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# Investigation of Unknown Neurogenetic Variants: An Integrated Computational Analysis Revealing Epilepsy-Associated Critical Variants in the GABRB3 Gene

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## Bilinmeyen Nörogenetik Varyantların Araştırılması: GABRB3 Genindeki Epilepsiyle İlişkili Kritik Varyantları Ortaya Koyan Bütünleşik Hesaplamalı Analiz

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**Abstract**

Alterations of GABA (A) receptors are linked to various disorders including epilepsy, which is diagnosed through a comprehensive approach including genetic screening. The functional consequence of many genetic variants in the β3 subunit of the GABA(A) receptor remain unknown. This presents a challenge for genetic testing and precision medicine. Addressing this obstacle, in the present study, we analyzed the 141 missense variants with unknown function in the *GABRB3* gene using a comprehensive in silico approach. Algorithmic computing based on the sequence homology and other features including the functional and disease-related analysis of missense variants revealed the prediction of the most pathogenic variants, mapped onto the different domains of the β3 subunit, with Y48C, D49E, D73H, M80R, D94E, H132Y, R142C, P169L, C175W and Y182H being located in the N terminus extracellular domain, S264F in the first transmembrane domain, G279R, T281A the second transmembrane region, R294Q is at the end of the second transmembrane domain, P298S in the linker between the second and the third transmembrane domains, and Y467S/H in the fourth transmembrane domains. These variants were generally associated with childhood absence epilepsy. Our results provide guidance for the laboratory research aiming for the identification of new pathogenic epilepsy mutations.

***Keywords:*** *Epilepsy, GABA (A) receptor, GABRB3, Variants of uncertain significance (VUS).*

**Öz**

GABA (A) reseptörlerindeki mutasyonlar, genetik testler de dahil olmak üzere bütünleştirici bir yaklaşımla teşhis edilen epilepsi gibi çeşitli hastalıklarla bağlantılıdır. Öte yandan, β3 alt ünitesini kodlayan *GABRB3* geninin varyantları da dahil olmak üzere GABA (A) reseptör alt ünitesi gen varyantlarının büyük bir kısmının fonksiyonel sonucu bilinmemektedir ve bu durum genetik testler ve hassas tıp için bir zorluk teşkil etmektedir. Mevcut çalışmada, kapsamlı in siliko analizi kullanılarak GABRB3 geninin işlevi bilinmeyen 141 varyantı analiz edildi. Sekans homolojisine ve varyantların fonksiyonel ve hastalıkla ilgili analizi de dahil çeşitli özelliklere dayanan algoritmik hesaplamalar, β3 alt ünitesinin farklı protein alanlarında bulunan en patojenik varyantları ortaya çıkardı. Y48C, D49E, D73H, M80R, D94E, H132Y, R142C, P169L, C175W, ve Y182H, N -terminal hücre dışı alanda, S264F birinci transmembran alanında, G279R ve T281A ikinci transmembran alanında, R294Q, ikinci transmembran bölgesinin sonunda, P298S ikinci ve üçüncü transmembran alanları arasındaki bölgede ve Y467S/H dördüncü transmembran alanda bulunan patojenik varyantlar olarak tespit edildi. Bu varyantlar, çocukluk çağı absans epilepsisi ile ilişkili bulunmuştur. Sonuçlarımız, yeni patojenik epilepsi mutasyonlarının tanımlanmasına yönelik laboratuvar araştırmalarına rehberlik sağlamaktadır.

***Anahtar Kelimeler:*** *Epilepsi, GABA (A) reseptörü, GABRB3, Belirsiz Anlamlı Varyant (VUS).*

## 1. Introduction

Epilepsy is diagnosed in people, who experience unprovoked seizures (Fisher et al. 2014). This condition is one of the most common neurological disorders, impacting over 70 million people globally (Thijs et al. 2019). The historical view on epilepsy pathophysiology is based on the network hyperexcitability, deriving from the imbalance between excitation and inhibition (Tipton and Russek 2022). However, aberrant communication of excitatory and inhibitory neurons alone does not explain epileptogenesis straightforwardly as the role of complex spatiotemporal operation of multiple elements becomes evident (Agopyan-Miu et al. 2023, Çarçak et al. 2023, Du et al. 2022, Dudok et al. 2021, Huberfeld et al. 2011, Olsen and Avoli 1997). Nevertheless, the significance of inhibitory dysfunction, characterized by a deficit of GABAergic inhibition preserves its place (Cohen et al. 1964).

GABA (gamma-aminobutyric acid), the primary neurotransmitter responsible for the mediation of

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inhibition in the mammalian brain, shows its inhibitory effects via the GABA(A) receptors, the heteropentameric ion channels (Goetz et al. 2007). Upon GABA binding, GABA(A) receptors undergo a complex conformational change, leading to the movement of $Cl^-$ and $HCO_3^-$ ions in opposite directions. In mature neurons, $Cl^-$ moves into the cell, while $HCO_3^-$ moves out. The depolarizing effect of $HCO_3^-$ moving out of the cell is overridden by the $Cl^-$ influx, which causes strong hyperpolarization, as the permeability ratio of $HCO_3^-/Cl^-$ is about 0.2 to 0.4 (Kaila et al. 1997). The channel pore determining this permeability is made up of the oligomerization of five subunits assembled from a diverse subunit pool (α1-α6, β1-β3, γ1-γ3, δ, ∈, θ, π and ρ1-ρ3), with the most prevalent assembly being composed of two α subunits, two β subunits, and one additional subunit with a special arrangement (Arslan 2021, Goetz et al. 2007). For instance, GABA(A) receptors, including the β3, α1 and γ2 subunits, are known to be positioned counterclockwise in the cell membrane when viewed from the extracellular space, which is as follows; β3, α1, γ2, β3, α1 (Tretter et al. 1997, Phulera et al. 2018). On the other hand, it appears that receptors, comprised of different stoichiometry and arrangements may also exist (Botzolakis et al. 2016, Olsen and Sieghart 2008, Puthenkalam et al. 2016, Sente et al. 2022, Verdoorn 1994).

The effect of dysregulation of the GABA (A) receptor function is linked to the alterations in its specific subunits (Audenaert et al. 2006, Baulac et al. 2001, Carvill et al. 2014, Chen et al. 2017, Ding et al. 2010, Feng et al. 2023, Hernandez et al. 2023a, Huang et al. 2014, Johannesen et al. 2016, Kang et al. 2015, Kang and Macdonald 2016). For instance, the dysfunction of the β3 subunit alone is associated with a range of disorders including autism (Delahanty et al. 2011, Vien et al. 2015), schizophrenia (Liu et al. 2019), epilepsy syndromes (Absalom et al. 2022, Absalom et al. 2020, Epi4K Consortium et al. 2013, Lachance-Touchette et al. 2010, Macdonald et al. 2010, Maillard et al. 2022, Møller et al. 2017, Papandreou et al. 2016, Pavone et al. 2020, Urak et al. 2006). Interestingly, the mutations in the β3 subunit, associated with epilepsy correlates with phenotype severity (Johannesen et al. 2022, Lin et al. 2023, Maillard et al. 2022, Yang et al. 2022) and cellular pathology such as alterations in the receptor clustering (Shi et al. 2019).

Although the β3 subunit is more common and abundant in the prenatal and neonatal brain, it is a necessary component of the GABA (A) receptor in many brain regions such as neocortex and hippocampus in adults (Laurie et al. 1992, Wisden et al. 1992). The predominant synaptic GABA(A) receptor, widely distributed throughout the brain, consists of α1, β2, and γ2 subunits in a ratio of 2:2:1 (2α1/2β2/1γ2). In contrast, the less common synaptic GABA(A) receptor is composed of γ2, α3, and β3 subunits in a ratio of 1:2:2 (1γ2/2α3/2β3). Although these receptor subtypes are frequently found in similar neuroanatomical regions like the cortex and thalamus, they are typically restricted to distinct cells, tissues or nuclei (Sieghart and Sperk 2002). In fact, accumulating research suggests that β3 is functionally distinguished from other β subunits. The β3 subunit determines the ionic selectivity of the receptor channel, regulates phasic and tonic currents, and mediates slower inhibitory postsynaptic current (IPSP) kinetics (Garifulina et al. 2022, Jensen et al. 2002, Menzikov et al. 2021). Additionally, in vitro studies suggest that GABA(A) receptors containing the β3 subunit may exhibit dual functionality. This means they could function in two distinct modes: either as a chloride ion channel regulated by GABA or as a P-type ATPase transporting anions (Menzikov et al. 2020). Recent studies also suggest that β3 subunit is required during the emergence of interhemispheric circuits for sensory processing (Babij et al. 2023).

