,i] (62)	I288T[b] (62)	T281A[b,iv] (119)	T288I[b] (107)	S306F[b] (91)
	V287L[b] (29, 52)	L296S[b,i,iii,iv] (51)	T284K[b] (104)	R293P[b] (104)	T281I[b] (70)	T288N[b,i] (31)	R323Q[b,i,iv] (34, 90)
	T289P[b] (47)		T287P[b,i,iii,iv] (61)	R293W[a,b] (62)	L284M[b] (117)	L293H[u] (69)	R323W[b,i,iv] (34)
Loop TM2-3	K306T[b,i,ii] (47, 50)		K298G[b] (63)	P300L[b] (62)	P301L[b] (114)		K328M[a,i,iv] (92)
			I299L[b] (62)	Y301C[b] (106)	Y302C[b,i,ii] (114)		
			I299S[b] (62)	V302M[b] (62, 120)			
TM3	W315L[b,i,iii,iv] (51)	A332V[b,v] (46)	K303N[b] (62)	A304V[b] (62, 104)	A305T[b] (69)	N328D[b,i,iv] (68)	F343L[b,i,iv] (34)
	A322D[a,i,iii,iv] (39)		K303R[b] (62, 63)	V316I[a,b] (62, 104)	A305V[b,i,iv] (109)		
	S326fs328X[b,i,iv] (43)		A304T[b] (62)		L321P[b] (107)		
Loop TM3-4	K353delins18X[u,iv] (48)		F331_del[a1] (52, 63)	A398X[I] (59, 121)	E357K[a1,i,iv] (68)		I389V[a,i,iv] (90)
			F331S[b] (52, 63)		R429Q[a2] (114)		Q390X[a,i,iv] (99, 122)
			N350_del[a2] (63)				W429X[u,i,iv] (97)
			R354C[a2] (63)				S443delC[a,iii,iv] (87)

Note 1: Inheritance
a: *familial* (a1: maternal; a2:paternal); b: *de novo;* I: Indian population; u: unknown
Note 2: Functional consequence
i: reduced current; ii: reduced GABA potency; iii: reduced total expression; iv: reduced surface expression; v: gain of function

TABLE 2 Phenotypes of epilepsy-associated variants in $GABA_A$ Receptors

Phenotype	α1	β2	β3	γ2
DS/DS-like	S76R, R112Q, L146M, R214C, R214H, L215P, G251S, V287I, K306T	A159S, Y181F, F331_del, F331S	T157M, R232Q, T281I	Q40X, T90R, P302L, Q390X
WS(IS)	R112Q, P260S, P260L, M263T, M263I, T292I, L296S, W315L	T184I, R240T, F245S, P252L, I299S	L52V, I69T, E77K, M80L, N110D, L256Q, L278F, Y302C	
OS(EIEE)	P260L, T289P, T289A	K303N	T287I	
EME		I246T, T284K, T287P		
EOEE	R112Q, N115D, V287L, A332V	P252L, K298G, K303R	N110D, K127R, L170R, T185I, S254F, L256Q, T288N, L293H, A305V	
EIMFS	P280T		L124F, Y245H, S254F, T281A, L284M	S306F
LGS	T292I	I246T, P252L, I288S	D120N, E180G, Y302C, A305T, N328D	P83S
GEFS+/FS+	V74I	D108Y, V133M, M161L, N350_del	P54L, T157M, R429Q	Q40X, N79S, P83S, T90M, R136X, R323Q, K328M, Q390X, W429X
FS		R354C	T157M	R82Q, R136X, R177G, R177fs, K328M
CAE/JAE/EOAE	R214C, L267I, S326fs328X	V316I	P11S, S15F, G32R, V37G, E357K	R82Q, T90M, R177fs

Table continued on following page

TABLE 2 Phenotypes of epilepsy-associated variants in $GABA_A$ Receptors (Continued)

Phenotype	α1	β2	β3	γ2
MAE/JME/MSE	F104C, R214C, K306T, A322D	V262F	S76C, R111X, D120N, R142L, Y184H	R323Q
Unspecified EE	G251D	M79T, D125N, Y244H, P252A, L277S, T287P, I288S	Y182F, R232X, R232Q, Q249K, P253L, P301L, Y302C	A106T, I107T, P282T, P282S, R323W, F343L
DEE/EDD/GDD/NDD/NDDE		M79T, A112E, D125N, Y181F, Y183H, T184I, F224C, R240T, Y244H, F245S, P252A, P252T, V262F, L277S, V282A, T284K, R293P, K298G, Y301C, K303N, A304V	V78L, M80L, Q249H, P253S, T288I, L321P	
RE				G257R, R323Q, I389V