The β3 subunit is encoded by *GABRB3 gene.* This gene contains an alternative exon 1 (exon 1a) that codes for a variant signal peptide, producing a β3 subunit of the same length but with a modified mature peptide sequence (Macdonald et al. 2010). The two transcripts exhibit distinct expression levels and patterns in the fetal and adult brain, with exon 1a being more prominently expressed in the fetal brain. The mutations P11S and S15F, are located in exon 1a in the β3 subunit signal peptide encoded by *GABRB3* gene, while mutation G32R, is in exon 2 and is located in the mature β3 subunit peptide near the N terminal extracellular domain (ECD) (Macdonald et al. 2010). It was indicated that mutations in the *GABRB3* gene could potentially diminish the expression of GABA (A) receptors and the overall current amplitudes in cells. This effect is proposed to occur through modifications in the N-linked glycosylation of the β3 subunit (Delahanty et al. 2011, Tanaka et al. 2008). The reduction in the membrane expression of GABA (A) receptors containing the β3 subunit aligns with an epilepsy-related phenotype (Gurba et al. 2012). Animal studies show that mutations in the β3 Subunit have epilepsy phenotypes (Nwosu et al. 2023, Qu et al. 2023). Homozygous *GABRB3* knockout mice exhibited myoclonic and atypical absence seizures, as well as deficits in cognition, motor coordination, and somatosensory functions. In contrast, heterozygous *GABRB3* knockout mice showed heightened epileptiform EEG activity and

increased susceptibility to seizures, which exhibited responsiveness to antiepileptic medications (Homanics et al. 1997, Tanaka et al. 2010). Given the importance of the β3 subunit, described so far, the present study focuses on the analysis of the genetic variants of unknown significance (VUS) of the β3 subunit encoded by *GABRB3* gene. There are many studies in the literature regarding *GABRB3* receptor gene variants (Lin et al. 2023, Absalom et al. 2022, Johannesen et al. 2022, Maillard et al. 2022, Yang et al. 2022, Absalom et al. 2020, Hernandez et al. 2019). Many of these studies involve analyses of genetic variants identified by researchers in their own patient populations. However, these studies are not able to provide sufficiently rapid answers to questions related to increasingly frequent and unknown variants or VUS derived from diagnostic genetic testing (Joynt et al. 2021). Preparing and testing each variant under *in vitro* conditions, especially in clinical settings where the evaluation of findings from genetic test results competes with time during patient diagnosis, presents a significant challenge. Thus, the VUS, which represent the variants with conflicting or insufficient evidence for disease association (Joynt et al. 2021), pose a challenge for genetic testing, an important component of the epilepsy diagnosis, prognosis and treatment (Scheffer et al. 2017). For instance, as many as one-third of the individuals worldwide affected by epilepsy do not exhibit a satisfactory response to currently available treatments, that require evidence-based precision approaches which includes efficient genetic testing results and interpretation of VUS (Chen et al. 2018, Knowles et al. 2022). The American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) recommend a framework that is considered the standard for interpreting the clinical significance of genetic variants (Richards et al. 2015). This framework classifies variants as either “Pathogenic”, “Likely Pathogenic”, “Benign” or “Likely Benign” (Richards et al. 2015). The evidence for pathogenic variants are categorized as supportive “PP1-5”, moderate “PM1-6”, strong “PS1-4” and very strong “PVS1”. Among these standards, the use of in silico approach to predict variant consequences is regarded as supporting evidence for pathogenicity, aligning with criterion “PP3” (Richards et al. 2015). This criterion, along with others, can assist in assessing the pathogenicity of VUS, with implications for tailoring personalized diagnosis, prognosis and treatment for epilepsy (Traynelis et al. 2017, Trowbridge et al. 2021). Thus, the present study utilized an integrated in silico approach, which was performed on the *GABRB3* VUS to assess their functional consequence and clinical significance by using a range of algorithms. In silico analysis of the *GABRB3* gene variants may have different focal points. For instance, some studies may focus on the analysis of the predictive accuracy of in silico tools for the classification of GABA (A) receptor gene variants. Typically, these studies focus on the known pathogenic and benign variants of GA*BRA1, GABRA2, GABRB3* and *GABRG2* genes to test the predictive performance of specific in silico tools such as AlphaMissense (Cheng et al. 2023). Additionally, some in silico studies focus on predicting the effect of unknown variants (VUS) on the structure and function of specific GABA(A) receptor subunit genes including *GABRA1, GABRD*, *GABRG2* (Arslan 2023, Arslan 2024, Abdullah and Arslan 2024). Other studies (Kulandaisamy et al. 2019, Molnár et al. 2016, Partridge et al. 2004) which focus on a broad screening of the entire human transmembrane proteome are valuable for offering new characteristics as a utility for the identification of the common molecular patterns, which can be used to improve the prediction of pathogenic variants of transmembrane proteins. However, such studies have a different focus as described above and cannot replace the detailed gene-centric analysis of specific gene variants. Thus, to our knowledge, this is the first study specifically focusing on the effect of variants with unknown function in *GABRB3* gene, collected from ClinVar, publicly available database maintained by the National Center for Biotechnology Information (NCBI) that aggregates information about genetic variants and their relationships to clinical significance (Landrum et al. 2018). In the present study we analyzed the 141 missense variants with unknown function in *GABRB3* gene using comprehensive in silico approach.

## 2. Materials and Methods

### *2.1. Data mining*

The National Center for Biotechnology Information (NCBI) - ClinVar database (Int.Ref.-1) a freely accessible, public archive of reports on relationships between human variations and phenotypes, was used for data mining (Harrison et al. 2016, Landrum et al. 2018) in order the identify the missense VUS located in the coding region of the Homo sapiens gamma-aminobutyric acid type A receptor subunit beta3 (GABRB3), transcript variant 1, mRNA (NCBI Reference Sequence: NM_000814.6). This transcript variant 1 represents the MANE (Matched Annotation from the NCBI and EMBL-EBI) select which is annotated as gamma-aminobutyric acid receptor subunit beta-3 isoform 1 precursor [Homo sapiens] in the NCBI database (NCBI Reference Sequence: NP_000805.1). The gene symbol (GABRB3) was used for searching the corresponding variants. This led to the list of variants under different categories. To screen missense variants of

unknown clinical significance (VUS), we selected 'single nucleotide variations,' 'missense,' and 'uncertain significance' from the dropdown menu in ClinVar. The data were retrieved and transferred to an MS Office Excel file by 'use text import wizard'. Additionally, UniProt **(Int. Ref.-2)** database was used to retrieve the variant information (UniProt ID: P28472 GBRB3_HUMAN) for functional significance, including the variants in the ligand binding sites according to structural data of the β3 subunit (Sente et al. 2022)

### *2.2. Analysis of Variant Effect*

A combination of six different software tools [SIFT (Ng and Henikoff 2003), PolyPhen-2 (Adzhubei et al. 2013), PANTHER-PSEP (Tang and Thomas 2016), SNAP2 (Hecht et al. 2015), FATHMM-XF (Rogers et al. 2018), PhD-SNP (Capriotti et al. 2006)] was used in the study. The final decission of pathogenicity was based on the consensus results among these six tools, which can be divided in to two categories, i.e., algorithms computing the sequence homology or variant fitness effect and algorithms considering the multiple features including the functional & disease-related analysis of missense variants. For variant fitness Effect PhD-SNP, PANTHER-PSEP and SIFT prediction methods were used for a sequence homology-based analysis, while the others were used for analysis taking into account multiple features and combining parameters such as conservation and epigenomics (FATHMM-XF), sequence and structure-based features (PolyPhen-2), conservation and predicted structural features (SNAP2) (Katsonis et al. 2022). Using sequence homology, the SIFT server (Int. Ref.-3) evaluates the effect of amino acid substitution on the functional and phenotypic consequence relevant to its protein (Ng and Henikoff 2003). This evaluation is presented by a metric between 0 and 1, with a score ≤ 0.5 repreenting a substitution with damaging (disease) consequence. PolyPhen-2 (Int. Ref.-4) utilizes a Bayesian approach and focuses on the sequence and structure-based properties of the target region for amino acid substitution and categorizes these features (Adzhubei et al. 2013). Two options are derived from two data sets, namely HumDiv (trained using rare alleles causing Mendelian disease) which was used in the present study and HumVar data set (more common alleles and nonsynonymous SNPs). Results are given as "Probably Damaging", "Possibly Damaging", or "Benign". The PANTHER-PSEP (Tang and Thomas 2016) **(Int. Ref.-5)** is another tool used in the present study. It utilizes sequence homology to evaluate the variant effect on the protein function. The homologous sequences of the protein under investigation are gathered, arranged in order, and then a metric for how much they have stayed the same over millions of years is computed by considering the weighted occurrences of various amino acids found at the specific position in the alignment. The SNAP2 (Int. Ref.-6) is a classifier trained by neural network-based machine learning that evaluates the functional consequence of mutations. Besides the evolutionary conservation derived from multiple sequence alignment, structural properties including secondary structure and solvent accessibility are considered for accurate prediction (Hecht et al., 2015). Its output is presented as neutral or effect with a score between -100 and +100. The FATHMM-XF (Int. Ref.-7) assesses the variant effect in the context of human genome and heritable disorders (Rogers et al. 2018). Its evaluation metric is the p value which ranges from 0 to 1, with the p value >0.5, representing a damaging effect, while p value ≤ 0.5 represents a neutral or benign effect. The PhD-SNP (Int. Ref.-8), a support vector machine-based classification, combines multiple sequence alignment data and context of the variant in the protein sequence (Capriotti et al. 2006).

### *2.3. Analysis of Stability and Functional Consequence*

Two servers (I-Mutant and Mupro) were used to evaluate the possibility of variants causing stabilizing or destabilizing effects on the structural features of protein structure. The I-Mutant server (Int. Ref.-9) was used to determine the effect of VUS on protein stability (Capriotti et al. 2005), as it evaluates the impact of missense variants on the free energy change (ΔΔG). The outputs were reported using SVM (support vector machine) based binary classification (ΔΔG < 0: Decreased Stability and ΔΔG >0: Increased Stability). The MUpro web server (Int. Ref.-10) utilizes SVM approach, predicting the protein stability changes for single amino acid mutations, based on the sequence and structural data (Cheng et al. 2006). MUpro predicts the change in Gibbs free energy changes as a measure of protein stability changes. MUpro has binary classification: ΔΔG < 0 meaning a decreased stability and ΔΔG > 0 meaning an increased stability.

### *2.4. Analysis of Evolutionary Conservation*

Conservation predictions of amino acids in β3 subunit were evaluated by the ConSurf server (Int. Ref.-11) (Ashkenazy et al. 2016). The ConSurf is a homology-based approach that provides an evolutionary conservation score for each protein residue. ConSurf determines these scores based on values between 1 and 9, from the least conserved to the most conserved. A functional residue is considered when an amino acid is exposed and highly conserved. If a residue is conserved and buried, it represents a structural residue.

### *2.5. Prediction of Molecular Mechanisms*

In addition to the previous procedure described so far, we have also used the MutPred2 server (Pejaver et al. 2020) (Int. Ref.-12), which incorporates features such as structure, function, and conservation. It is used to validate the preliminary results obtained from previous steps since their predictive focus is molecular mechanisms associated with disease. MutPred2 is based on a machine learning approach and it ranks the evaluation of disease effect according to "pathogenicity score (g)". The g value ≥ 0.5 represents a pathogenic effect of the variant. To increase specificity, $g > 0.75$ and $p < 0.01$ were taken as thresholds for pathogenicity prediction.

### *2.6. Structural Properties and Modeling*

UCSF Chimera (Pettersen et al. 2004) was used to locate the specific residues on the β3 subunit three-dimensional structure and display it with highlighted colors. The HOPE server was used for the structural comparison of most pathogenic variants with the corresponding wild-type (WT) residues (Venselaar et al. 2010). A diagram showing the cell membrane localization of the β3 subunit together with variant annotation was generated by using Protter server (Omasits et al. 2014).

## 3. Results

### *3.1. Overview*

In this study, the integrated bioinformatic analysis (**Figure 1**) was performed for the assessment of the variant effect of the missense variants with unknown function in the coding region of the Homo *sapiens* gamma-aminobutyric acid receptor subunit beta-3 (GABRB3) gene. Following the data mining in the first step, a combination of softwares based on the sequence homology, structural homology and other properties, were used for the prediction of pathogenicity. Thus, as a first step of data mining, among the 152 single nucleotide polymorphisms with unknown function retrieved from the ClinVar database, only missense variants or VUS (n =141) were included in the analysis. The study has first focused on the analysis of this 141 VUS in terms of variant fitness effect besides other characteristics to identify the variants manifesting highest degree of pathogenicity. These characteristics include, sequence homology derived from the multiple sequence alignment (SIFT), evolutionary preservation (PANTHER-PSEP) and multiple sequence alignment combined with local sequence alignment (PhD-SNP). In addition, servers, which use multiple characteristics such as sequence, conservation and structure prediction (SNAP2, PolyPhen-2) as well as meta-predictor FATHMM-XF, utilizing more than thirty features including data from ENCODE and Epigenomics were utilized (**Figure 1, 2a**). This has led to selection of 43 variants. This was followed by the evaluation of variant effects on protein stability and molecular mechanisms as a validation. In addition, determination of conservation scores was performed in this step (**Figure 1, 3a**), leading to the identification of 21 variants (Supplementary file 3). After filtering out the variants lacking association with epilepsy, 17 variants were selected for modeling (**Figure 1, 3b**).

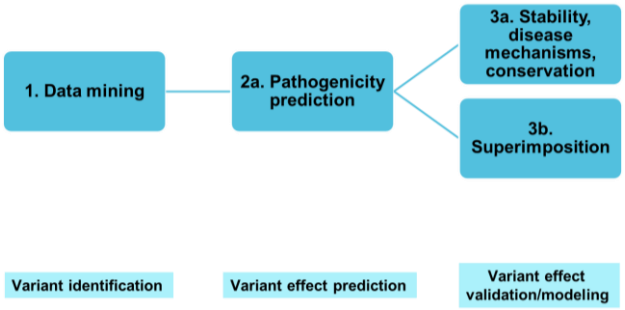


**Figure 1.** Overview of the study design.

### *3.2. Data mining*

*GABRB3* gene variants (n=852) were retrieved from the ClinVar database (accessed in December, 2022) as summarized in Figure 2. Only missense variants or single nucleotide mutations represented in the NCBI Reference Sequence: NM_000814.6, i.e., gamma-aminobutyric acid receptor subunit beta-3 (GABRB3) / Isoform 1 precursor (*Homo sapiens*), were selected for the analysis in the study. Among these variants, there were 465 single nucleotide polymorphisms (SNPs), 4 indel variants, 32 insertion, 161 duplication and 190 deletion (**Figure 2A**).

Among the total of 465 SNPs, 238 of them were missense, 61 of them were in three prime or five prime untranslated regions (3´UTR and 5´UTR), 55 of them belonged to ncRNA, 13 of them were nonsense, and 3 of them were splice site variants (**Figure 2B**). The ClinVar filtering of 238 missense SNPs for clinical significance have led to the identification of 152 VUS, besides to 6 bening, 16 likely bening, 42 likely pathogenic, and 17 pathogenic variants. In addition, there were 14 variants with conflicting interpretations (**Figure 2C**). Among the 152 VUS, 141 unknown missense variants VUS were included in the analysis (Supplementary file 1).