Abbreviation: CAE, childhood absence epilepsy; DS, Dravet syndrome; DEE, developmental and epileptic encephalopathy; EDD, epileptogenic developmental disorders; EE, epileptic encephalopathy; EIEE, early infantile epileptic encephalopathy (or OS, Ohtahara syndrome); EIMFS, epilepsy of infancy with migrating focal seizures; EME, early myoclonic encephalopathy; EOEE: early onset epileptic encephalopathy; FS, febrile seizure; FS+, febrile seizure plus; GDD, global development delay; GEFS+, Genetic epilepsy syndrome with febrile seizures plus; JAE, Juvenile absence epilepsy; JME, Juvenile myoclonic epilepsy; LGS, Lennox-Gastaut syndrome; MAE, epilepsy with myoclonic-atonic seizures; MSE, Myoclonic status epilepsy; NDD, neurodevelopmental disorder; NDDE, neurodevelopmental disorder with epilepsy; RE, rolandic epilepsy; WS, West syndrome (or, IS, infantile spasms).

They shield TM2 from lipid bilayers. Interestingly, variants located at TM2 and TM3 could significantly affect receptor surface expression. For instance, A322D, a missense variant in TM3 of *GABRA1*, is associated with CAE and JME (35). This variant significantly reduces its cell surface expression and thus the whole-cell current. The variable long ICD loop between TM3 and TM4 contains key binding sites for regulatory proteins, which are involved in receptor posttranslational modifications and trafficking (17). The *familial* variant R429Q at ICD of *GABRB3* is associated with Dravet syndrome-like symptoms, even though it does not significantly reduce receptor current (36).

EPILEPSY-ASSOCIATED VARIANTS IN THE α1 SUBUNIT

The α subunit of $GABA_AR$ is the requisite subunit as it forms the GABA binding sites and BZD binding site. Among all six members, the *GABRA1* gene (located on chromosome 5q34) is the most widely expressed α subunit. The predominately composed α1β2γ2 receptors contribute around 43-60% of all $GABA_ARs$ in the adult brain (37). It has been well characterized that many epilepsy-related variants in $GABA_ARs$ affect protein folding, assembly, and trafficking of the subunits, leading to impaired channel function, such as altered kinetics and conductance (12, 38). At the early developmental stage, GABA needs to depolarize neuronal cells due to the high concentration of chloride at the intracellular side. Therefore, variants in *GABRA1* may not only impact the channel inhibitory function but also disrupt early brain development. Many variants in *GABRA1*, the majority of which are missense variants, have been associated with CAE, JME, DS, GEFS+, and WS (Table 1, 2 and Figure 2).

The positions of the α1 variants in the NTD domain are illustrated in Figure 2A, whereas those in the TMD domains and their connecting loops are illustrated in Figure 2B. The first case linking the *GABRA1* variant with epilepsy was reported in 2002: a missense *familial* variant, A322D in α1, was identified in individuals

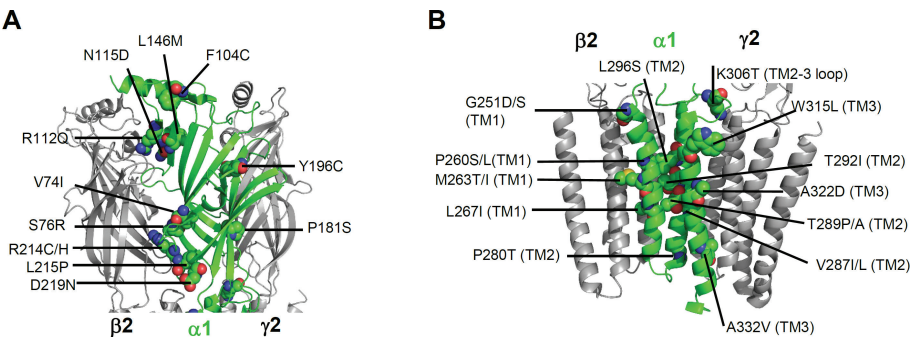

Figure 2. Epilepsy-associated variants in the α1 subunit of $GABA_A$ receptors. Gene name: *GABRA1*.Viewed as the structure constructed from 6X3S.pdb. **A,** Positions of the variants in the N-terminal domain are presented as sphere models. **B,** Positions of the variants in the transmembrane (TM) domain are presented as sphere models. TM4 was omitted to visualize the positions more clearly.