### *3.3. Variant Effect Analysis*

The 141 VUS were comparatively analyzed by six algorithms (SIFT, PolyPhen-2, PhD-SNP, PANTHER-PSEP, SNAP2 and FATHMM-XF) (Figure 3). As explained in the Methods section, a score below 0.05 in the SIFT program predicts the substituted amino acid to be pathogenic or deleterious.

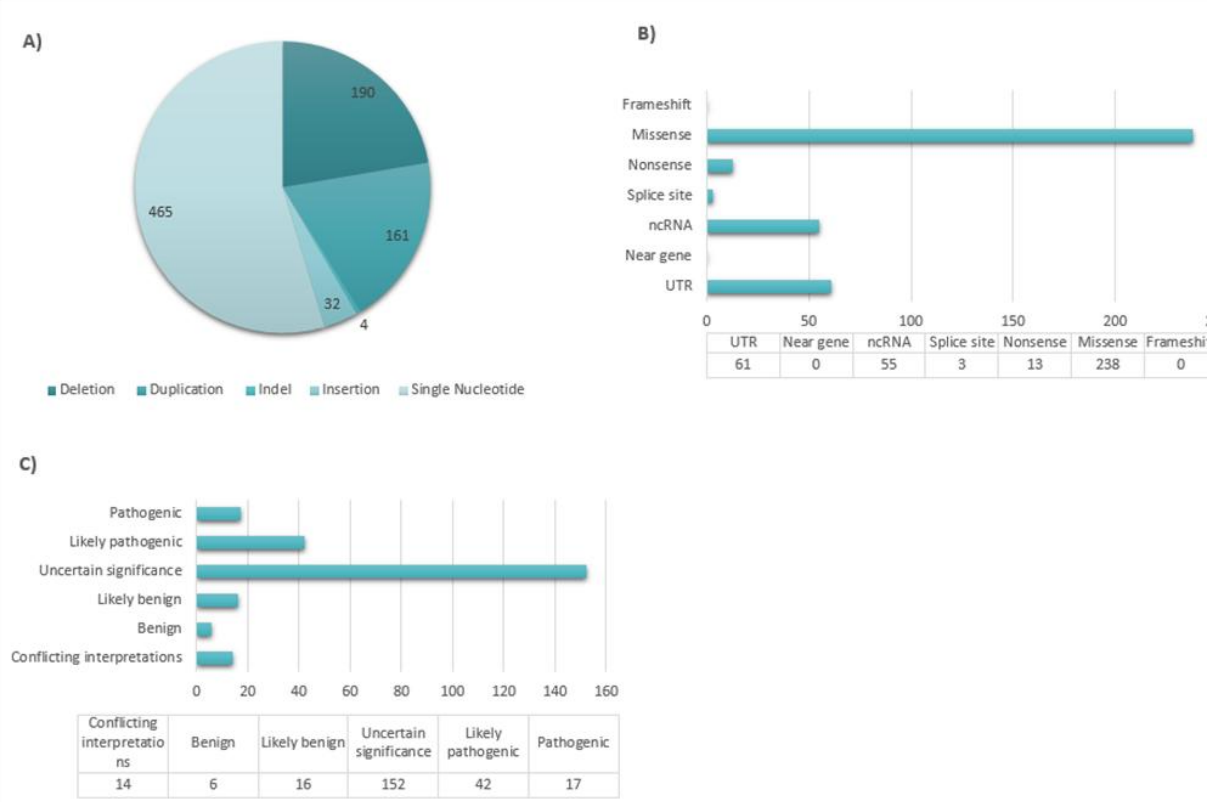


**Figure 2.** Summary of data retrieved from ClinVar. (A) Types of variations in the *GABRB3* gene; (B) Molecular consequences of *GABRB3* gene variations; (C) Classification of missense variations in the GABRB3 gene based on clinical significance.

Thus, the SIFT algorithm predicted 73 variants as deleterious while 64 variants were predicted as tolerated, and 4 variants as low confidence. The support vector machine-based classifier PhD-SNP (Capriotti et al. 2006), offers two options in the result output: Disease or Neutral. In our study, we classified all disease-resulting amino acid variations as pathogenic. We did not consider the reliability index (RI) score that comes with the output since the binary classification better align with our study design, which includes the use of multiple in silico tools, leading us to prioritize clarity over the nuanced information provided by the RI values.

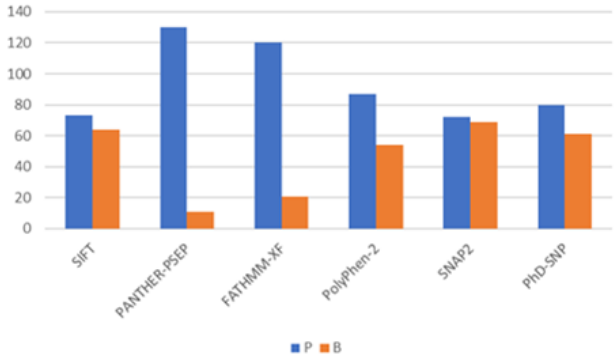


**Figure 3.** Pathogenicity evaluation of GABRB3 variants by six tools. Blue bars represent pathogenic (P), likely pathogenic or deleterious variants. Orange represents neutral or benign variants (B).

Thus, out of 141 VUS, 80 were found to be disease-related, and 61 were found to be neutral. We determined the Pdel score of PANTHER-PSEP as 0.74 ≤ probably damaging ≤ 1 to include in our study. This corresponds to a preservation time (P. Time) of over 750 years. By including different prediction algorithms in our study, we further supported these results.

Thus, we have also used PolyPhen-2 which utilizes phylogenetic and structural data of the protein encoded by *GABRB3* to predict possible structural and functional impacts of the *GABRB3* missense variants. PolyPhen-2 outputs were given as benign, probably damaging or possibly damaging. PolyPhen-2 prediction results consist of a score starting from zero/neutrality and increasing towards positive numbers as a damaging effect. Among 141 VUSs, 69 probably damaging, 18 possibly damaging and 54 benign variants were identified. (accessed on March 15th, 2023). These predictions were scored (p-value) in the range of 0-1 in FATHMM-XF. If the p-value is > 0.5, it is predicted as pathogenic, and if the p-value is ≤ 0.5, it is predicted as benign. Thus, 114 VUS were predicted as pathogenic, 6 were not found (nf), and 21 were predicted as benign (accessed in 19.03.2023). In this study, we included SNAP, another algorithm developed to determine the impact of single amino acid substitutions on protein function. We selected results from SNAP that

had an accuracy rate of over 50 % and 72 were found to be pathogenic, and 69 were found to be not. Among the variant residues tested, the ones with the highest probability of pathogenic effect in all six softwares were reserved for further analysis (n=43) (See also Supplementary file 2).

### *3.4. Analysis of Stability, Conservation and Functional Consequence*

Deformation of a protein's scaffolding is thought to be one of the major causes of molecular pathophysiology in humans (Bromberg and Rost 2009). Thus, the VUS predicted as pathogenic (n=43) were analyzed for the variant effect on the protein stability and functional consequence by I-Mutant 2 and MUpro servers. I-Mutant assessment revealed that all of the studied set of 43 VUS decreased the stability of the β3 subunit. The prediction conditions were 25°C and PH=7, and the resulted the calculation of DDG, which stands for the change in Gibbs free energy (ΔΔG) due to the variant. DDG > 0 is associated with a stabilizing effect while DDG < 0 is associated with the destabilizing mutation. Similarly, MUpro server has been used for the prediction of stability changes due to the variants. Both of the programs predicted all 43 GABRB3 variants as decreased stability (ΔΔG <0: Decreased Stability and ΔΔG >0: Increased Stability) (Supplementary file 3).

The multiple sequence alignment of *H. sapiens* β3 subunit would show the highly conserved amino acid residues. By default, Consurf searches for homologues from the UNIREF database (Ashkenazy et al. 2016, Glaser et al. 2003, Suzek et al. 2015) "a clustered version of the UniProt database" (UniProt Consortium 2023, Suzek et al. 2015). The process yields homologous sequences for comparative multiple sequence alignment, enabling the calculation of amino acid conservation in the β3 protein, ranked on a color-coded scale from 1 to 9, with 9 indicating the highest level of conservation (**Figure 4**). The conservation score ≥ 8 revealed 25 amino acids of the GABRB3 are highly conserved (Supplementary file 3). Additionally, the pathogenicity of variants of unknown significance or VUS of GABRB3 and probable molecular mechanisms were evaluated by MutPred2. As described in the Methods section "2.5. Prediction of Molecular Mechanisms", more stringent conditions of selection criteria to increase specificity has led to the identification of 21 VUS with Mutpred2 score $g > 0.75$ and $p < 0.01$ (see also Supplementary file 3). Since we wanted to focus on epilepsy syndromes, we have removed the VUS, lacking any indication relevant to epilepsy and this has led to a list of 17 missense variants (**Table 1**).

### *3.5. Mapping the variants on the GABRB3 protein*

The potential impact of the variants should be assessed in the context of their respective variant locations in the secondary as well as tertiary protein structure. For instance, the diagram showing the variant locations in the protein domains (**Figure 5A**), and the corresponding β3 subunit in the three-dimensional visualization of the receptor (**Figure 5B and 5C**) and its critical residues (**Figure 5D**), where the pathogenic variants are predicted should be studied by comparative modeling. Nine of the 17 variants (Table 1) which were predicted as pathogenic were located in the N-terminus extracellular domain (ECD) (Y48C, D49E, D73H, M80R, D94E, H132Y, R142C, P169L, C175W, Y182H), one in the TM1 (S264F), two in the TM2 (T281A, R294Q), two in between the TM2 and TM3 region (R294Q, P298S) and two in the TM4 (Y467S, Y467H) (**Figure 5A**).

### *3.6. Superimposition of the wild type and the variants*

One way to verify the functional consequence of a variant is to simulate its superimposition with the WT residue in the 3D protein model as shown in **Figure 6**. In the molecular context, starting with the N-terminal domain, the variant amino acid (C) is smaller and less hydrophilic than the WT amino acid (Y), which is a highly conserved functional (exposed) residue at the position 48 (see also Supplementary file 3, Table 1, see also Figure 4).

**Table 1**. Summary of the results. Critical variants (predicted as pathogenic) identified in the study and listed according to amino acid position starting from the C terminal domain of the β3 subunit encoded by GABRB3 gene. (CAE: Childhood Absence Epilepsy; DEE: Developmental Epileptic Encephalopathy).

| Variants predicted as pathogenic | ClinVar Variation ID | Condition |
|---|---|---|
| Y467S | 1999008 | CAE |
| Y467H | 1038688 | CAE |
| P298S | 2011043 | CAE |
| R294Q | 2419058 | CAE |
| T281A | 548619 | DEE, CAE |
| G279R | 1042138 | CAE |
| S264F | 1701763 | DEE, CAE |
| Y182H | 2010195 | CAE |
| C175W | 2103323 | CAE |
| P169L | 537288 | CAE |
| R142C | 1696530 | DEE, CAE |
| H132Y | 1038248 | CAE |
| D94E | 1320972 | CAE |
| M80R | 559623 | CAE |
| D73H | 409958 | CAE |
| D49E | 834156 | CAE |
| Y48C | 1474854 | CAE |


**Figure 4.** Conservation analysis of β3 subunit of GABA (A) Receptor

This could potentially result in a loss of external interactions and will affect hydrogen bond formation, as shown in the **Figure 6A**, where native amino acid residues are represented in blue, while amino acid variants (predicted pathogenic variants) are illustrated as red in the enlarged overlays.

As depicted in **Figure 6B**, the variant residue (E) is bigger than the WT residue at position 49 and this can disturb the multimeric interactions of this conserved and exposed residue (Supplementary file 3, Table 1, see also Figure 4).

**Figure 6C** shows the superimposition of D73H. The WT residue is positioned on the protein surface, as also listed in Table 1 and the mutation can alter the inter and intra molecular interactions of the protein. There is also a difference between the charges, the WT having the negative charge and the mutant being the neutral. **Figure 6D** shows the superimposition of M80R. There is a size difference between the mutant residue and the WT, which is also a highly conserved structural residue (Supplementary file 3, Table 1).

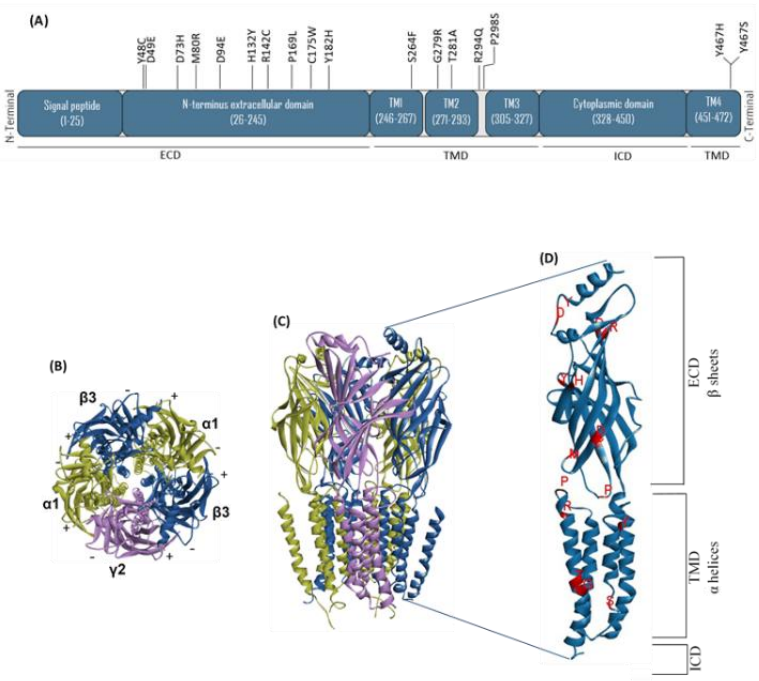


**Figure 5.** The position of variants in the three-dimensional structure of β3. (A) The schematic representation of β3 protein with respect to domain specific location of 17 genetic variants predicted pathogenic. (B) three-dimensional reconstruction of GABA (A) receptor assembled from α1, γ2 the β3 subunits (top view). (C) three-dimensional reconstruction of GABA (A) receptor, assembled from α1, γ2 the β3 subunits showing the position of β3 subunit (side view). (D) three-dimensional reconstruction of the β3 subunit of the GABA(A) receptor (expanded version, side view) presenting the positions of amino acids (written in red color), at which the pathogenic variants were detected. Image not to scale.

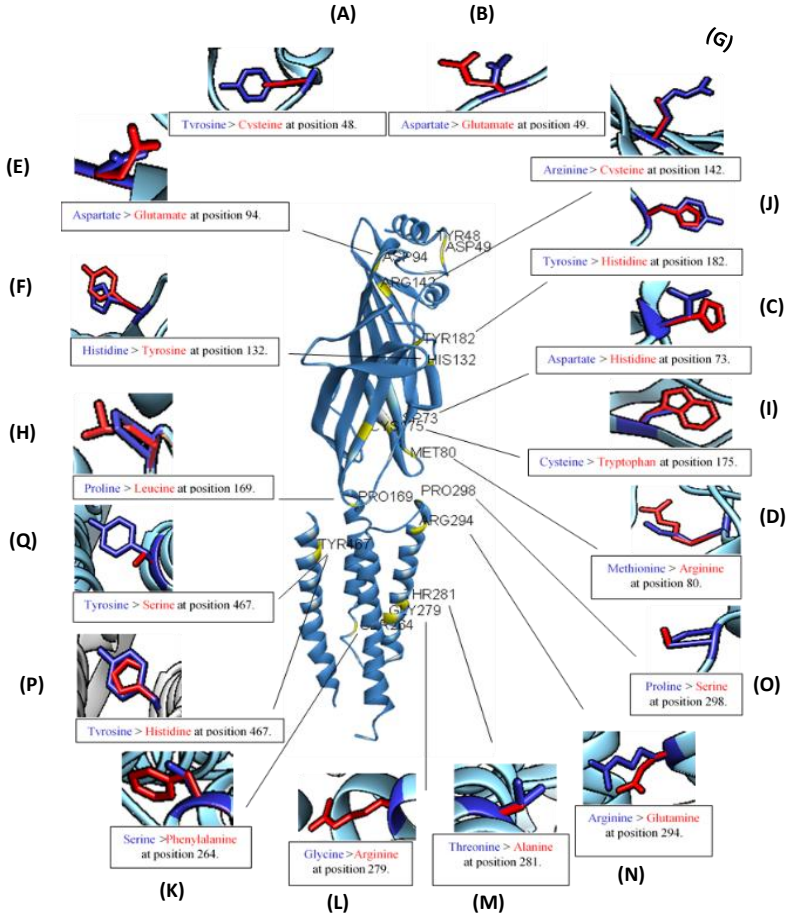


**Figure 6.** Superimposition of wild type and pathogenic variants shown in the three-dimensional structure of β3 subunit. Wild type residues are shown in blue and pathogenic variants are shown in red.