with JME (39). This variant, introducing a negatively charged aspartate into TM3, reduces both total and surface α1 subunit expression level as well as the peak GABA-evoked current amplitude when co-expressed with β2 and γ2 subunits (35, 39, 40). Molecular mechanism studies showed that this variant destabilizes TM3 and impairs subunit folding, leading to excessive degradation of misfolded subunits through endoplasmic reticulum associated degradation (ERAD) (41, 42). Four years later, a *de novo* frameshift variant in TM3 of *GABRA1* (S326fs328X) was identified in a patient diagnosed with CAE. The variant, inducing a premature stop codon in TM3, leads to no detection of surface α1 and GABA-evoked current when co-expressed with β2 and γ2 subunits, indicating the abnormal trafficking. The premature stop codon results in protein degradation through nonsense mediated mRNA decay (NMD), and the remaining of the misfolded proteins is degraded through ERAD (43). In a large screen for *de novo* variants in patients with EE, T292I in TM2 was found in association with WS and LGS (44). This variant significantly reduces the surface expression of α1 subunit and induces a faster desensitization rate (45). A332V in TM3 near the subunit interface was found in association with early-onset epileptic encephalopathies (EOEE). Compared with wild type, this variant does not change the surface and total expression levels when expressed with β3 and γ2 subunits in HEK293T cells. However, functional analysis in oocytes showed that the GABA activation potency in variant-containing receptors is higher than that in wild type, demonstrating a novel gain-of-function variant mechanism. The variant does not affect the peak current amplitude but accelerates the channel desensitization (46).

Variants in *GABRA1* were identified in association with JME and CAE. For example, a *familial* variant F104C at NTD, which is close to the agonist binding site, was found in a patient with JME. Compared with the wild type receptor, the variant-containing receptor only reaches 24% current response to 1 mM GABA and decreases GABA sensitivity (47). A *familial* variant D219N, located at the β+/α1– interface near the TM1 entrance, was found in a Canadian population with idiopathic generalized epilepsy (IGE) (48). The reported functional consequences of this variant are slightly different: the surface expression of variant α1 is either reduced to half or unchanged compared with wild type; the peak current amplitude is either reduced to 30% or unaltered (45, 48, 49). Consistently, this variant leads to faster desensitization.

EOEEs are severe epilepsy phenotypes with early infantile onset, including WS, Ohtahara syndrome (OS) (early infantile epileptic encephalopathy), DS, and early myoclonic encephalopathy (EME) (8). Two *de novo* variants (R112Q and N115D) at NTD, which were likely part of the binding domain, were found in several EOEE cases (29, 47, 50). R112Q was also identified in cases associated with DS and IS. *In vitro* studies showed that both variants decrease GABA potency without changing peak GABA-evoked current amplitude or total and surface expression levels when co-expressed with β3 and γ2 subunits (51). S76R and L146M, located at the NTD of α1, were identified in DS and DS-like syndromes. Three more missense variants (R214C, R214H, and L215P), which are localized just on the edge of NTD, were identified in patients with DS. This highly conserved subdomain is known to couple the ligand binding-induced conformational changes with the channel pore. R214H was reported as a loss-of-function variant in oocytes, leading to a significant reduction (up to 50%) of GABA-evoked current amplitude (47). Both R214C and L215P result in around 60% decrease of macroscopic GABA-evoked currents when

co-expressed with β2 and γ2 subunits; however, L215P, but not R214C extends the desensitization and activation of $GABA_A$Rs (52). Interestingly, the variant R214C reduces the surface and total α1 levels when co-transfected with β2 and γ2 subunits in HEK293T cells, consistent with the mechanistic studies showing that R214C leads to subunit misfolding and ERAD (53).