As the WT amino acid was buried in the protein, the bigger variant probably will not fit. The WT amino acid was not charged, while the charge of the mutant amino acid is positive. There will be a difference in the hydrophobic interactions formed by WT and mutant amino acids. Thus, this mutation will likely cause loss of hydrophobic interactions in the corresponding region of the protein. **Figure 6E** shows the superimposition of D94E: The mutant amino acid is bigger than the WT residue, which is highly conserved (**Figure 4,** Supplementary file 3). Variants of a 100% conserved residue are usually considered as damaging for the protein (conservation score is 9, **Table 1**).

The superimposition of H132Y is given in Figure **6F**. The mutant residue is bigger than the WT residue. The hydrophobicity of the WT and mutant amino acid differs. The variant interactions will lead to the removal of hydrogen bonds and respective interactions. **Figure 6G** shows the superimposition of R142C. There is both size and charge difference between the mutant amino acid and WT amino acid, with mutant amino acid being smaller than the WT amino acid, and neutral compared to WT residue which is positively charged. In addition, there is a hydrophobicity difference, the mutant residue being more hydrophobic than the WT. These factors will cause the loss of intermolecular interactions such as hydrogen bonding. **Figure 6H** shows the superimposition of P169L. The WT residue is a proline. Proline residues are inflexible, leading to a distinct conformation that could be crucial in this context. Consequently, mutations at this site may disrupt the specific conformation induced by proline. Also, mutant residue has a longer side chain thus has larger molecular size compared to WT residue. The superimposition of C175W is shown in the **Figure 6I**. The WT amino acid was smaller and was concealed in the core of the protein. Also, cysteine bonds of the WT residue will be lost which altogether suggest an unstable state. **Figure 6J** shows the superimposition of Y182H. There is a size disparity between the WT and mutant, with the mutant residue being smaller than the WT. This mutation will create a void in the protein's core. There is also a difference in hydrophobicity of the WT and mutant residues, which will cause the loss of hydrophobic interactions. The variant amino acid residue is in a highly conserved position (Supplementary file 3, Table 1, see also Figure 4). **Figure 6K** shows the superimposition of S264F: The mutant amino acid is larger than the WT amino acid. Leading a difference in size, which can impact on the molecular interactions. The bigger variant amino acid probably will not fit in the frame interactome. WT residue is less hydrophobic than WT amino acid and this can affect the hydrophobic interactions and hydrogen bond formation. **Figure 6L** shows the superimposition of G279R. The variant residue is larger in size compared to the WT residue. This size difference could impact its interactions with surrounding molecules. While the WT residue was nestled within the protein's core, the variant residue, being larger, might not fit properly. Additionally, the WT amino acid exhibits greater hydrophobicity than the variant amino acid. These discrepancies in hydrophobicity could influence the protein's interactions with the lipid bilayer. Moreover, whereas the WT residue is electrically neutral, the variant residue carries a positive charge.

This alteration introduces a charge in a previously buried residue, potentially affecting protein folding. **Figure 6M** shows the superimposition of T281A, revealing that the variant residue is smaller than the WT residue. This reduced size might render the new residue inadequate for forming multimer contacts. Additionally, the variant residue exhibits higher hydrophobicity compared to the WT residue. Consequently, this disparity in hydrophobicity could influence hydrogen bond formation and affect hydrophobic interactions. In **Figure 6N**, the superimposition of R294Q illustrates that the variant residue is smaller than the WT residue. The size distinction between the WT and mutant residues results in the new residue being positioned unfavorably to form the hydrogen bonding. Furthermore, the WT residue carries a positive charge, while the variant is neutral. This difference in charge is likely to disrupt the ionic interaction established by the original WT residue. **Figure 6O** displays the superimposition of P298S, revealing that the variant amino acid is smaller than the WT amino acid. The WT amino acid proline, is highly conserved and exhibits greater hydrophobicity compared to the variant residue. Prolines are renowned for their rigidity, contributing to a unique backbone conformation potentially essential at this specific position. However, the mutation carries the risk of disturbing this distinctive conformation. In **Figure 6P**, the superimposition of Y467H demonstrates that the variant amino acid is smaller than the WT amino acid. This difference in size could impact interactions with the lipid membrane. Additionally, the variant is more hydrophilic or less hydrophobic than the WT amino acid. This disparity in hydrophilic/hydrophobic states could affect hydrogen bond formation and interactions with membrane lipids. Furthermore, the size discrepancy between the residues prevents the new residue from occupying the same position as the original WT residue, thus impeding the formation of the same hydrogen bond. **Figure 6Q** illustrates the superimposition

of Y467S, where the variant residue appears smaller than the WT residue, potentially leaving an empty space in the protein core. This size difference could also influence the intermolecular contacts and interactions. Overall, these modeling results are in line with our previous findings derived from the evaluation of homology and structural-based algorithms, thus further confirming the pathogenicity of the 17 VUS or unknown variants.

### 3.7. Comparison of the predicted variants with experimentally known mutations in the β3 subunit structural domains

Comparison of the predicted variants (VUS or unknown variants predicted as pathogenic in the present study) with experimentally validated known mutations in the structural domains of the β3 subunit may suggest important insights for the pathological mechanisms and epilepsy phenotype. The frequent presence of pathogenic variants in specific structural domains of the GABA (A) receptors, share common functional characteristics which suggests correlation between structure and function (Hernandez et al. 2019). These "epileptogenic structural cassettes" emerge as the foundation for the correlation between structure and function (Hernandez et al. 2019). Thus, our results will be better understood when epilepsy mutations analysis from experimental findings are structurally integrated with our findings. *GABRB3* mutations causing the structural distortion of the specific structural domains of β3 subunit, acknowledged as a primary cause of severe pediatric epilepsy syndromes as well as more moderate epilepsy disorders like childhood absence epilepsy (Shi et al. 2019, Hernandez et al. 2019). Many familial and *de novo* mutations (missense) in the GABRA1, GABRA2, GABRA5, GABRB1, GABRB2, GABRB3, and GABRG2 subunits of GABA (A) receptors have been identified in individuals with a wide variety of genetic epilepsy syndromes such as childhood absence epilepsy (CAE), Early Onset Absence Epilepsy (EOAE), epilepsy with myoclonic-atonic seizures (MAE) and epileptic encephalopathies (EEs), including West Syndrome (WS) or infantile spasms (IS), Lennox-Gastaut syndrome (LGS) (Hernandez et al. 2019). These mutations are located in the N -terminal domain, neurotransmitter (NT) binding site, and four transmembrane domains (TM1, TM2, TM3, TM4) as well as other regions associated with various channel functions. Among them, there exist over 25 mutations in the GABRB3 gene (Hernandez et al. 2019, Fu et al. 2022), which corresponds to missense pathogenic mutations including G32R (familial, NT binding, CAE), V37G (familial, NT binding, EOAE), S76C (de novo, NT binding, MAE), N110D (de novo, NT binding, IS/WS), D120N (de novo, NT binding LGS/MAE), L124F (de novo, NT binding, EOAE), K127R (de novo, NT binding, EOAE), R142L (familial, NT binding, MAE), T157M (familial, NT binding, DS), L170R (de novo, NT binding, EIEE), E180G (de novo, NT binding, LGS), Y182F (de novo, NT binding, EE), Y184H (de novo NT binding MAE), T185I (de novo NT binding EOEE), R232Q (unknown NT binding EE), Q249K (de novo, M1 pore, EE), P253L (de novo M1 pore EE), S254F (de novo M1 pore EOEE), L256Q (de novo, M1 pore, EE/WS), T287I (de novo, M2 pore, EIEE), T288N (de novo, M2 pore, EIEE), L293H (unknown, M2 pore, EOEE), P301L (de novo, M2-M3 coupling, FE/EE), Y302C (de novo, M2-M3 coupling LGS/EE/FE), A305V (de novo, M2-M3 coupling, EIEE), A305T (de novo, M2-M3 coupling, LGS), R429Q (familial, M3-M4, intracellular, FS). The diagram (**Figure 7**) shows these mutations in comparison with our findings, the predicted pathogenic variants of β3 subunit (encoded by GABRB3 gene). Known missense epilepsy mutations (blue) and unknown missense variants (green) predicted as pathogenic in the present study are shown in the β3 subunit. There is a frequent incidence of these mutations in the N terminal ECD and transmembrane domains (TMDs).

The extracellular N-terminal is shared by the missense pathogenic variants (G32R**,** V37G**,** S76C**,** N110D**,** D120N**,** L124F**,** K127R**,** R142L**,** T157M**,** L170R**,** E180G**,** Y182F**,** Y184H**,** T185I, R232Q) as shown by the blue color and predicted pathogenic variants Y48C, D49E, D73H, M80R, D94E, H132Y, R142C, P169L, and Y48C, D49E, D73H, M80R, D94E, H132Y, R142C, P169L, and Y182H as shown by the geen color (Figure 7). TM1 region is shared by four missense pathogenic mutations (Q249K, P253L, S254F, L256Q) and the predicted variant S264F identified in our study. The TM2 region is shared by three mutations (T287I, T288N, L293H) as shown by the blue color and two predicted variants (T282A) as shown by the green color representing our findings (**Figure 7**).

The extracellular TM2-TM3 linker is shared by pathogenic mutations P301L & Y302C and predicted variants R294Q and P298S. We have also identified Y467S/H and predicted it as pathogenic but the majority of our predicted variants are located in the other domains which are aligned with the experimental findings (Hernandez et al. 2019). Indeed, there are approximately 400 *GABRB3* mutations known to be pathogenic (Shi et al. 2019) however, since our study focuses only on the pathogenicity prediction of the unknown missense variants, only missense variants from those experimentally shown to be pathogenic in the literature (Hernandez et al. 2019) and our predicted missense variants identified as pathogenic are highlighted for comparison in the **Figure 7.**

## 4. Discussion and Conclusions

In the present study, by comprehensive analysis, we studied variants with unknown function or VUS in the coding region of the *GABRB3* gene encoding for the β3 subunit of GABA(A) receptor. Our evaluation, based on the integrated algorithmic assessment, has led to the identification of highly conserved 17 variants as the most pathogenic, possibly leading to the loss of essential molecular interactions with structural and/or functional consequences impacting on the receptor function. The summary of results are given in Table 1.

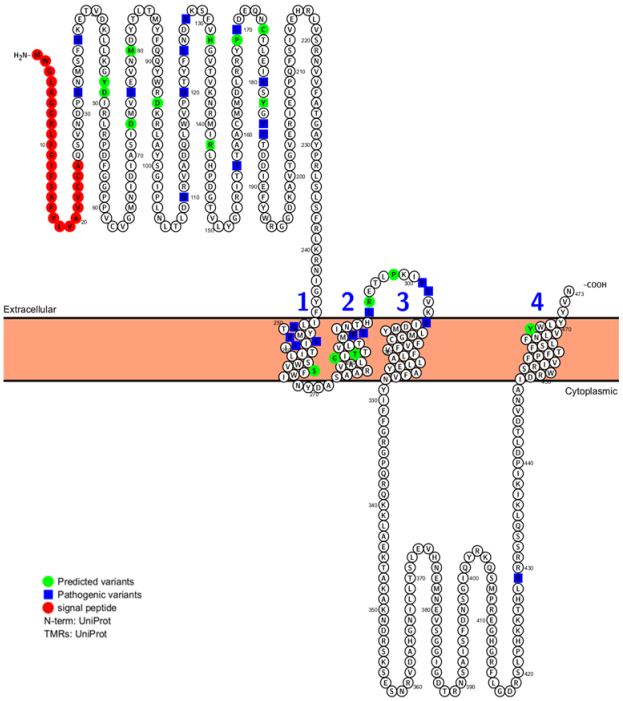


**Figure 7.** Protein diagram comparing the experimentally validated missense epilepsy mutations of *GABRB3* from literature and predicted variants of the *GABRB3* identified in the present study. The β3 subunit encoded by the *GABRB3* gene is shown in its cell membrane position. The experimentally validated pathogenic mutations (blue) and the predicted pathogenic variants (green) identified in the present study share common structural domains, including the ECD followed by the the signal peptide (red), the four transmembrane domains (TM; 1-4), the large cytoplasmic loop (TM3-TM4 loop) and the extracellular linker between the second and the third transmembrane domains (TM2-TM3 linker).

The predicted 17 variants were further studied by mapping them onto the epileptogenic structural cassettes, where well known epilepsy mutations overrepresented with functional consequence and association with epilepsy phenotype severity.

Among the pathogenic variants identified in our study, Y48C, D49E, D73H, M80R, D94E, H132Y, R142C, P169L, and Y182H were located in the ECD, T281A and R294Q were located in the TM2, P298S in the TM2-TM3 linker, and Y467S and Y467H in the TM4. Our multilevel pathogenicity analysis of β3 subunit variants are significant for the specific context of GABA (A) receptor variant localization linked to the relevant molecular cascades required for the receptor biogenesis, maintenance and plasticity. The specific sequences of the different subunit domains are linked to the specific functions relevant to membrane localization, clustering, trafficking, kinetics, regulation and pharmacology (Alldred et al. 2005, Arslan 2006, Arslan et al. 2014, Arslan 2015, Christie et al. 2006, Essrich et al. 1998, Goetz et al. 2007, Haucke et al. 2012, Schweizer et al. 2003, Wong et al. 2015, Yuan et al. 2022). The N-terminal ECD sequences of GABA(A) receptor subunits play a crucial role in expression, assembly, and intracellular trafficking (Luscher et al. 2011). Various mechanisms can contribute to this, such as mutations in this region preventing subunits from reaching the plasma membrane, leading to intracellular degradation. Besides, it was proposed that unique properties of subunit interfaces together with the α1 subunit N-linked glycans lead to an ordered assembly, by which an α1β3 dimer combines with an α1β3γ2L trimer to form a hetero-pentameric receptor (Sente et al. 2022). Also, β subunit is necessary for receptor cell surface expression (Lorenz-Guertin and Jakob, 2018), while the TM4 of the γ2 subunit is required for the postsynaptic clustering of the receptor (Alldred et al. 2005). TM3-TM4 loop (ICD) of this latter subunit is dispensable as shown by membrane targeting studies of chimeric subunit constructs, made up of α/γ2 subunits (Alldred et al. 2005) or δ/γ2 subunits (Arslan et al. 2014). These suggest that depending on the variant's amino acid localization in a subunit, different effects will occur. These will impact the receptor's function, modulation, and plasticity. As a result, our predicted results mapped on to the domains of the β3 subunit will serve as a basis to test these specific mechanisms. This is in line with the pathophysiological data from epilepsy patient mutations, which represent another aspect of our results. Identifying the specific structural domain linked to epilepsy mutations and assessing their effect on receptor dynamics have become key themes in GABA (A) receptor dysfunction in epilepsy. This is due to the structure-function relationship, which is relevant to pathogenesis, such as altered channel gating (Hernandez et al. 2019, Hernandez et al. 2023a). Furthermore, data from epilepsy patient mutations, which have revealed the emergence of epileptogenic structural cassettes with in the GABA (A) receptor subunits, appear to be associated with the severity of epileptic symptoms (Hernandez et al. 2021, Hernandez 2023b, Hernandez and Macdonald 2019, Maillard et al.