Two *de novo* variants (L296S in TM2 and W315L in TM3) in α1 subunit were identified in individuals associated with WS (51). Both variants, facing the channel pore, lead to significant reductions in total and surface expression of α1 subunits due to impaired biogenesis and trafficking deficiency. Consistently, both variants result in ~60% reduction of GABA-evoked current amplitude. However, both variants increase the GABA potency by about 5-fold and enhance Zn^{2+} sensitivity (51). The *de novo* variant P260L in TM1 was identified in patients related to OS to WS, affecting the same position as the previously reported P260S (29). Both P260S and P260L reduce the GABA-evoked current without decreasing GABA potency. Two adjacent variants (M263T and M263I) in TM1 were also diagnosed with WS. All these TM1 variants localized at the interface between β+/α1– are likely to impair the transduction of binding energy to channel gating (29). The *de novo* variant V287L in TM2 was identified in association with unclassified EOEE (29), whereas the same location variant V287I was found in CAE and DS (52). The difference in amino acid changes at this position could feature slightly different phenotypes. V287I reduces current amplitude and desensitization without changing activation and deactivation; besides, V287I does not alter total and surface subunit levels (52). More EOEE-associated variants have been identified, such as OS-associated T289P and T289A and DS-related G251S and K306T (47).

Overall, these studies indicated that the loss of function of $GABA_A$Rs caused by epilepsy-associated variants can arise from different molecular mechanisms, such as channel gating defect, impaired protein biogenesis in the ER (protein misfolding, inefficient assembly, ER retention and ERAD), trafficking deficiency, and NMD.

EPILEPSY-ASSOCIATED VARIANTS IN THE β2/β3 SUBUNIT

Both β2 and β3 subunits are widely distributed in the brain (54). The *GABRB2* gene has the same location as *GABRA1* on chromosome 5q34, whereas the *GABRB3* gene is located on 15q12. Rodent data indicated that the β2 subunit is increasingly expressed during development, and the β2 subunit is considered as the major constituent of $GABA_A$Rs in the adult brain. Loss of β2 subunits in the brain is not lethal in the mouse model (55), but receptors containing variant β2 subunits are more likely to cause loss of partial functions (25, 56). The β3 subunit is the major component of $GABA_A$Rs at the developing stage, but its expression declines postnatally (57). Unlike other $GABA_A$R subunits, the β3 subunit can traffic to cell surface when expressed alone as a homopentamer, suggesting the unique role of the β3 subunits on $GABA_A$R trafficking (58).

Epilepsy with β2 variants

Before the last decade, no record had connected *GABRB2* variants with epilepsy. A study in 2007 did not find an association between *GABRB2* with epilepsy when

common variants across 279 prime candidate genes from the European and Australian populations associated with epilepsy were examined. In a large EE screening project conducted by Epi4K in 2013, no *GABRB2* variants were identified in 315 cases with LGS and WS (44). The only evidence connecting *GABRB2* with epilepsy is a nonsense variant A398X in *GABRB2* in the North Indian population (59). Later, a missense variant M79T in the β2 subunit was identified as the first *de novo* variant associated with epilepsy in 2014 (60). Then the T287P variant in β2 was reported in association with myoclonic encephalopathy in 2017; this variant protein is rapidly degraded, leading to reduced surface receptor expression and impaired channel function (61). Since then, the epilepsy phenotypic spectrum has been expanded with various symptoms due to various *GABRB2* variants.

The positions of the β2 variants in the NTD domain are illustrated in Figure 3A, whereas those in the TMD domains and their connecting loops are illustrated in Figure 3B. To date, data about mechanisms and functional consequences are still missing for the majority of epilepsy-causing *GABRB2* variants. Only limited pathogenic variants have been investigated. The β2 variant Y181F at NTD and F331S at

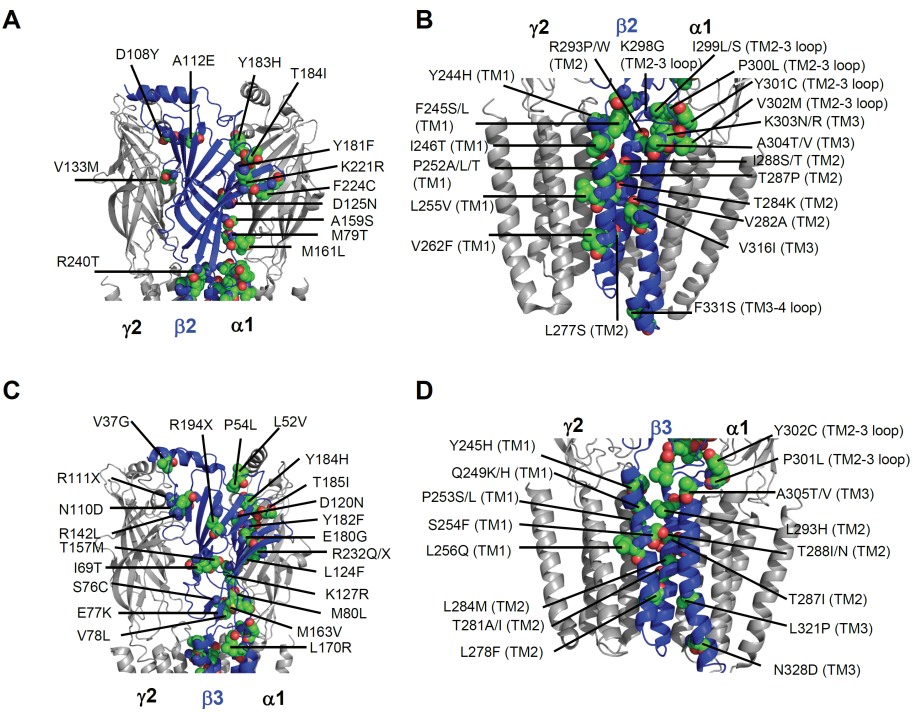

Figure 3. Epilepsy-associated variants in the β2 and β3 subunits of $GABA_A$ receptors. A, Positions of the variants in the N-terminal domain of β2 (gene name: *GABRB2*), viewed in the structure constructed from 6X3S.pdb, are presented as sphere models. **B,** Positions of the variants in the transmembrane (TM) domain of β2 are presented as sphere models. TM4 was omitted to visualize the positions more clearly. **C,** Positions of the variants in the N-terminal domain of β3 (gene name: *GABRB3*), viewed in the structure constructed from 6HUK.pdb, are presented as sphere models. **D,** Positions of the variants in the transmembrane (TM) domain are presented as sphere models. TM4 was omitted to visualize the positions more clearly.

the TM3-TM4 loop were associated with DS. Unlike most DS-associated α subunit variants, both β2 variants do not reduce the peak GABA-evoked current amplitudes. F331S does not impact the channel gating or kinetics, whereas Y181F alters the channel kinetics from activation to desensitization. Besides, one in-frame deletion variant at the same position of F331, which was also associated with DS, reduces the current amplitude and alters the desensitization and deactivation rate (52). A *de novo* T287P variant was identified in a severe case of early myoclonic encephalopathy (EME). T287P at TM2 facing the pore reduces the expression of both total and surface β2 subunits and results in lower channel current amplitude (61). Mapping the location of the functional sites of reported *GABRB2* variants showed that four *de novo* variants, I246T and P252L in TM1, V282A and I288S in TM2, alter conserved amino acids in these positions. All four variants reduce the amplitude of GABA-evoked current, thus considered as loss-of-function variants; however, both I246T and V282A result in a higher GABA potency at low GABA concentrations (from 0.1 to 10 μM) (62).

Through the investigation of case reports and literature, forty-seven *GABRB2* variants have been identified with a wide range of epilepsy types and syndromes. Overall, variants in TM1, TM2, and TM2-TM3 loop tend to lead to more severe phenotypes than variants in NTD and TM3 (Table 1 and 2) (62). But there are exceptions: A159S and Y181F at NTD were identified in association with Dravet-like syndromes and DS (52, 62), and the in-frame deletion variant F331_del in TM3 was associated with DS (63).

Epilepsy with β3 variants

The spectrum of *GABRB3* variants-associated epilepsy phenotypes is expanding. These variants spread all over the β3 subunit structure (Figure 3C and Figure 3D). So far, the functional consequences for many pathogenic *GABRB3* variants are still unclear. Heterozygous β3 knock-out mice exhibit absence-like seizures (64). Since the β3 subunit is abundant in the developing brain, a substantial number of *GABRB3* variants has been associated with severe early onset epilepsies, including LGS and a broad phenotypic range of EOEEs. Some other *GABRB3* variants were associated with relatively benign CAE and myoclonic atonic seizures (MAE) or autism (65).