2022, Yang et al. 2022). For instance, data from patients diagnosed with epilepsy syndromes carrying mutations in *GABRA1, GABRB2, GABRB3, and GABRG2* genes show that mutations located in the TMDs were linked to more severe epilepsy phenotypes, whereas variants in the extracellular N terminal ECD appeared to correlate with milder phenotypes (Maillard et al. 2022). This correlation of phenotype with the structural localization of variant is further confirmed with specific emphasis on *GABRB3* in that the variant location is associated with the clinical features, including age of epilepsy onset, epilepsy type, and degree of intellectual disability (Johannesen et al. 2022). Thus, *GABRB3* variants located in the ECD were associated with milder phenotypes with an onset of myoclonic, atonic, or absence seizures at a median age of 12 months, while *GABRB3* variants located in the TMDs manifested more severe phenotypes such as early onset focal/multifocal epilepsy and severe intellectual disability. There was also an association with resistance to antiseizure medications (Johannesen, 2022). Thus, our predicted variants mapped on the β3 subunit domains represent critical value in this context. Combining these with our data, we propose that among the pathogenic variants identified in our study, the ones located in the ECD (Y48C, D49E, D73H, M80R, D94E, H132Y, R142C, P169L, C175W, Y182H) may represent a milder phenotype compared to those located in the TMDs (S264F in the TM1, T281A, R294Q in the TM2 and Y467S, Y467H in the TM4).

Accumulating evidence suggests that epilepsy mutations are linked to a form of cellular pathogenesis. This involves a reduction in cell surface expression, often due to decreased receptor trafficking and increased retention in the endoplasmic reticulum, caused by protein conformational defects (Fu et al. 2022, Lorenz-Guertin et al. 2018). Correcting alterations in receptor biogenesis by addressing the homeostatic deficiency of receptor subunits with proteostasis regulators is a promising therapeutic strategy for treating genetic epilepsies (Di et al. 2021, Fu et al. 2018). A detailed analysis of receptor subunit mutations will be crucial in this context and represents one of the strengths of our study. The β3 subunit, encoded by the *GABRB3* gene, plays a central role in GABA(A) receptor oligomerization and trafficking. Indeed, the β3 subunit, without co-assembly, can form homo-pentamers and traffic to the cell membrane due to the critical amino acids (glycine 171, lysine 173, glutamate 179, and arginine 180) that are specific to the β3 subunit. Thus, It was suggested that altered presence of receptors at synapses is a mechanism for *GABRB3* mutations associated with epilepsy syndromes (Shi et al. 2019). As a defect of the oligomerization and trafficking, a decrease in the cell surface expression of the respective GABA (A) receptors is observed. This defect is reflected by the reduction of γ2 containing GABA (A) receptors as membrane targeting of γ2 subunit requires the β subunit encoded by *GABRB3* gene (Shi et al. 2019). These cascades of events show the critical position of the *GABRB3* gene in GABA (A) receptor channelopathies. Thus, extension of our findings for the predicted pathogenic variants with in vitro and vivo analysis will help better elucidate GABA (A) receptor mutations in the mechanism of epilepsy pathogenesis.

Additionally, we have structurally aligned our predicted results with patient mutations in the protein diagram. This comparative illustration of predicted variants with experimentally validated epilepsy mutations is crucial, as it allows for estimating the structural and functional correlations of the predicted variants based on experimental findings. However, a more robust estimation requires the availability of additional experimental data and the development of a reliable model to predict the functional mechanisms.

This study has several limitations. Firstly, relying on predictive algorithms for generalizations based on single studies may not be advisable. Secondly, selecting appropriate cutoffs is crucial to balance sensitivity and specificity, ensuring accurate variant classification. In our study, we used either default or relatively higher cutoff to prioritize confident predictions of pathogenic variants, reducing false positives and including variants with significant disease impact. Although this higher cutoff enhances specificity, it may sacrifice some sensitivity, potentially missing truly pathogenic variants. However, for the study's objectives, focusing on fewer but more confidently predicted variants aligns better with the chosen higher cutoff. Consequently, there's a possibility that some pathogenic variants of *GABRB3* were not captured, with true positives potentially falling slightly below the cutoff or misclassified.

Thirdly, the study specifically concentrated on missense mutations within the protein coding region of the α1 subunit gene. Yet, other variations in crucial areas like splice sites or untranslated regions (UTRs) of *GABRB3* could also be critical for epilepsy or other conditions. Additionally, in our study we have focused on the canonical sequence but there are other transcript isoforms (Macdonald et al. 2010) of the *GABRB3* that require examination. Analyzing the whole spectrum of *GABRB3* variants would give a better overview prioritizing causal variants influencing human phenotypes.

In silico analysis of *GABRB3* gene variants can have different areas of focus. For instance, some studies may examine the predictive accuracy of in silico tools for classifying GABA(A) receptor gene variants. These studies typically evaluate known pathogenic and benign variants of GABRA1, GABRB2, GABRB3, and GABRG2 genes (Wang et al. 2024) to assess the performance of specific in silico tools, such as AlphaMissense (Cheng et al. 2023). Other in silico studies aim to predict the impact of unknown variants (VUS) on the structure and function of specific GABA(A) receptor subunit genes, including GABRA1, GABRD, and GABRG2 (Arslan 2023, Arslan 2024, Abdullah and Arslan 2024). Other studies have a focus on improvement of variant effect prediction by presenting an analysis of the common patterns of pathogenicity in the entire transmembrane protein families in the human transmembrane proteome (Molnár et al. 2016). While these broad screening approaches of the human transmembrane proteome are valuable for offering a predictive view of transmembrane protein-related disease pathogenesis, their focus is different and thus they cannot replace the detailed gene-centric analysis of specific gene variants, such as those in *GABRB3* presented in our study.

As next-generation sequencing becomes more prevalent, the challenge of classifying variants with unknown significance grows, emphasizing the importance of in silico approach for variant interpretation (Abdullah and Arslan 2024, Arslan 2023, Arslan 2024, Dakal et al. 2017, Katsonis et al. 2022, Richards et al. 2015). The present study utilized an integrated in silico approach, which was performed on the *GABRB3* VUS to assess their functional consequence and clinical significance by using a range of algorithms. As, the utilization of multiple lines of in silico analysis to predict variant consequences is regarded as supporting evidence for pathogenicity, our results align with criterion PP3 of ACMG guidelines (Richards et al. 2015). This criterion, along with others, can assist in assessing the pathogenicity of VUS, with implications for tailoring precision and personalized treatments for epilepsy (Traynelis et al. 2017, Trowbridge et al. 2021). Consequently, our results shed light on epilepsy disease mechanisms and offer insights for personalized interventions. However, the relationship between epilepsy syndromes and GABA (A) receptor variants remains complex, warranting further research for effective interventions. Finally, our integrative study has implications for guiding wet lab research by potentially enabling better-planned experiments.

**Declaration of Ethical Standards**

This study is based on the master's thesis entitled "Virtual functional profiling of variants of unknown significance in the GABRB3 gene associated with epilepsy'' (Thesis number: 10612321), completed on January 19, 2024, under the supervision of Asst. Prof. Dr. Ayla Arslan. It is hereby declared that the preparation of this study adhered to scientific and ethical standards, and that all sources consulted have been duly cited in the bibliography.

**Credit Authorship Contribution Statement**

Author 1: Data collection, analysis and data presentation. Drafting and editing the manuscript. The majority of the results presented in this study is based on the masters thesis of the Author 1.

Author 2: Conception, design and supervision of the study. Data analysis and presentation. Interpretation and validation of the data. Drafting, editing and revision of the original manuscript.

**Declaration of Competing Interest**

The authors have no relevant financial or non-financial interests to disclose.

**Data Availability**

All data used in the present study were retrieved from public databases and mentioned in the Methods section of the manuscript. Analysis of the data details were provided as Supplementary files.

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