The positions of the β3 variants in the NTD domain are illustrated in Figure 3C, whereas those in the TMD domains and their connecting loops are illustrated in Figure 3D. Three *familial GABRB3* variants (P11S, S15F, and G32R) were identified in patients associated with CAE (66). P11S and S15F are located in the signal peptide (exon 1a), whereas G32R resides in the mature NTD (exon 2). *In vitro* studies showed that all three variants reduce the current due to abnormal N-linked glycosylation. P11S decreases β3 subunit surface expression level, consistent with an epilepsy phenotype (65). However, G32R increases β3 subunit surface expression level by enhancing the formation of α1β3 or homomeric β3 receptors, but G32R reduces the expression of functional α1β3γ2L receptors in HEK293T cells. G32R could perturb allosteric structures at the β–/γ+ interface to disrupt β3 oligomerization (67). To date, five *GABRB3* variants (S76C, R111X, D120N, R142L, and Y184H) were associated with MAE (36). These variants form a completely different subgroup of *GABRB3*-related epilepsy phenotype. D120N was also diagnosed in a more severe epilepsy phenotype, LGS (44), which will be discussed in the next part.

Moreover, a rare *familial* variant E357K was identified in a patient associated with Juvenile absence epilepsy (JAE). E357K, located in the ICD, reduces β3 surface expression and decreases GABA-evoked whole-cell current (68).

In 2013, a large spectrum of EE study by Epi4K consortium and Epilepsy Phenome/Genome Project (EPGP) identified four *de novo* variants in *GABRB3* associated with severe EEs, the LGS and WS. Three variants (N110D, D120N, and E180G) are located in the NTD, and one variant (Y302C) is located in the ICD (44). None of these variants were reported to reduce β3 surface expression. The LGS-associated variants (D120N, E180G, and Y302C), located at the β+/α– interface, reduce GABA-evoked peak current and GABA potency, whereas the WS-associated variant (N110D), located at the β–/α+ interface, only alters channel gating (26). Besides, E180G reduces single-channel opening time, and Y302C shows slow activation but fast deactivation. Overall, all four variants disrupt the partial function of the receptor, and three LGS-associated variants cause more severe consequences compared to WS-associated variant. Two more *de novo* missense variants of *GABRB3* (N328D and A305T in TM3) were associated with LGS. N328D possesses a different mechanism compared with previously reported LGS-associated variants. Although N328D has a similar negative effect on the channel current, a more pronounced effect is that this variant disrupts the oligomerization and assembly of the receptors, thus reducing the total and surface expression level of the subunit (68). Currently, data is lacking on the functional consequence of the A305T variant (69).

De novo variants are one of the major causes of EOEE. Recent studies have associated a number of *de novo* missense variants in *GABRB3* with EOEE. In 2017, a study reported functional effects from three variants (L170R, A305V, and T288N), located in major structural locations directly related to the transduction of the binding-coupling pathway. L170R (at Cys-loop in NTD) and A305V (in the beginning of TM3) are located at the interface between NTD and the pore, whereas T288N is located in TM2. All three variants reduce peak whole-cell currents to different extents; however, only L170R and A305V variants decrease the subunit surface expression level, suggesting that these two variants cause the ER retention and decrease the trafficking efficiency (31). Recently, a cohort of *GABRB3* variants were characterized, and two variants (N328D and E357K) were compared in depth: these variants cause reduced α1β3γ2 receptor clustering at synapse, indicating the importance of β3 subunits in the synaptic inhibition (68). Up to now, of all *GABRB3* variants, three were associated with more deleterious DS or DS-like phenotype: one *familial* variant (T157M) and two *de novo* variants (R232Q and T281I) (36, 69, 70). Recently, two new variants (E77K and T287I) were reported associated with an atypical gain-of-function molecular phenotype. E77K in NTD was associated with WS, and T287I in TM2 was with OS. Even though their locations and related epilepsy phenotypes are different, they share similar functional effects on $GABA_A$Rs: both variants result in a significant increase in GABA potency without changing desensitization and current amplitude (71).

EPILEPSY-ASSOCIATED VARIANTS IN THE γ2 SUBUNIT

Similar to *GABRA1* and *GABRB2*, *GABRG2* is located on chromosome 5q34. γ2 is the requisite subunit for the postsynaptic clustering of $GABA_A$Rs. As mentioned

above, α and β subunits are essential in the assembly of $GABA_A$Rs, which were confirmed by data from α and β subunit knockout mice (64, 72, 73). By contrast, lack of γ2 subunits would only impair the channel function partially by reducing GABA binding sites (74). Surprisingly, among all subunit genes, variants in *GABRG2* are the most commonly linked to the etiology of epilepsy. Abundant evidence validated that, along with its interacting $GABA_A$R-associated protein (GABARAP), the γ2 subunit plays an important role in the translocation of receptors from Golgi to the plasma membrane, as well as in receptor clustering and synaptic maintenance (75–78). The majority of variants identified in *GABRG2* are associated with relatively benign FS or CAE, although some variants are associated with more severe genetic epilepsy syndromes, like GEFS+, or even worse phenotype like DS. Given the critical role of γ2 and many recently updated epilepsy-associated variants, we included the broad spectrum of pathogenic *GABRG2* variants with their resulting epilepsy syndromes. These variants, including missense, nonsense, and frameshift variants, are located throughout the primary γ2 sequence (Table 1 and 2). The positions of the γ2 variants in the NTD domain are illustrated in Figure 4A, whereas those in the TMD domains and their connecting loops are illustrated in Figure 4B.

VARIANTS WITH MODERATE SEVERITY OF EPILEPSY SYNDROMES

Among the *GABRG2* variants that were identified in individuals with FS, CAE, or mild generalized epilepsy, most are missense variants, and one is frameshift variant (R177fs). One heterozygous missense variant R82Q in the distal NTD was identified in a large family of several individuals with FS and CAE (79). This *familial* variant serves as a great model to investigate the pathogenic effect, functional consequences, and mechanism for the CAE and FS-associated γ2

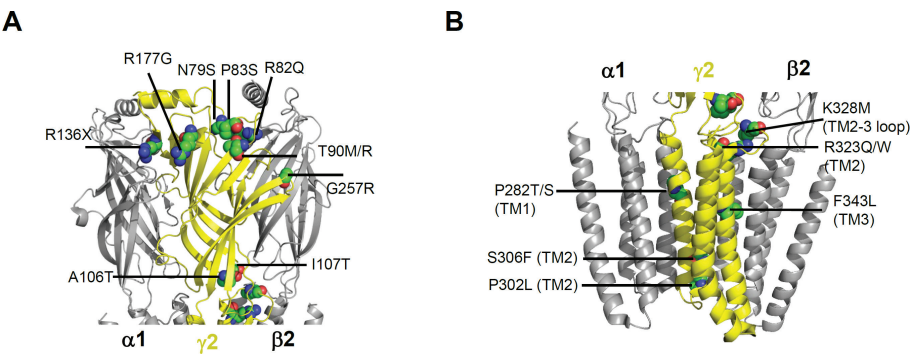

Figure 4. Epilepsy-associated variants in the γ2 subunit of $GABA_A$ receptors. Gene name: *GABRG2*. Viewed in the structure constructed from 6X3S.pdb. **A,** Positions of the variants in the N-terminal domain are presented as sphere models. **B,** Positions of the variants in the transmembrane (TM) domain are presented as sphere models.

NTD variants. R82Q decreases BZD sensitivity to $GABA_A$Rs (79). Moreover, R82Q reduces γ2 surface expressions mainly by impairing γ2 and β2 subunits oligomerization and causing their ER retention (80–82). In addition, R82Q could alter the endocytosis or the subunit composition on the cell surface (82, 83). In 2007, the heterozygous R82Q γ2 mice became one of the first *in vivo* animal models to investigate CAE phenotype, onset behavior, and treatment (84). Similar to R82Q, R177G is a *familial* variant associated with FS (85). R177G alters current kinetics and reduces BZD sensitivity. In addition, R177G impairs the receptor trafficking and causes glycosylation arrest. Interestingly, compared to homozygous expression of R177G (60% surface reduction), the heterozygous expression results in even larger reduction of γ2 surface level (90% reduction) (86). One deletion variant in *GABRG2* (S443delC), identified with FS and mild generalized epilepsy, affects the subunit total and surface expression level (87).

Conversely, some *GABRG2* variants were associated with slightly more severe EE. N79S and P83S are *familial* variants associated with GES (48, 88). Both N79S and P83S contribute to the γ2+/β2– interface and impair $GABA_A$R assembly in the endoplasmic reticulum (ER). P83S results in a significant reduction (60–90%) of the subunit surface expression due to strong ER retention, whereas N79S only reduces 12% of surface expression due to deficient trafficking (89). Six *de novo* variants (A106T, I107T, P282S, R323W, R323Q, and F343L) were found in patients associated with unclassified EE (34). They are all located in functionally important regions: A106T and I107T are in the NTD binding site, occupying the γ+/β– interface, P282S in TM1, R323W and R323Q in TM2, and F343L in TM3. Overall, these variants reduce the subunit surface expression level and decrease the channel function. R323W and R323Q in TM2 lead to accelerated deactivation, whereas the rest four result in accelerated activation and prolonged deactivation (34). Besides, G257R, I389V, and R323Q were identified in Rolandic epilepsy (RE) (90). The *familial* variant G257R reduces trafficking and the cell surface expression level. P282T, an EE-related variant, shares the same location and functional consequence with P282S (91).

VARIANTS WITH SEVERE EPILEPSY SYNDROMES

Considering the severity of epilepsy phenotypes, several variants in γ2 subunits have been identified in patients with GEFS+. Some of the previously mentioned missense variants, especially those located near the distal NTD such as N79S, P83S, and T90M, could exhibit worse epileptic phenotypes, such as GEFS+ during development. K328M, a heterozygous variant located at the γ+/β– interface, is a missense variant associated with an AD generalized GEFS+ (92). Functional studies demonstrated that K328M impairs channel gating properties instead of perturbing the trafficking (93, 94). A γ2(K328M) knock-in mouse model demonstrates GEFS+ and premature sudden death (95). Two nonsense variants, R136X and W429X, were associated with FS and GEFS+ (96, 97). Both variants cause ER retention, decrease total subunit protein level, and reduce the forward trafficking of functional receptors.

Two nonsense *GABRG2* variants (Q40X and Q390X) were associated with DS, one of the most devastating forms of epilepsy (98, 99). Q40X causes a premature

stop at the 5' end, showing essentially no surface expression. Q390X leads to the aggregation of γ2 subunits in the ER, resulting in strong ER retention when expressed in HEK293 cells. In addition, Q390X induces trafficking defect in a temperature-dependent manner, and thus this variant was associated with temperature-induced seizure. Currently, only two missense variants in *GABRG2* (T90R and P302L) were associated with DS. One *de novo* variant T90R, located in the same position as T90M, exhibits several seizure phenotypes. T90R decreases receptor surface expression level and GABA-evoked current due to inefficient receptor assembly and strong ER retention (52). On the other hand, P302L, facing the conduction pathway within the pore in TM2, only leads to a small reduction (~24%) of the receptor surface expression level, but reduces the channel function (up to 76%) by increasing the desensitization and reducing GABA potency (31).

CONCLUSION

$GABA_A$Rs are widely expressed throughout the brain (54), and their functional defect contributes to the initiation of seizures. The α1β2/β3γ2 receptor, the most abundant receptor subtype in the brain, is responsible for fast decaying anion conductance at synapses. Over 150 *de novo* or *familial* variants in these major $GABA_A$R subunits have been identified to be associated with mild or deleterious genetic epilepsy (Table 1 and 2). The vast majority of these variants lead to the loss of function of $GABA_A$ receptors with various functional consequences through a number of molecular mechanisms, including impaired protein biogenesis in the ER (protein misfolding, assembly defect, ER retention, and excessive ERAD), NMD, and gating defects. Since the functional defects of $GABA_A$ receptors largely depend on specific pathogenic variants, it is critical to develop personalized treatment for precision medicine. Given the progress of genetic sequencing capacity, genetic screening would provide valuable information for effective treatment plan. Over 30% of epilepsy patients are resistant to conventional anti-epilepsy drug treatment partially because current treatments focus on relieving symptoms instead of targeting causative factors (100). Therefore, novel therapeutic strategy is urgently needed with the consideration of the underlying disease-causing mechanism. Since many of the pathogenic variants in $GABA_A$ receptors lead to impaired protein biogenesis in the ER, reduced receptor surface trafficking, and thus loss of function due to protein conformational defects, correcting proteostasis deficiency is a promising therapeutic strategy to treat such genetic epilepsies (12, 101). Indeed, a number of investigations demonstrated that enhancing the protein folding and surface trafficking of pathogenic $GABA_A$ receptors containing various subunit variants is sufficient to restore their functions (38, 40, 49, 102, 103), suggesting a therapeutic potential of the proteostasis maintenance strategy.

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